



Assessment of Antiretroviral Drugs by LC-MS/MS: A Review of Bioanalytical Strategies

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Abstract

Antiretroviral drugs (ARVs) are essential in the prevention of HIV/AIDS. ARV drugs combat the viral infection of HIV by targeting and inhibiting critical steps and its replication. Specific quantification of these drugs in biological matrices is crucial for therapeutic drug monitoring, pharmacokinetic and pharmacodynamic studies, bioequivalence testing, and drug development. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has become the method of choice for estimating ARVs due to its superior sensitivity, specificity, and ability to handle complex biological matrix like plasma, urine, dried blood cells, saliva, blood etc. These biological matrices are dominant for these analyses, and are prepared by using protein precipitation, liquid-liquid extraction or solid-phase extraction depending on the specific complement of analytes.

This review article is aimed to give an extensive assessment of different bioanalytical strategies which have been reported for quantification of ARV drugs in biological matrix. This review also summarizes regulatory considerations for quantification of antiretroviral drugs from biological matrix by LC-MS/MS from 2019 to 2025. Special emphasis is placed on sample preparation techniques, chromatographic conditions, detection strategies.

Keywords

LC-MS/MS, ARV drugs, FDC, Biological Matrix, Method development, Validation

1. INTRODUCTION

AIDS (Acquired Immuno Deficiency Syndrome) is a life-threatening disease caused by HIV, which weakens the immune system. HIV-infected individuals require lifelong antiretroviral therapy (ART). Antiretroviral drugs are used for the treatment of HIV. Antiretroviral drugs (ARVs) are classified into six categories based on their mechanism of action: Nucleoside/Nucleotide reverse transcriptase Inhibitors (NRTIs or NtRTIs), Non-nucleoside reverse transcriptase Inhibitors (NNRTIs), Protease Inhibitors (PIs), Fusion Inhibitors, Entry Inhibitors and Integrase strand transfer Inhibitors ^[1]. Current international guidelines recommend a daily combination of three or more antiretroviral drugs, known as Highly Active Antiretroviral Therapy (HAART) for treatment of HIV. A standard HAART regimen typically includes two or more drugs from two different ARV classes in fixed dose combination (FDC). Now a days HAART treatment becomes most beneficial to reduce illness and death rate ^[2-3].

Presently, more than 100 ARV drugs for the treatment of HIV have been approved by the Food and Drug Administration (FDA). Quantitative analysis of ARV drugs in biological matrix is important to determine bioavailability, drug metabolism and other pharmacokinetic parameters like absorption, distribution, metabolism and excretion^[4]. The antiretroviral drugs work by targeting different stages of the HIV life cycle, such as viral replication and maturation^[5-7]. HIV binds surface of CD4 cell of host and after fusion it multiply and enters into CD4 cell. HIV integrate its viral DNA into the DNA of the CD4 cell. Highly Active Antiretroviral therapy (HAART) is recommended for the HIV treatment. The antiretroviral drug development has been marked by the introduction of novel formulations and the identification of new drug classes to address issues such as drug toxicities and resistance^[8]. The early regimens of antiretroviral therapy were complex, requiring multiple drugs to be taken at different intervals throughout the day, leading to challenges in long-term adherence. However, advancements in drug development have led to the simplification of treatment regimens, with many patients now being able to control their HIV with as little as one pill once a day. Bioanalytical methods are vital for measuring antiretroviral drug levels in biological samples, supporting drug monitoring, adherence checks, and treatment evaluation^[9]. Various bioanalytical techniques such as Liquid Chromatography, HPTLC, Gas Chromatography, Immunofluorescence assay, GC-MS, HPLC-UV, LC-MS, LC-MS/MS etc. are widely used to quantify the drug in biological matrix such as plasma, serum, blood, urine, saliva etc. Liquid Chromatography is widely used in pharmaceutical field to estimate the drugs and metabolites^[10].

Bioanalytical method development and validation is the important step in bioanalysis. It determines the concentration of drug in biological matrix. Advanced techniques like High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS) are widely used for the bioanalysis of drugs in both preclinical and clinical studies. Bioanalysis of drugs and their metabolites in a biological matrix is carried out using different extraction methods like solid phase extraction (SPE), liquid-liquid extraction (LLE), microextraction and protein precipitation. From these extraction methods samples are spiked with reference standards and quality control samples^[11]. The high sensitivity of LC-MS/MS has facilitated the exploration of drug deposition in alternative biological matrices such as dried blood spots, saliva, peripheral blood mononuclear cells, and breast milk^[12].

2. BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION

Bioanalysis is important for the identification and quantification of analytes in various biological matrices. Developing and validating analytical/Bioanalytical methods is a crucial step in the pharmaceutical industry, particularly during the development of antiretroviral drugs. Drugs can be detected, identified, quantified, and characterized using analytical methods. The accuracy and reliability of these methods guarantee that the final product complies with quality standards for purity, potency, safety, and efficacy^[13].

Bioanalytical method development is the set of procedure to determine the concentration of unknown compound or novel compound in a biological matrix. Multiple analytical methods are used to determine physical and chemical properties of analyte, concentration, matrix sample, instrument and analysis cost, speed and time of analysis^[14].

2.1 Sample Collection and Preparation

The living media that contain the analyte are usually blood, plasma, urine, serum, etc. Blood is usually collected from human volunteers/ subjects by vein puncture with a hypodermic syringe up to 5-7 ml. The venous blood is withdrawn into tubes with an anticoagulant, generally ethylenediaminetetraacetic acid and heparin is used as anticoagulant. Plasma is obtained by centrifugation at 4000 rpm for 15 minutes. Around 30-50% of the volume is collected. The aim of sample preparation is to clean up the sample before analysis. Material in biological samples that can affect with analysis, the chromatographic column or the detector includes endogenous macromolecules, proteins, salts, small molecules, and metabolic by products. The sample preparation is also useful for transfer of the analyte from the biological matrix into a solvent, which is suitable for instillation into the chromatographic system. General methods for sample preparation such as liquid/liquid extraction, solid-phase extraction (SPE) and protein precipitation, chromatography, and ligand binding assay (LBA)^[15].

2.2 Biological Sample Processing

Sample preparation is often regarded as the most critical and challenging step in bioanalysis, as each biological matrix presents its own distinct complexities. The development of bioanalytical sample preparation techniques has grown increasingly challenging over the years due to the demand for greater sensitivity, accuracy, and faster analysis, particularly when dealing with complex biological fluids such as blood, serum, plasma, saliva and urine^[16]. The primary goal of sample preparation is to isolate the analyte of interest by removing unwanted substances without causing significant analyte loss. The complexity of biological matrices necessitates effective sample preparation^[17,18]. The process comprises shown in fig.1

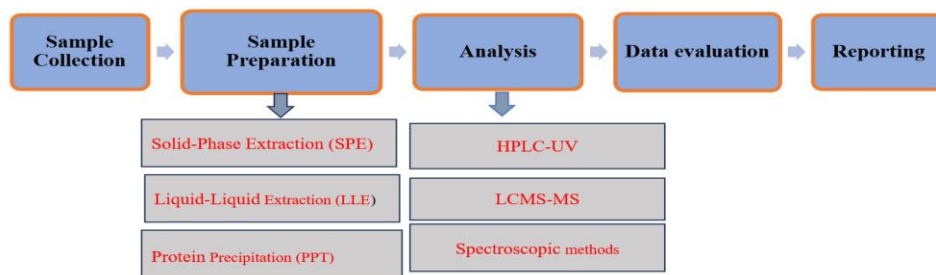


Fig.1: An Bioanalytical work flow for LC-MS/MS can include several steps prior to the actual analysis

2.3. Sample Preparation Strategies

Various chemical and separation techniques are used for sample preparation in Analytical/Bioanalytical chemistry. Sample preparation is an important step for the extraction of analyte from biological matrix. Solid-phase extraction (SPE), protein precipitation (PPT), and liquid–liquid extraction (LLE) is widely reported techniques for the preparation of human biological matrix in the analysis of antiretroviral (ARV) drugs^[19,20]. Fig.2 shows the work flow of extraction techniques.

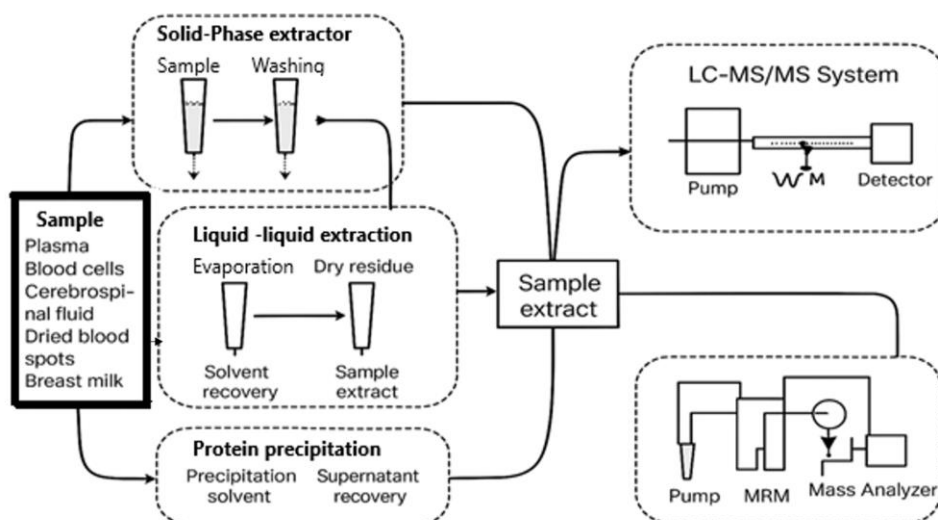


Fig.2 Extraction techniques of Biological sample by LC -MS/MS

2.4 Selection of Chromatographic Conditions

Efficient separation and accurate quantification of active pharmaceutical substances (APS) require proper chromatography conditions. The success of the method is greatly depended on the choice of column, use of mobile phase composition and gradient elution ^[21].

➤ Column Choice:

The selection of appropriate stationary phase can also help to improve the efficiency of method development. The column selection is depended on polarity, molecular weight and sample matrix. Generally, C18 (octadecyl silane) or C8(octyl silane) reverse phase columns are used to separate ARVs. The column dimensions, such as length and particle size affect the resolution and sensitivity. By using a shorter column with a suitable stationary phase, the run time can be reduced ^[22,23]

➤ Mobile Phase Composition:

The mobile phase composition plays a crucial role in analysis. The peak shape, retention time and selectivity can be influenced by selecting different solvent combinations. The preparation and delivery of the mobile phase in LC-MS/MS analysis is done by employing either isocratic or gradient elution flow ^[22-25].

2.5 Bioanalytical Method Validation

Once a method is developed, it has to undergo validation in accordance with internationally recognized guidelines. This process confirms that the method is fit for its intended purpose, capable of generating accurate and reliable data for the analyte in the selected biological matrix. Validation also ensures that the method can be successfully applied to the analysis of real patient samples ^[26].

Validation of bioanalytical method is essential to employ well-characterized and fully validated analytical methods to yield reliable results which can be satisfactorily interpreted. It is recognized that analytical methods and techniques are constantly undergoing changes and improvements. It is also important to emphasize that each analytical technique has its own characteristics, which will vary from analyte to analyte. Specific validation criteria are needed for methods intended for analysis of each analyte (drug and/or metabolite). The process in which specific bioanalytical methods are developed are validated by various validation parameters like Carry over effect, Anticoagulant effect, Selectivity, Hemolysis effect, Linearity, Limit of Detection, Limit of Quantification, Accuracy, Precision, Recovery, Stability studies according to the U.S. Food and Drug Administration (USFDA) or International Council for Harmonisation (ICH) guidelines ^[27].

3. DEVELOPED BIOANALYTICAL METHODS

The majority of the methods for the quantitation of ARV drugs in biological matrices involves LC-MS/MS assay. Numerous LC-MS/MS methods have been reported for the analysis of ARV drugs in biological matrices. The majority of the mobile phase consisted of a mixture of acetonitrile and buffer. Isocratic or gradient elution were used depending on the number of ARV drugs to be analysed. Before injection of samples to the chromatographic system, sample preparation techniques like liquid-liquid extraction, solid-phase extraction and protein precipitation are used for extraction of analyte from biological matrix.

Prathipati et al (2019) created the LC-MS/MS method that simultaneously determines Tenofovir, Emtricitabine, Elvitegravir, and Rilpivirine in dried blood spots. A Restek Pinnacle DB Biph (2.1 × 50mm, 5µm) column with isocratic mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) (45:55 v/v), 0.250 mL/min used as flow rate. Linearity was observed in the range of 10–2000 ng/mL for Tenofovir, Emtricitabine, Elvitegravir and Rilpivirine ^[28]. The Cenicriviroc is dual – acting inhibitor (antagonist CCR2/CCR5) used to treat HIV. The quantification of Cenicriviroc in human plasma was achieved by Penchala et al (2019) using a new LC-MS/MS method. A reverse-phase C18 XBridge column (50 × 2.1 mm; 3.5 µm) is used for chromatographic separation, which involves utilizing 0.1% formic acid and acetonitrile in a gradient elution and having a flow rate of 400 µl/min. Calibration curve ranged from 5 to 1000 ng/mL for plasma and from 0.241 to 15.0 ng/mL for cerebrospinal fluid ^[29].

An UPLC-MS/MS method for simultaneous quantification of Maraviroc, Nevirapine, Rilpivirine, Dolutegravir, Raltegravir, Cobicistat, Darunavir, Ritonavir, Atazanavir, Efavirenz, Elvitegravir and Etravirine in peripheral blood mononuclear cells (PBMCs) was developed and validated by Nicolò *et al* (2020). The samples were analysed using an Acquity® UPLC HSS T3 column (2.1 × 150 mm, 1.8 µm), with a gradient flow 0.4 mL/min for two mobile phases MP-A: 0.05% v/v FA in water and MP-B: 0.05% v/v FA in acetonitrile (MP-A:MP-B;70:30). The linearity range (calibration curve range) was established between the lowest limit of quantification (LLOQ) and the upper limit of quantification (ULOQ). The LLOQ value for Maraviroc, Rilpivirine, Dolutegravir, Raltegravir, Cobicistat, Atazanavir, Ritonavir, Elvitegravir, Etravirine drugs was observed in the range 0.039 – 10 ng/sample, and for Nevirapine, Darunavir, and Efavirenz the range was 0.390 – 100 ng/sample [30].

Perrine Courlet (2020) designed an UPLC-MS/MS method to quantify the concentration of Bictegravir, Cabotegravir, Doravirine and Rilpivirine in plasma sample of HIV people. Xselect HSS T3 (2.1 × 75 mm, 3.5 µm) analytical column is used for chromatographic analysis. Water and 0.1% Formic acid as mobile phase A, Acetonitrile and 0.1% Formic acid as mobile phase B with flow rate 300 µL/min. No matrix effect is observed during the validation. The developed method exhibits good performances for linearity concentration ranges 30 to 9000 ng/mL for Bictegravir, Cabotegravir and Doravirine and from 10 to 1800 ng/mL for Rilpivirine [31]. Vadlamani and Ravindharnath (2020) created a new, easy, receptive, and precise LC-MS/MS technique for Efavirenz and impurities. A Hypersil C-18 column (250 × 4.6 mm, 5 µm) with 0.01 M ammonium acetate buffer and methanol as a mobile phase and 1 ml/min flow rate utilized for chromatographic separation. The linearity observed in the concentration range of 2.5-78 ppb [32].

Deqing Xiao *et al* (2020) developed an LC-MS/MS method for the determination of Tenofovir in human plasma. The prodrugs Tenofovir disoproxil fumarate (TDF) and Tenofovir alafenamide (TAF) were quantified using an Acquity UPLC HSS PFP column (2.1 × 50 mm, 1.8 µm) with 10 mM ammonium formate in water (pH 2.5) as Mobile Phase A and 100% methanol as Mobile Phase B. The mobile phases were delivered at a combined flow rate of 0.5 mL/min. The method was thoroughly validated in terms of linearity, sensitivity, accuracy, precision and selectivity. the linearity range for the validated LC-MS/MS method observed was 0.300 – 300 ng/mL for tenofovir. The developed method was proved robust, reproducible and fit for purpose for clinical applications, ensuring accurate assessment of TFV pharmacokinetics [33].

Chengalva and Kuchana (2020) investigate a novel UPLC-MS/MS method for the Simultaneous Estimation of Lamivudine, Tenofovir Disoproxil Fumarate, Doravirine and Efavirenz in tablet form. Analysis is performed on Acquity Ethylene Bridged Hybrid (BEH) Phenyl column (50 × 2.1 mm, 1.7 µm) with UV detection at 238 nm with mobile phase water and acetonitrile in the ratio 50:50 (v/v) was pumped at a flow rate 0.4 ml/min. The developed method shows the excellent linearity 7.5-45 µg/ml for lamivudine and Tenofovir disoproxil fumarate, 2.5- 15 µg/ml for Doravirine and 15-90 µg/ml for Efavirenz [34]. Alieu Amara *et al* (2021) developed a highly sensitive LC-MS/MS method for the simultaneous quantification of Molnupiravir (MPV) and its active metabolite β-d-N4-hydroxycytidine (NHC) in human plasma and saliva. The chromatographic separation was achieved using a Waters XBridge C18 column (3.5 µm, 100 × 2.1 mm). The mobile phase consisted of 1 mM ammonium acetate (pH 4.3 with acetic acid) as phase A and 1 mM ammonium acetate in acetonitrile as phase B. A gradient elution was performed at a flow rate of 350 µL/min. The method was validated over a linear concentration range of 2.5–5000 ng/mL for both plasma and saliva matrices [35].

Morsy *et al* (2021) formulated a novel LC-MS/MS method for determination of Favipiravir in human plasma. Protein precipitation extraction technique is used for plasma sample preparation by using methanol. The chromatographic analysis is carried out on Eclipse plus C18 column (50 × 4.6 mm, 3.5 µm) using isocratic elution mode. Methanol and 0.2 % acetic acid (20:80, v/v) as a mobile phase with flow rate 0.6 mL/min. The linearity observed in the range of 100.0–20000.0 ng/mL. The proposed method was effectively applied for the pharmacokinetic evaluation of Favipiravir [36].

A sensitive, fast and simple LC-MS / MS method for the quantitation of favipiravir in human serum was investigated by Eryavuz Onmaz *et al* (2021) The analysis was done using a reverse-phase Phenomenex C18 (50 × 4.6 mm, 5 µm) column. A gradient mixture of 0.1% formic acid in water and 0.1% formic acid in methanol was used as the mobile phase. The method showed good linearity in the concentration range

of 0.048 to 50 µg/mL for negative ion mode and 0.062 to 50 µg/mL for positive ion mode. The total analysis time was 3.5 minutes^[37]. Tanuja and

Ganapaty (2022) investigate a novel and reliable LC-MS/MS method for the estimation of Bictegravir (BIC), Tenofovir Alafenamide Fumarate (TNF) and Emtricitabine (EMT) in plasma by using protein precipitation extraction technique. The plasma drug concentration is determined by using The Zorbax XDB C 18 analytical column (2.1 × 50 mm, 5 µm). Mobile phase consists of Acetonitrile: Formic acid (0.1%) in water (70:30, v/v), at a flow rate of 0.15 mL/minute. The concentration rang found linear between 2- 500 ng/mL^[38]. Gouda *et al* (2022) validated LC-MS/MS method for determination of antiviral prodrug Molnupiravir in human plasma. Proposed method was simple and fully validated by using protein precipitation technique. The chromatographic separation was carried out on Agilent Zorbax Eclipse plus C18 column (4.6 × 150 mm, 5 µm) and isocratic elution system comprised of 0.2% acetic acid: methanol (5:95, v/v) as a mobile phase that was pumped at a flow rate of 0.9 mL/min. The linearity was observed in the range of 20.0–10000.0 ng/mL^[39].

Manikanta and Jayakumari (2022) developed LC-MS/MS method for quantification of Darunavir in human plasma by using protein precipitation extraction technique. Agilent, Zorbax, and XDB C18 (2.1 x 50 mm ID, 5 µm) analytical column was selected for analysis. Acetonitrile: 2 Mm Ammonium Formate with 0.1% formic acid in water (70:30 v/v) used as a mobile phase. An injection volume of 10 µL, flow rate used 0.120 mL/min and total run time 3.0 min. Linearity range was observed 10.00 to 2000.00 ng/ml^[40]. Yan Wu *et al* (2022) reported a highly sensitive LC-MS/MS method for simultaneous estimation of six antiretroviral drugs as Lamivudine, Zidovudine, Nevirapine, Efavirenz, Ritonavir and Lopinavir in human hair. Analysis is performed on a Platisil ODS C18 column (150 × 4.6 mm, 5 µm), protected by a C18 guard cartridge (10 × 4.6 mm, 5 µm). Methanol and 0.004 mol/L ammonium acetate in tri-distilled water (95:5, v/v) was used as the mobile phase and flow rate was kept 300 µL/min. The developed method show's good reliability and linearity 6 – 5000 pg/mg for Lamivudine, 10 – 5000 pg/mg for Zidovudine, 6 – 50000 pg/mg for Nevirapine, 12–50000 pg/mg for Efavirenz, 8–5000 pg/mg for Ritonavir and 8–12500 pg/mg for Lopinavir. The limits of quantification (LOQ) ranged between 6 and 12 pg/mg for all six analytes^[41].

Patel *et al* (2023) proposed a sensitive and rapid LC-MS/MS method for simultaneous quantification of Emtricitabine (FTC), Tenofovir alafenamide (TAF) and Dolutegravir (DTG) in human plasma. The chromatographic separation is done using Kinetex biphenyl 100 A column C18 (100 × 4.6 mm, 5 µm) with mobile phase of 0.1% v/v formic acid in water. Plasma drug is extracted by using solid phase extraction. Developed Method is validated according to FDA Guidelines. The developed method has been validated with a linear range of 20 to 5000 ng/ml for Emtricitabine, 2 to 500 ng/ml for Tenofovir alafenamide and 30 to 7500 ng/ml for Dolutegravir^[42]. Bioanalytical method development and validation of Tenofovir in human plasma is proposed by Salunke *et al* (2023). Solid phase extraction technique is used for extraction of analyte and chromatographic analysis is carried out on Thermo C18 (4.6 X 150 mm, 5 µm) column. The chromatographic run with 1 ml/min flow-rate using mobile phase A (0.1% Formic acid in methanol) and mobile phase B (0.1% Ammonia in water) with an initial ratio of (85:15 v/v). The LLOQ and LOQ for Tenofovir was found were 5 ng/mL and 15 ng/mL^[43].

Priyadarshini and Devi (2023) developed and validated a simple and novel LC-MS/MS method for quantification of tenofovir and emtricitabine in human plasma by simple solid phase extraction technique. The chromatographic separation is performed on A Kinetex PFP, (50 X 4.6 mm, 5 µm) Phenomenex column, using 5 mM Ammonium Formate: Acetonitrile: Methanol 60:25:15 (v/v/v) mobile phase. Flow rate was used 1.0 ml/min with an injection volume of 10 µl. The calibration curves were linear over the range of 5-500 ng/ml for tenofovir and 8 - 4000 ng/ml for emtricitabine^[44]. Kumar *et al* (2023) establish a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method for Amantadine, Lamivudine and Acyclovir in rat plasma. Analysis was carried out by using Phenomenex Kinetex Polar C18 (50 × 4.6 mm, 2.6 µm) column. The mobile phase composed of solvent-A which contains 100% water and solvent-B as Methanol with 0.05% acetic acid having 1 ml/min flow-rate. The method was linear 57–5,000 ng/ml for all drugs^[45]. Challenger *et al* (2023) investigated and validated a robust LC-MS/MS method for quantification of Favipiravir in human plasma. Analysis was performed on a Synergi Polar-RP (150 × 2.1 mm, 4 µm) column. 0.2% formic acid in water and 0.2% formic acid in methanol used as mobile phase in gradient elution with flow rate 600 µL/min. For extraction of sample Protein precipitation technique is used. The linearity is found over the range 500–50,000 ng/mL^[46]. Vemuri *et al* (2024) estimate

a LC-MS/MS method for Bictegravir in human plasma. The separation was carried out on Zorbax SB-C8 (150 × 4.6 mm, 3.5 µm) column. Mobile phase A contains Methanol: Buffer (5:95%). Mobile phase B contains Acetonitrile: Methanol: Tetrahydrofuran (50:10:25%) With flow rate of 1.2 mL/min. Linearity was obtained 0.17 g/mL to 2.25 g/mL ^[47].

Rao *et al* (2024) developed and validated an analytical method for the determination of Favipiravir (FVPR) in pure and tablet dosage forms by LC/MS-MS Technique. The Chromatographic separation was carried out on Waters X-Bridge Phenyl Hexyl column (150 x 4.6 mm, 3.5 µm). The mobile phase used a mixture of buffer and Acetonitrile in a 60:40 v/v ratio with flow rate 1 mL/min. The developed method is validated according to ICH guidelines with better outcome. The linearity rang observed in the range of 2-40 ng/mL ^[48]. Katthala and Gubbiyappa (2024) investigated a new LC-MS/MS Method for the estimation of Ritonavir in Plasma Samples. Isolation of analyte is done on HYPURITY ADVACE (50 × 4.6 mm, 5 µm) analytical column with mobile phase methanol and ammonium acetate 5 mM buffer (85:15% v/v). The linearity observed in the concentration range of 8.004 to 1600.001 ng/mL for ritonavir. The developed method is simple, novel and can be used for industries, forensic labs, quality control labs, and bioavailability studies ^[49].

Kanjarla and Katta (2024) Constructed a novel UPLC-MS/MS method for Simultaneous Quantification of Doravirine, Lamivudine, and Tenofovir Disoproxil Fumarate in Human Plasma. An agilent zorbax XDB C18 analytical column (2.1 × 50 mm, 3.5 µm) was used for chromatographic separation, and its isocratic mobile phase consists of acetonitrile and buffer (5 mM of ammonium formate with 0.1 % formic acid) in the ratio 80:20, v/v, at a flow rate of 0.120 mL/min. The method was validated throughout concentration ranges of 2.5–1000 ng/mL ^[50]. Mounika and Akila (2025) developed an LC-MS/MS Method for the Determination of Lamivudine, Tenofovir & Efavirenz in human plasma. Conditions like Xterra, C18 (150 x 4.6 mm, 5 µm) column with a mobile phase composed of 10 mM Ammonium Formate: Methanol: Acetonitrile, 50:25:25 (v/v/v), at a flow rate of 1.0 mL/min used for separation. The calibration ranges are 10-3018 ng/mL, 5-500 ng/mL and 20-6021 ng/mL for Lamivudine, Tenofovir disoproxil fumarate and Efavirenz respectively ^[51]. West *et al* (2025) invented a novel and robust UPLC-MS/MS method for the simultaneous quantification of a panel of nine antiretrovirals: Abacavir, Bictegravir, Cabotegravir, Dolutegravir, Doravirine, Emtricitabine, Lamivudine, Raltegravir, and Tenofovir in human plasma. The chromatographic separation was achieved with a Waters CORTECS T3 column (2.1 × 100 mm; 1.6 µm) along with CORTECS T3 Vanguard pre-column (2.1 x 5 mm, 1.6 µm) The solvent flow rate was 0.300 mL/min, and the mobile phases were 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The developed method shows the LLOQ at 100 ng/mL and linearity ranges 100-5000 ng/mL for all drugs ^[52].

A novel simultaneous analytical quantification of Lopinavir and Ritonavir in drug products by using LC-MS/MS is done by Paduri *et al* (2025). Acquity BEH C18 column with ACN and 0.1% Formic acid in water (80:20 v/v) as mobile phase. retention periods of 1.89 min for LPV and 1.85 min for RTV. The linearity ranges 10-150 ng/mL for both drugs ^[53]. A Novel Micro-LC-MS/MS Method for the Quantification of Tenofovir and its Active Metabolite Tenofovir-Diphosphate in Biological Matrices for Therapeutic Drug Monitoring investigated by Tarcomnicu *et al* (2025) Separation of biological samples is done on a microbore amino Luna NH2, (150 × 0.3 mm, 3.0 µm) 100 A, Phenomenex, column. Gradient elution with flow rate 10 µL/min and injection volume using 2 µL using water with 0.025% ammonia as mobile phase A and acetonitrile with 0.025% ammonia as a mobile phase B. The LOQ obtained for Tenofovir is 0.25 mg/mL and for Tenofovir-Diphosphate is 0.5 mg/mL ^[54]. Yang *et al* (2025) introduces a new LC-MS/MS method for the simultaneous determination of simnotrelvir and ritonavir in human serum and bronchoalveolar lavage fluid. Kinetex F5 (3.0 × 100 mm, 2.6 µm, Phenomenex) column was used for better sensitivity and separation efficiency. 10 mM ammonium acetate and 20 mM ammonium acetate used as mobile phase with different gradient elution. The linearity of was good in the concentration ranges 2.5-5000 ng/mL for simnotrelvir, 0.1-1000 ng/mL for ritonavir and 1 - 1000 µg/mL for urea ^[55].

4. DISCUSSION

The present review offers a comprehensive and critical assessment of various bioanalytical strategies that have been developed and reported for the quantification of antiretroviral (ARV) drugs in different biological matrices. The various strategies highlight the methodological advancements, analytical challenges and validation approaches employed in LC–MS/MS-based bioanalysis, involved in therapeutic drug monitoring and pharmacokinetic studies of ARV drugs.

5. CONCLUSION

An attempt has been made to understand and explain the bioanalytical method development and validation for antiretroviral drugs (ARV) from a quality assurance point view. Basic Steps in Chromatographic method development, sample pre-treatment, different bioanalytical sample extraction technique and method validation parameters were discussed in this article. Various method development and essential validation characteristics for bioanalytical methodology have been discussed with a view to improve the standard and acceptance criteria in this field of bioanalysis research particularly for ARV drugs. LC-MS/MS has revolutionized the field of bioanalysis, particularly in the estimation of antiretroviral drugs. LC-MS/MS is the gold standard for pharmaceutical analysis in complex matrices due to high sensitivity, specificity and robustness make it indispensable for pharmacokinetic studies, therapeutic monitoring, and drug development. The rising use of antivirals, developing robust and sensitive analytical methods are crucial.

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6. REFERENCES

1. Daskapan, A., van Hateren. K., Stienstra, Y., Kosterink, J., van der Werf, T., Touw, D., Alffenaar, J. (2018). Development and validation of a bioanalytical method for the simultaneous determination of 14 antiretroviral drugs using liquid chromatography-tandem mass spectrometry. *Journal of Applied Bioanalysis*, 4(2): 37-50.
2. Önal A. (2006). Analysis of Antiretroviral Drugs in Biological Matrices for Therapeutic Drug Monitoring. *Journal of Food and Drug Analysis*, 14(2):99-119.
3. Gouget, H., Noé, G., Barrail-Tran A, Furlan V. (2020). UPLC–MS/MS method for the simultaneous quantification of Bictegravir and 13 others antiretroviral drugs plus cobicistat and ritonavir boosters in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*, 181(113057):1-8.
4. Fical, L., Khalikova, M., Vlčková H., Lhotská, I., Hadysová, Z., Vokřál, I., Červený, L., Švec, F., Nováková L. (2021). Determination of Antiviral Drugs and Their Metabolites Using Micro-Solid Phase Extraction and UHPLC-MS/MS in Reversed-Phase and Hydrophilic Interaction Chromatography Modes., *Molecule*, 26(8):1-22.
5. Nuwagaba, J., Li, J., Ngo, B., Sutton R. (2025). 30 years of HIV therapy: Current and future antiviral drug targets. *Virology*, 603 (110362) :1-7.
6. Saag, M., Gandhi, R., Hoy, J., Landovitz, R., Thompson, M., Sax, P., Smith, D., ... & Volberding P. (2020). Antiretroviral Drugs for Treatment and Prevention of HIV Infection in Adults 2020 Recommendations of the International Antiviral Society–USA Panel. *Journal of American Medical Association*, 324(16);1651-1669.
7. Shin, Y., Park, C., Yoon C. (2021). An Overview of Human Immunodeficiency Virus-1 Antiretroviral Drugs: General Principles and Current Status. *Infection and Chemotherapy*, 53(1):29-45.
8. Cunha, R., Simões, S., Carvalheiro, M., Azevedo Pereira, J., Costa, Q., Ascenso, A. (2021). Novel Antiretroviral Therapeutic Strategies for HIV. *Molecules*, 26 (17):1-19.

9. Charbe, N., Zacconi, F., Amnerkar, N., Ramesh, B., Tambuwala, M., Climenti, E. (2019). Bio-analytical Assay Methods used in Therapeutic Drug Monitoring of Antiretroviral Drugs-A Review. *Current Drug Therapy*.14(1):16-57.
10. Deshpande, M., Kasture, V., Mohan, M., Chavan, M. (2018). Bioanalytical Method Development and Validation: A Review, Chapter in a book: Recent Advances in Analytical Chemistry;1-20.
11. Chaudhari, S., Taktode, A., Kotgale, N., Adhao, V. (2024). Bioanalytical Method Development and Validation for Estimation of Active Pharmaceutical Substance by LC-MS/MS: A General Review on Bioanalysis. *International Journal of Research and Analytical Reviews*. 11(1):69-81.
12. Taylor, P., Tai, C., Franklin, M., Pillans, P., (2011). The current role of liquid chromatography-tandem mass spectrometry in therapeutic drug monitoring of immunosuppressant and antiretroviral drugs. *Clinical Biochemistry*.44(1):14–20.
13. Awachat, S., Kalbande, S., Raut, S., Changade, T., Sarnaik, S., Rodge, K., Kale, R. (2025). Analytical method development and validation of antiviral drugs for HIV. *International Journal of Science and Research Archive*,15(01):1618-1646.
14. Tijare, L., Rangari, N., Mahajan, U. (2016). A Review on Bioanalytical Method Development and Validation. *Asian Journal of Pharmaceutical and Clinical Research*, 9(3): 6-10.
15. Pia Dima, A., De Santis, L., Verlengia, C., Lombardo, F., Lenzi, A., Mazzarino, M., Botre, F., Paoli, D. (2020). Development and validation of a liquid chromatography-tandem mass spectrometry method for the simultaneous determination of phthalates and bisphenol a in serum, urine and follicular fluid. *Clinical Mass Spectrometry*,18:54-65.
16. Ingle, R., Zeng, S., Jiang, H., Fang, W. (2022). Current developments of bioanalytical sample preparation techniques in pharmaceuticals. *Journal of Pharmaceutical Analysis*, 12(4) :517-529.
17. Pawar, S., Dr. Gaware, V. (2025). A Review on: Bioanalytical Method Development and Validation. *Asian Journal of Pharmaceutical Research and Development*, 13(3): 167-179.
18. Gope, E., Pottendla, S., Yaparathi, S. (2025). A Review on Analytical Methods for Detection and Quantification of Novel Antiviral Drugs. *Journal of Pharma Insights and Research*, 3(3): 231-241.
19. Sichilongo, K., Mwando, E., Sepako, E., Massele, A. (2018). Comparison of efficiencies of selected sample extraction techniques for the analysis of selected antiretroviral drugs in human plasma using LC-MS. *Journal of Pharmacological Toxicological Methods*, 89:1-8.
20. Checa, A., Oliver, R., Hernández-Cassou, S., Saurina, J., (2009). Determination of HIV drugs in biological matrices: A review. *Analytica Chimica Acta*, 647(1):1-13.
21. Wong, A., Xiang, X., Ong, P., Mitchell, E., Syn, N., Wee, L., Kumar, A., Yong, W., Sethi, G., Goh, B., Ho, P., Wang, L. (2018). A Review on Liquid Chromatography-Tandem Mass Spectrometry Methods for Rapid Quantification of Oncology Drugs. *Pharmaceutics*, 10(4):1-20.
22. Rappold, B. (2022). Review of the Use of Liquid Chromatography-Tandem Mass Spectrometry in Clinical Laboratories: Part I-Development. *Annals of Laboratory Medicine*, 42(2) :121-140.
23. Wal, P., Kumar, B., Bhandari, A., Rai, A., Wal, A. (2010). Bioanalytical Method Development – Determination of Drugs in Biological Fluids. *Journal of Pharmaceutical Science and Technology*, 2(10): 333-347.
24. Whitmire, M., Ammerman, J., De Lisio, P., Killmer, J., Kyle, D., Mainstone, E., Porter, L., Zhang, T. (2011). LC-MS/MS bioanalysis method development, validation, and sample analysis: points to consider when conducting nonclinical and clinical studies in accordance with current regulatory guidances. *Journal of Analytical and Bioanalytical Techniques*, 1(1).
25. Reddy, K. T. K., Gandla, K., Babu, P. V., Chakravarthy, M. V. K., Chandrasekhar, P., Sagapola, R. (2022). A critical review on bioanalytical method development and validation of few oncology drugs by using LC-MS-MS. *Journal of Pharmaceutical Negative Results*, 13(S01):16-27.
26. Kumar, S., Gautam, D. Talwan, P., Dogra, P., Patial, B., Rana, R. (2023). A Review on Bioanalytical Techniques of Anti-HIV Drugs. *Journal for Basic Sciences*, 23(3):669-683.
27. Kaza, M., Karaźniewicz-Łada, K., Kosicka, K., Siemiatkowska A, Rudzki Piotr, J. (2019). Bioanalytical method validation: new FDA guidance vs. EMA guideline. Better or worse? *Journal of Pharmaceutical and Biomedical Analysis*,165:381-385.

28. Prathipati, P., Mandal, S., Destache, C. (2018). LC-MS/MS method for the simultaneous determination of tenofovir, emtricitabine, elvitegravir, and rilpivirine in dried blood spots. *Biomedical Chromatography*, 32(9):1-16.
29. Dilly Penchala, S., Alagaratnam, J., Challenger, E., Amara, A., Else, L., Winston, A., Khoo, S. (2020). The development and validation of a novel LC–MS/MS method for the quantification of cenicriviroc in human plasma and cerebrospinal fluid. *Biomedical Chromatography*, 34(1):1-8.
30. De Nicolò, A., Ianniello, A., Ferrara, M., Avataneo, V., Cusato, J., Antonucci, M., et al. (2021). Validation of a UHPLC-MS/MS Method to Quantify Twelve Antiretroviral Drugs within Peripheral Blood Mononuclear Cells from People Living with HIV. *Pharmaceuticals*, 2021; 14(12):2-16.
31. Courlet, P., Saldanha, S., Cavassini, M., Marzolini, C., Choong, E., Csajka, C., et al. (2020). Development and validation of a multiplex UHPLC-MS/MS assay with stable isotopic internal standards for the monitoring of the plasma concentrations of the antiretroviral drugs bictegravir, cabotegravir, doravirine, and rilpivirine in people living with HIV. *Journal of Mass Spectrometry*, 55(46):1-13.
32. Vadlamani, A., Ravindhranath, K. (2020). A Novel LC-MS/MS Method Development and Validation for Ultra-trace Level Determination of Three Genotoxic Efavirenz Impurities. *Indian Journal of Pharmaceutical Science*, 82(3):429-434.
33. Xiao, D., Ling, K., Tarnowski, T., Majeed, S., German, P., Kearney, B., Zhao, Y., Chen, Y., Ma, L., Zhang, T. (2020) An LC-MS/MS method for determination of tenofovir (TFV) in human plasma following tenofovir alafenamide (TAF) administration: Development, validation, cross-validation, and use of formic acid as plasma TFV stabilizer. *Analytical Biochemistry*, 593:1-11.
34. Chengalva, P., Kuchana, M. (2020). Development and Validation of Ultra Performance Liquid Chromatographic Method for the Simultaneous Estimation of Lamivudine, Tenofovir Disoproxil Fumarate, Doravirine and Efavirenz in Bulk and Pharmaceutical Formulations. *Indian Journal of Pharmaceutical Science*, 82(6):1006-1014.
35. Amara, A., Penchala, S., Else, L., Hale, C., FitzGerald, R., Walker, L., Lyons, R., Fletcher, T., Khoo, S. (2021). The development and validation of a novel LC-MS/MS method for the simultaneous quantification of Molnupiravir and its metabolite β -d-N4-hydroxycytidine in human plasma and saliva. *Journal of Pharmaceutical and Biomedical Analysis*, 206 :1-7.
36. Morsy, M., Nouman, E., Abdallah, Y., Zainelabdeen, M., Darwish, M., Hassan, A., Gouda, A., Rezk, M., Abdel-Megied, A., Marzouk, H. (2021). A novel LC-MS/MS method for determination of the potential antiviral candidate favipiravir for the emergency treatment of SARS-CoV-2 virus in human plasma: Application to a bioequivalence study in Egyptian human volunteers. *Journal of Pharmaceutical and Biomedical Analysis*, 199:1-8.
37. Eryavuz Onmaz, D., Abusoglu, S., Onmaz, M., Yerlikaya, F., Unlu, A. (2021). Development and validation of a sensitive, fast and simple LC-MS / MS method for the quantitation of favipiravir in human serum. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 1176:1-8.
38. Tanuja, A., Ganapaty, S. (2022). Bio-analytical Method Development and Validation for Simultaneous Determination of Bictegravir, Emtricitabine, and Tenofovir Alafenamide Fumarate in Human Plasma by LC-MS/MS. *Indian Journal of Pharmaceutical Education and Research*, 56(4):1190-1205.
39. Gouda, A., Marzouk, H., Rezk, M., Salem, A., Morsi, M., Nouman, E., Abdallah, Y., Hassan, A., Abdel-Megied, A. (2022). A validated LC-MS/MS method for determination of antiviral prodrug molnupiravir in human plasma and its application for a pharmacokinetic modeling study in healthy Egyptian volunteers. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 1206:1-10.
40. Manikanta, Y., Jayakumari, S. (2022). LC-MS/MS method development and validation for the determination of antiviral drug in human plasma. *International journal of health sciences*, 6(S6):7604–7612.
41. Wu, Y., Chu, L., Yang, H., Wang, W., Zhang, Q., Yang, J., Qiao, S., Li, X., Shen, Z., Zhou, Y., Liu, S., Deng, H. (2021). Simultaneous Determination of Six Antiretroviral Drugs in Human Hair Using an LC-ESI+-MS/MS Method: Application to Adherence Assessment. *Therapeutic Drug Monitoring*, 43(6): 756–765.

42. Patel, R., Dave, N., Mehta, D. (2023). Development and Validation of LC-MS/MS Method for Simultaneous Estimation of Emtricitabine, Tenofovir Alafenamide and Dolutegravir in Human Plasma. *International Journal of Pharmaceutical Sciences Review and Research*; 83(1): 5-12.
43. Salunke, S., Wankhede, S., Medhe, S. Nimje, H., Ranjan, S., Kendre, V., Bhaskar, P. (2023). Method Development and Validation for Tenofovir an Antiretroviral Drug in Plasma by LC-MS/MS Technique. *Journal of Research in Pharmacy*, 27(5): 1790-1798.
44. Priyadarshini, I., Akila, D. (2024). Development and Validation of an LC-MS/MS Method for the Determination of Tenofovir and Emtricitabine. *International Journal of Applied Pharmaceutics*, 16(2):116-123.
45. Kumar, L., Azeemuddin, M., Tulasi, T., Vijayakumar, M., Babu, U., Pai, K. (2023). A validated LC-MS/MS method for studying the pharmacokinetic interaction of Immusante® and antiviral drugs in rats. *Journal of Applied Pharmaceutical Science*, 13 (08): 125-131.
46. Challenger, E., Penchala, S., Hale, C., Fitzgerald, R., Walker, L., Reynolds, H., Chiong, J., Fletcher, T., Khoo, S., Else, L. (2023). Development and validation of an LC-MS/MS method for quantification of favipiravir in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*, 233:2-7.
47. Vemuri, D., Gundla, R., Raghupathi, J., Jeedimalla, N., Burle, G., Katari, N., Jonnalagadda, S. (2025). Development of LC/LCMS method for estimation of impurities in anti-HIV drug (Bictegravir) using Analytical Quality by Design (AQbD) approach. *Chinese Journal of Analytical Chemistry*, 53(2):1-10.
48. Rao, N., Adimulapu, A., Kumari, M., Rao, P., Abbineni, A., Thathapudi, D. (2024). Development and Validation of a Sensitive, Fast and Simple LC-MS/MS Method for the Quantitation of Favipiravir Pure and Tablet Dosage Forms. *Indian Journal of Pharmaceutical Education and Research*, 58(3):984-990.
49. Katthala, P., Gubbiyappa, S. (2024). An LC-MS Method Development and Validation for then Estimation of Ritonavir in Plasma Samples. *International Journal of Pharmaceutical Quality Assurance*, 15(1):443-449.
50. Kanjarla, N., Katta, B. (2025). Simultaneous Quantification of Doravirine, Lamivudine, and Tenofovir Disoproxil Fumarate in Human Plasma by UPLC-MS/MS: Method Development and Validation. *Turkish Journal of Pharmaceutical Sciences*, 22(3):191-206.
51. Mounika, V., Akila, D. (2025). Development and Validation of an LC-MS/MS Method for the Determination of Lamivudine, Tenofovir & Efavirenz in human plasma. *Journal of Neonatal Surgery*, 14 (19): 851-864.
52. West, R., Oberly, P., Riddler, S., Nolin, T., Devanathana, A. (2024). Development and validation of an ultra-high performance liquid chromatography-tandem mass spectrometry method to quantify antiretroviral drug concentrations in human plasma for therapeutic monitoring. *Journal of Pharmaceutical and Biomedical Analysis*, 240:1-22.
53. Paduri, A., Malothu, N., Kantlam, C., Guntupalli, C. (2025). LC-MS/MS Method for Simultaneous Estimation of Lopinavir and Ritonavir in Bulk and Dosage Form. *Indian Journal of Pharmaceutical Education and Research*, 59(1):292-299.
54. Tarcomnicu, I., Iacob, S., Anuta, V., Neaga, E., Otelea, D. (2025). Novel Micro-LC-MS/MS Method for the Quantification of Tenofovir and Its Active Metabolite Tenofovir-Diphosphate in Biological Matrices for Therapeutic Drug Monitoring. *Pharmaceutics*, 18(6):2-15.
55. Yang, X., Lin, W., Zhao, Y., Zhao, C., Zhou, F., Wang D., Yue, Z., Pei, L., Jia, M., Cao, B., Cao, L., Wang, H. (2025). Development and validation of a LC-MS/MS method for the simultaneous determination of simnotrelvir and ritonavir in human serum and bronchoalveolar lavage fluid. *BMC Chemistry*, 19(1):1-12.