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# Analytical Method Development and Validation Techniques for Antiviral Drugs: A Comprehensive Review

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**Abstract**
The global burden of viral infectious diseases — including HIV/AIDS, hepatitis B (HBV), hepatitis C (HCV), influenza, herpes simplex virus (HSV), and the recently emerged SARS-CoV-2 — has created an urgent need for efficacious antiviral pharmacotherapy and, equally, for robust analytical methods capable of monitoring these drugs with precision and reliability. Antiviral drugs are structurally diverse, often administered at low doses, and subject to complex metabolic and degradation pathways; these characteristics make reliable quantitative analysis both critical and technically challenging. The present review provides a comprehensive account of the major analytical methodologies that have been developed and validated for antiviral drug quantification in bulk, pharmaceutical formulations, and biological matrices. Techniques discussed include reversed-phase high-performance liquid chromatography (RP-HPLC), ultra-high-performance liquid chromatography (UHPLC), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), high-performance thin-layer chromatography (HPTLC), UV-visible spectrophotometry, and capillary zone electrophoresis (CZE). Special emphasis is given to method validation parameters as governed by the International Council for Harmonisation guidelines Q2(R2) and the newly complementary Q14, along with the FDA and EMA bioanalytical guidance documents (ICH M10). The review also addresses the growing importance of analytical quality by design (AQbD) in method development, the role of forced degradation studies in establishing stability-indicating capability, and the emerging paradigm of green analytical chemistry as evaluated through metrics such as GAPI, AGREE, NEMI, and the Analytical Eco-Scale. Challenges specific to simultaneous multi-analyte quantification, including the structural diversity of antivirals used in combination therapy for HCV and COVID-19, are discussed alongside current solutions reported in recent literature. This review consolidates evidence from peer-reviewed Scopus-indexed journals published between 2014 and 2026 and aims to serve as a reference framework for researchers undertaking analytical method development for antiviral pharmaceuticals.

***Keywords:*** *RP-HPLC; antiviral drugs; ICH Q2(R2); ICH Q14; method validation; LC-MS/MS; green analytical chemistry; AQbD; stability-indicating method; favipiravir; remdesivir; sofosbuvir; HIV; COVID-19*

## I. Introduction

Viral infections continue to constitute one of the foremost public health challenges globally, responsible for millions of deaths annually. The World Health Organization estimates that over 38 million people are living with HIV worldwide, while more than 290 million individuals are chronically infected with hepatitis B virus (HBV), and approximately 58 million carry chronic HCV infection. The SARS-CoV-2 pandemic that emerged in late 2019 underscored with devastating clarity the global vulnerability to novel viral pathogens. In response to these threats, the pharmaceutical industry has developed an extensive arsenal of antiviral agents spanning diverse mechanistic classes: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs), polymerase inhibitors, and direct-acting antivirals (DAAs) for HCV. More recently, RNA-dependent RNA polymerase (RdRp) inhibitors such as favipiravir, molnupiravir, and remdesivir have entered clinical practice for COVID-19 management.

The analytical characterization of these diverse molecules presents formidable challenges. Antiviral drugs are often structurally similar yet functionally distinct; some, such as combination HIV regimens containing tenofovir disoproxil fumarate (TDF), emtricitabine (FTC), and dolutegravir (DTG), must be simultaneously quantified in a single analytical run for quality control purposes. Others, such as sofosbuvir and its metabolite GS-331007, require bioanalytical methods sensitive enough to detect nanogram-per-

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milliliter plasma concentrations. Rigorous analytical method development and validation (AMDV) is therefore not only a scientific necessity but also a regulatory imperative underpinned by guidelines such as ICH Q2(R2), ICH Q14, ICH M10, FDA Guidance on Bioanalytical Method Validation (2018), and the EMA's bioanalytical guideline (2011).

Until the adoption of ICH Q2(R2) in November 2023, the field had operated under the framework of ICH Q2(R1) (2005) for nearly two decades. The revised guideline expands coverage to non-linear methods, multivariate calibration models, and biotechnology-derived products, while explicitly linking validation with the entire analytical procedure lifecycle — a concept further elaborated in ICH Q14. These regulatory evolutions demand that analysts and quality assurance professionals remain conversant with the updated technical requirements.

This review systematically evaluates analytical method development and validation techniques for antiviral drugs based on peer-reviewed literature published in high-impact, Scopus-indexed journals. The coverage spans spectrophotometric methods, chromatographic platforms (HPLC, UHPLC, HPTLC), hyphenated mass spectrometric approaches, and electrochemical sensors. The review additionally discusses AQbD as a scientifically sound and regulatory-aligned framework for method development, stability-indicating methods as tools for demonstrating specificity, and green analytical chemistry as an ethical imperative for sustainable pharmaceutical analysis.

## II. Overview and Classification of Antiviral Drugs Relevant to Analytical Method Development

Antiviral drugs act through diverse mechanisms, and their physicochemical characteristics directly govern the selection of analytical platforms. A thorough understanding of drug properties — including aqueous solubility, log P (partition coefficient), pKa, molecular weight, chromophore availability for UV detection, and thermal/photolytic stability — is a prerequisite for rational method development.

### 2.1 Nucleoside and Nucleotide Analogues

This class encompasses agents such as acyclovir, tenofovir, lamivudine, emtricitabine, ribavirin, favipiravir, sofosbuvir, and remdesivir. These molecules incorporate modified sugar or base moieties that mimic endogenous nucleosides and competitively inhibit viral polymerases. From an analytical standpoint, they are generally hydrophilic, possess moderate UV absorbance owing to pyrimidine or purine base chromophores, and demonstrate stability-related challenges due to their susceptibility to hydrolytic degradation. Favipiravir ($C_5H_4FN_3O_2$, MW 157.10 g/mol), for instance, absorbs at approximately 323 nm, which confers an analytical advantage in selectivity. Remdesivir ($C_{27}H_{35}N_6O_8P$, MW 602.58 g/mol), an adenosine phosphoramidate prodrug, exhibits greater lipophilicity and requires gradient HPLC conditions for adequate chromatographic resolution from its metabolite GS-441524.

### 2.2 Protease Inhibitors

HIV protease inhibitors including ritonavir, lopinavir, darunavir, saquinavir, and atazanavir are structurally complex peptidomimetics with high molecular weights and significant lipophilicity. These properties demand the use of methanol- or acetonitrile-rich organic mobile phases in reversed-phase HPLC, often with acidic pH modifiers. The co-formulation of lopinavir with ritonavir (as pharmacokinetic booster) — and more recently the Paxlovid combination of nirmatrelvir and ritonavir for COVID-19 — mandates simultaneous multi-drug assay methods. Ritonavir's complex UV spectrum, with absorption around 240 nm, requires careful selection of detection wavelength to avoid interference.

### 2.3 Non-Nucleoside Reverse Transcriptase Inhibitors

NNRTIs such as efavirenz, nevirapine, rilpivirine, and doravirine bind allosterically to HIV reverse transcriptase. These molecules generally possess aromatic ring systems conferring strong UV absorbance, facilitating spectrophotometric and HPLC-UV detection. Efavirenz, with lambda-max around 246 nm, has been widely characterized by RP-HPLC methods using C18 stationary phases and phosphate buffer-based mobile phases.

### 2.4 Direct-Acting Antivirals for HCV

The therapeutic revolution in HCV treatment has been driven by DAAs including sofosbuvir (NS5B polymerase inhibitor), daclatasvir, ledipasvir, velpatasvir (NS5A inhibitors), and simeprevir (NS3/4A protease inhibitor). These agents are typically administered as co-formulated fixed-dose combination products, creating analytical challenges of simultaneous multi-

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drug quantification. Their differing UV absorption characteristics — sofosbuvir at 261 nm, daclatasvir at 317 nm, and simeprevir at 380 nm — have been exploited in UV spectrophotometric multi-component methods while also demanding sophisticated gradient HPLC protocols.

### 2.5 COVID-19 Antivirals

The COVID-19 pandemic stimulated rapid development of antiviral agents including favipiravir, remdesivir, molnupiravir (an NHC prodrug), and the Paxlovid combination. These drugs represent a diverse structural landscape, and their rapid clinical deployment meant that validated analytical methods were initially sparse. The published literature since 2020 demonstrates a surge in HPLC-UV, LC-MS/MS, and spectrophotometric methods for these agents, many incorporating AQbD principles and green chemistry assessment.

## III. Analytical Method Development: Strategies and Considerations

Method development is the iterative process through which an analytical procedure is designed, optimized, and shown to be fit for its intended purpose before formal validation. ICH Q14, adopted in November 2023 as a companion guideline to Q2(R2), represents the first harmonized international guidance dedicated specifically to analytical procedure development. Q14 introduces the concept of an Analytical Target Profile (ATP) — a prospective summary of the analytical procedure's quality characteristics — and promotes a risk-based, science-driven approach aligned with Quality by Design principles.

### 3.1 Reversed-Phase High-Performance Liquid Chromatography

RP-HPLC remains the workhorse of pharmaceutical analysis due to its versatility, sensitivity, and broad applicability across drug classes of varying polarity. The method development process for antiviral drugs typically involves selection of the stationary phase, optimization of mobile phase composition, determination of pH, flow rate, column temperature, and detection wavelength.

C18-bonded silica columns (octadecylsilyl; ODS) are the predominant choice, with column dimensions of 150–250 mm length, 4.6 mm internal diameter, and 3–5 µm particle sizes commonly reported. For simultaneous analysis of multiple antivirals with diverse physicochemical properties, gradient elution — typically employing binary combinations of aqueous phosphate or formate buffers with acetonitrile or methanol — is preferred over isocratic systems. The landmark study by Nassar et al. (2025) demonstrated that an isocratic method employing methanol:water (70:30 v/v, pH 3.0 adjusted with 0.1% ortho-phosphoric acid) on a Hypersil BDS C18 column (150×4.6 mm, 5 µm) at 1 mL/min and UV detection at 230 nm could simultaneously resolve five COVID-19 antivirals — favipiravir, molnupiravir, nirmatrelvir, remdesivir, and ritonavir — with retention times of 1.23, 1.79, 2.47, 2.86, and 4.34 minutes respectively, and linearity from 10 to 50 µg/mL ($r^2 \geq 0.9997$ for all analytes).

A parallel approach by Gamal and colleagues employed a Box-Behnken Design (BBD)-assisted RP-HPLC method for four repurposed COVID-19 antivirals (favipiravir, sofosbuvir, ledipasvir, remdesivir). The BBD facilitated systematic exploration of critical method parameters — mobile phase composition, pH, and flow rate — within an experimental design framework, enabling identification of the design space and establishment of a robust analytical procedure with minimal experimentation. This QbD-embedded approach yielded an isocratic method with satisfactory system suitability, greenness, and practicality assessments.

For HCV antivirals, Elshobaki et al. developed a versatile HPLC-DAD method for the simultaneous determination of daclatasvir, ledipasvir, sofosbuvir, and ribavirin using a Thermohypersil BDS-C8 column with a phosphate buffer (pH 7.5)/methanol gradient at 1.5 mL/min, achieving linearity across 0.5–500 µg/mL depending on the analyte with LOD values of 0.10–0.66 µg/mL. The method demonstrated specificity by chromatographic separation from seven process-related sofosbuvir impurities and its major metabolite GS-331007, making it a valuable stability-indicating tool.

### 3.2 Ultra-High-Performance Liquid Chromatography and Hyphenated Techniques

UHPLC systems operate at substantially higher pressures (above 600 bar) and employ sub-2 µm particle-size columns, enabling significantly faster analyses, sharper chromatographic peaks, and improved sensitivity compared with conventional HPLC. For antiviral drugs requiring high-throughput bioanalytical quantification — such as in pharmacokinetic studies — UHPLC-MS/MS has become the gold standard. Harahap et al. demonstrated the utility of UHPLC-MS/MS for

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simultaneous analysis of favipiravir and remdesivir in volumetric absorptive microsampling (VAMS), a minimally invasive blood-sampling technique, achieving sensitive and precise quantification in a matrix that substantially simplifies sample collection logistics.

Habler and colleagues reported a two-dimensional isotope-dilution LC-MS/MS method capable of simultaneously quantifying seven repurposed COVID-19 drugs — remdesivir (and its metabolite GS-441524), chloroquine, hydroxychloroquine, lopinavir, ritonavir, favipiravir, and azithromycin — in human serum. The use of isotope-labeled internal standards and two-dimensional chromatographic separation addressed the matrix complexity inherent in clinical serum samples, yielding sub-µg/mL detection limits for all analytes.

### 3.3 UV-Visible Spectrophotometry

UV-visible spectrophotometry, while inherently less specific than chromatographic techniques, offers practical advantages of simplicity, low cost, rapid analysis, and accessibility, making it particularly suitable for quality control applications in resource-limited settings. Single-component spectrophotometric methods exploit the characteristic lambda-max of each analyte; sofosbuvir at 261 nm in ethanol or methanol systems, daclatasvir at 317 nm in methanol, and simeprevir at 380 nm.

For multi-component mixtures with overlapping spectra, advanced mathematical techniques have been applied. Derayea et al. developed three simultaneous spectrophotometric methods for sofosbuvir and simeprevir — the iso-absorptive point method, ratio subtraction, and dual wavelength approaches — validated in accordance with ICH guidelines with accuracy between 97 and 103%. Similarly, Attimarad and colleagues employed partial least squares (PLS) and simultaneous equations to resolve the spectral overlap between velpatasvir (λmax 255 nm) and sofosbuvir (λmax 244.8 nm) in their combined pharmaceutical dosage form.

### 3.4 High-Performance Thin-Layer Chromatography

HPTLC offers a complementary platform to HPLC, enabling simultaneous multi-sample analysis, parallel processing, and visual documentation of separations. For antiviral drugs, HPTLC methods have been developed primarily for tablet dosage form assays where high sample throughput is required. Pre-coated silica gel 60 F aluminum sheets are commonly employed, with mobile phases typically comprising combinations of toluene, ethyl acetate, methanol, and ammonia in varying proportions. The developed spots are quantified using densitometric scanning at the analyte's absorption maximum. HPTLC methods for anti-HCV combinations including sofosbuvir-velpatasvir and HIV regimens have been published and validated per ICH Q2(R1) guidelines.

### 3.5 LC-MS/MS for Bioanalytical Applications

Liquid chromatography–tandem mass spectrometry represents the pinnacle of sensitivity and selectivity for antiviral drug quantification in biological matrices. The technique exploits multiple reaction monitoring (MRM) in triple-quadrupole MS configurations, where precursor ions are fragmented to produce characteristic product ions, providing analyte-specific identification that minimizes matrix interference. Sample preparation strategies, including protein precipitation (PPT), liquid-liquid extraction (LLE), and solid-phase extraction (SPE), are optimized to achieve acceptable matrix factor values and analyte recovery as mandated by ICH M10 (2022).

For HIV treatment monitoring, LC-MS/MS methods have been described for simultaneous quantification of tenofovir, emtricitabine, dolutegravir, cabotegravir, and rilpivirine in dried blood spots (DBS) and VAMS, which are increasingly recognized as patient-friendly sampling modalities for therapeutic drug monitoring programs in HIV clinic settings.

### 3.6 Electrochemical and Other Emerging Techniques

Electrochemical methods including voltammetry and amperometry have been explored for antiviral drug analysis, particularly for favipiravir and remdesivir, using glassy carbon electrodes (GCE) in anionic surfactant media, and modified screen-printed electrodes for molnupiravir. While these methods offer advantages of simplicity and low reagent consumption, their selectivity in complex matrices remains a limitation compared with chromatographic techniques, and they are generally applied as complementary tools rather than primary methods in pharmaceutical quality control.

## IV. Analytical Method Validation: ICH Q2(R2) Framework and Key Parameters

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Method validation provides documented evidence that an analytical procedure consistently produces results that are accurate, precise, specific, and reliable within the intended range of application. The ICH Q2(R2) guideline, adopted in November 2023 alongside ICH Q14, represents a landmark update to the international harmonized framework for analytical procedure validation.

### 4.1 Evolution from ICH Q2(R1) to ICH Q2(R2)

The original ICH Q2(R1) (2005) established the foundational validation characteristics — specificity, linearity, range, accuracy, precision, LOD, LOQ, and robustness — and has guided pharmaceutical analysis for nearly two decades. A gap analysis recently published in the Journal of Pharmaceutical Sciences (2025) identified 56 specific omissions, expansions, and additions between Q2(R1) and Q2(R2), providing a structured toolkit for organizations updating their validation systems. Key changes in Q2(R2) include: explicit coverage of non-linear calibration models relevant to immunoassays and potency assays; expanded guidance for multivariate analytical procedures; terminology updates (e.g., 'trueness' in place of 'accuracy' for the mean recovery parameter, while 'accuracy' now encompasses both trueness and precision); and alignment with the life-cycle concept introduced in Q14 through the Analytical Control Strategy (ACS). The revised guideline also strengthens the link with ICH Q9 (Quality Risk Management) and ICH Q11 (Development and Manufacture of Drug Substances).

### 4.2 Validation Parameters

Table 1 summarizes the key validation parameters mandated by ICH Q2(R2), their definitions, and representative acceptance criteria applicable to assay methods for antiviral pharmaceuticals.

**Table 1: Summary of ICH Q2(R2) Validation Parameters and Acceptance Criteria for Antiviral Drug Assay Methods**

| Validation Parameter | Definition | Acceptance Criterion |
|---|---|---|
| Specificity/Selectivity | Ability to assess the analyte unequivocally in the presence of components likely to be present (impurities, degradants, matrix) | Resolution ≥ 2.0; no interference peaks at analyte retention time |
| Linearity | Ability to obtain test results that are directly proportional to the concentration of the analyte | $r^2 \geq 0.999$; slope, intercept, and residuals acceptable |
| Accuracy (Trueness) | Closeness of test results to the true value; assessed via recovery studies | % Recovery 98–102% (assay); 80–120% (bioanalytical) |
| Precision | Degree of agreement among independent results | % RSD ≤ 2% (assay); ≤ |

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|---|---|---|
| | under prescribed conditions: repeatability, intermediate precision, reproducibility | 15% (bioanalytical); ≤ 20% at LLOQ |
| Limit of Detection (LOD) | Lowest amount of analyte that can be detected but not necessarily quantitated | Signal-to-noise ratio ≥ 3:1 or based on standard deviation of response |
| Limit of Quantitation (LOQ) | Lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy | Signal-to-noise ratio ≥ 10:1 or % RSD ≤ 10%; % recovery 80–120% |
| Range | Interval between upper and lower concentration of analyte with demonstrated suitable levels of precision, accuracy, and linearity | Typically 80–120% of nominal concentration; LOQ to 120% for impurities |
| Robustness | Measure of capacity to remain unaffected by small, deliberate variations in method parameters | Evaluated via Plackett-Burman or full factorial design; results within ± 2% RSD |

#### 4.2.1 Specificity and Selectivity

Specificity is arguably the most critical validation parameter, as it ensures that the method measures only the intended analyte. For antiviral drugs, this requires demonstrating that the method is unaffected by known degradation products, process-related impurities, metabolites, and formulation excipients. For HPLC methods, specificity is assessed by injecting forced degradation samples and demonstrating baseline separation from the analyte peak; peak purity assessment using photodiode array (PDA/DAD) detection provides additional assurance. Nagulancha et al. confirmed the specificity of their stability-indicating HPLC method for baloxavir marboxil by chromatographic resolution from five process-related impurities and identification of an unknown oxidative degradant by LC-MS, demonstrating mass spectral characterization as an integral component of specificity evaluation.

#### 4.2.2 Linearity and Range

Linearity is demonstrated by preparing a minimum of five calibration standards spanning the intended concentration range and assessing the goodness of fit of the response–concentration relationship. For antiviral assay methods, the calibration range typically extends from 80% to 120% of the nominal analytical concentration. The correlation coefficient ($r^2$) should be ≥ 0.999 for robust methods, as demonstrated in the multi-antiviral HPLC study by Nassar et al. ($r^2 \geq 0.9997$) and the HCV DAA method by Elshobaki et al. Residual analysis — plotting deviations of actual responses from the fitted line — provides a sensitive indicator of non-linearity that should be included in validation reports per Q2(R2) recommendations.

#### 4.2.3 Accuracy and Precision

Accuracy (trueness) for pharmaceutical assay methods is assessed by analyzing samples at three concentration levels (typically 80%, 100%, and 120% of nominal) in triplicate, with percentage recoveries expected within 98–102%. Precision encompasses repeatability (intra-day), intermediate precision (inter-day, typically across two analysts and two days), and reproducibility (between laboratories). For the five-drug COVID-19 HPLC method, Nassar et al. reported percentage RSD values below 1.1% for all analytes across both repeatability and intermediate precision assessments, with trueness values between 99.59% and 100.08%. These exemplary results underscore the achievability of stringent precision criteria when method conditions are thoroughly optimized before validation.

#### 4.2.4 Limit of Detection and Limit of Quantitation

LOD and LOQ may be determined by visual evaluation of signal-to-noise ratios (3:1 and 10:1 respectively), by the standard deviation and slope method as specified in ICH Q2(R2), or from calibration curve statistics. For impurity determination methods, LOQ must be ≤ 0.05% of the drug substance concentration for ICH Q3A/Q3B compliant analysis. The multi-drug COVID-19 HPLC method reported LOD values of 0.415–0.946 µg/mL and LOQ values of 1.260–2.868 µg/mL — values well suited for pharmaceutical quality control

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where typical analytical concentrations are in the 10–50 µg/mL range.

#### 4.2.5 Robustness

Robustness evaluation — typically by deliberate variation of method parameters such as mobile phase pH (± 0.2 units), organic modifier percentage (± 2%), flow rate (± 0.1 mL/min), and column oven temperature (± 5°C) — documents the procedure's reliability under small perturbations likely encountered during routine use. Experimental designs such as the Plackett-Burman design or fractional factorial designs efficiently screen multiple factors simultaneously. Q2(R2) links robustness evaluation to the establishment of the Analytical Control Strategy, requiring that parameters found to be critical during robustness testing be controlled within defined ranges in the validated method.

## V. Stability-Indicating Method Development for Antiviral Drugs

A stability-indicating method (SIM) is a validated analytical procedure with demonstrated capability to distinguish, quantify, and resolve the drug substance or drug product from its degradation products generated under stress conditions, such that a change in drug content over time can be accurately measured. The FDA defines a SIM as a validated quantitative analytical procedure that accurately measures the active ingredient without interference from degradation products, process impurities, or formulation excipients.

Forced degradation studies — conducted as per ICH Q1A(R2) guidelines — subject the drug to acidic hydrolysis (typically 0.1 N HCl at 60°C), alkaline hydrolysis (0.1 N NaOH at 60°C), oxidative degradation (3% H O at room temperature), photolytic degradation (ICH Q1B conditions: UV exposure ≥ 200 Wh/m², visible light ≥ 1.2 million lux-hours), and thermal stress (usually 60°C or above). These conditions are designed to generate degradation products at levels that permit method characterization without completely destroying the drug. The percentage degradation under each stress condition is reported, and the chromatographic method is confirmed as stability-indicating if it resolves degradation products from the parent compound, as confirmed by peak purity analysis.

Marzouk et al. published a comprehensive stability-indicating HPLC-DAD method for favipiravir validated for COVID-19 pharmaceutical formulations. The authors subjected favipiravir to all five ICH Q1A stress conditions and demonstrated that the HPLC-DAD method resolved the parent drug from all generated degradation products, supported by mass spectrometric characterization of the degradants. The method was further applied to in-vitro dissolution profiling — an important application that requires a stability-indicating method to distinguish dissolved drug from degradation products in the dissolution medium.

Similarly, the HPLC method for baloxavir marboxil by Nagulancha et al. incorporated identification of an unknown impurity formed under oxidative stress using LC-MS fragmentation data, exemplifying how modern stability-indicating methods integrate structural characterization capabilities. For HIV antiretroviral combinations, stability-indicating methods capable of simultaneously quantifying tenofovir, emtricitabine, and efavirenz while resolving known degradation products have been reported by multiple groups using C18 columns with acetonitrile/phosphate buffer gradients.

## VI. Analytical Quality by Design in Antiviral Method Development

Analytical Quality by Design (AQbD) is a systematic approach to analytical method development that begins with predefined objectives and emphasizes understanding and control of method parameters through the application of risk assessment, design of experiments (DoE), and statistical modeling. The AQbD framework, elaborated in ICH Q14, represents the analytical counterpart of pharmaceutical QbD principles articulated in ICH Q8(R2) and Q9, Q10, Q11, and Q12.

The core workflow of AQbD comprises: (1) definition of the Analytical Target Profile (ATP) articulating the method's intended purpose and performance requirements; (2) identification of Critical Quality Attributes (CQAs) of the analytical procedure, such as specificity, accuracy, and precision; (3) risk assessment to identify Critical Method Attributes (CMAs) and Critical Method Parameters (CMPs) — those factors most likely to affect CQAs; (4) DoE-based screening and optimization experiments to characterize the method operable design region (MODR) or analytical design space; and (5) implementation of an Analytical Control Strategy to ensure the method remains in a state of control throughout its lifecycle.

In the context of antiviral drug analysis, Gamal and colleagues employed a Box-Behnken Design with three independent variables — organic modifier percentage, buffer pH, and flow rate — to

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simultaneously optimize the RP-HPLC method for favipiravir, sofosbuvir, ledipasvir, and remdesivir. The BBD generated polynomial response surface models for resolution, peak asymmetry, and theoretical plate count, enabling identification of optimal conditions within the experimental design space. This approach yielded a robust isocratic method validated across ICH parameters and assessed for greenness using multiple environmental evaluation tools. The AQbD approach is valued not only for producing robust methods but also for providing regulatory bodies with enhanced process understanding documentation, which supports more flexible regulatory filings under enhanced approaches described in Q14.

For nevirapine, an NNRTI for HIV, an AQbD-assisted stability-indicating chromatographic method was developed using ACE C18 column conditions optimized through a full factorial design. Guard bands were established from measurement uncertainty estimates, leading to specification tightening for the nevirapine assay from a conventional 90–110% to 92.1–107.9%, demonstrating how AQbD provides quantitative justification for method specifications directly linked to patient risk. The Analytical QbD approach has also been reported for capillary electrophoretic methods and spectrophotometric procedures, confirming its applicability across analytical platforms.

## VII. Green Analytical Chemistry and Sustainability in Antiviral Drug Analysis

The 12 principles of Green Analytical Chemistry (GAC), proposed by Gałuszka et al. as the analytical counterpart to Anastas and Warner's Green Chemistry principles, advocate for minimizing the quantity and toxicity of reagents and solvents, reducing waste generation, preferring in-situ analysis, minimizing energy consumption, and employing safer, more degradable materials. In pharmaceutical analysis, where organic solvents such as acetonitrile and methanol are consumed in large volumes during routine HPLC analysis, the adoption of GAC principles has both environmental and economic significance.

Several standardized tools have been developed to objectively assess the greenness of analytical methods. The National Environmental Methods Index (NEMI), one of the earliest tools, uses a binary yes/no pictogram format to indicate whether the method meets criteria related to hazardous reagents, waste generation, corrosivity, and overall environmental impact. The Analytical Eco-Scale (ESA), introduced by Van Aken et al., assigns penalty points across multiple greenness criteria — reagent quantity and hazard, waste production, energy consumption, and operator safety — with scores above 75 indicating excellent greenness. The Green Analytical Procedure Index (GAPI), a semi-quantitative tool using a three-color (green/yellow/red) pictogram of 15 pentagons, evaluates each stage of the analytical procedure from sample collection through waste treatment, providing a visual overview of environmental impact.

The most recent tool, AGREE (Analytical GREEnness), uses a circular pictogram based on all 12 GAC principles, generating a numerical score between 0 and 1 where values approaching 1 indicate maximal greenness. For sample preparation assessment, the complementary AGREEprep and ComplexGAPI tools have been developed. A 2024 modification of GAPI, termed MoGAPI, provides improved granularity in assessment. Kumar and Pasha demonstrated, for example, that an HPLC method for the HIV PIs ritonavir and ombitasvir employing an eco-friendly methanol-based mobile phase — assessed using AGREE, GAPI, and AMGS (Analytical Method Greenness Score) tools — achieved acceptable greenness scores compared with acetonitrile-based methods, validating the environmental benefit of solvent substitution.

For COVID-19 antivirals, Nassar et al. included comprehensive greenness evaluation of their five-drug RP-HPLC method using AGREE, AGREEprep, and MoGAPI, alongside practicality assessments by BAGI (Blue Applicability Grade Index) and CACI (Cost Analytical Chemistry Index), establishing a holistic analytical sustainability framework. This approach, integrating greenness, practicality, and cost alongside traditional ICH validation parameters, represents the emerging standard for publishable analytical method development manuscripts targeting high-quality international journals.

## VIII. Bioanalytical Method Validation for Antiviral Drugs in Biological Matrices

Bioanalytical methods quantify drug concentrations in biological fluids — plasma, serum, urine, cerebrospinal fluid, dried blood spots — for pharmacokinetic, pharmacodynamic, bioequivalence, and therapeutic drug monitoring studies. These methods are governed by regulatory frameworks distinct from those for pharmaceutical formulation analysis. The ICH M10 Bioanalytical

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Method Validation guideline, adopted in May 2022, harmonizes the requirements of the FDA Guidance for Industry (2018) and the EMA Guideline on Bioanalytical Method Validation (2011), standardizing calibration curve requirements, quality control (QC) sample acceptance criteria (within ±15% of nominal; ±20% at LLOQ), stability evaluations (freeze-thaw, bench-top, long-term, and stock solution stability), matrix effect assessment, and incurred sample reanalysis (ISR) for clinical samples.

For antiviral bioanalysis, LC-MS/MS in MRM mode is the method of choice due to the demand for high sensitivity (LLOQ values in the nanogram-per-milliliter range), selectivity in biological matrices containing endogenous interferents, and broad dynamic range. Sample preparation typically involves protein precipitation with acetonitrile, LLE with ethyl acetate or methyl tert-butyl ether, or SPE using C18 or mixed-mode cation exchange cartridges. The inclusion of stable isotope-labeled internal standards (SIL-IS) — isotopologues of the analyte bearing $^{13}C$, $^{1}N$, or $^{2}H$ labels — corrects for inter-sample variability in extraction efficiency and ionization suppression, and is considered best practice.

For favipiravir, Hailat et al. developed and validated an LC-MS/MS method for quantification in spiked human plasma, achieving an LLOQ of 0.2 µg/mL with linearity from 0.2 to 8.0 µg/mL using weighted least-squares regression (1/x weighting factor) to address heteroscedasticity at lower concentrations. The method met ICH M10 standard curve requirements and QC sample precision and accuracy criteria, and was applied to a pharmacokinetic profile study in simulated COVID-19 patient samples.

The emerging use of volumetric absorptive microsampling (VAMS) and dried blood spot (DBS) sampling for antiviral therapeutic drug monitoring has stimulated the development of UHPLC-MS/MS methods optimized for these microvolume matrices. Harahap et al. validated a VAMS-UHPLC-MS/MS method for favipiravir and remdesivir, demonstrating suitability for outpatient monitoring without the requirement for venous blood collection, an important practical advantage for COVID-19 patients in ambulatory care settings.

## IX. Challenges and Advances in Simultaneous Multi-Analyte Antiviral Drug Analysis

The clinical management of HIV, HCV, and COVID-19 routinely involves co-administration of multiple antiviral agents with distinct physicochemical properties, which presents significant analytical challenges. Simultaneous chromatographic resolution of structurally diverse analytes requires careful optimization of mobile phase selectivity, gradient profiles, and column chemistry.

For HIV combination therapy (e.g., TDF + FTC + DTG), the three components differ substantially in molecular weight (328, 247, and 419 g/mol respectively), log P values, and UV absorption maxima. Stability-indicating methods for five-drug antiretroviral combinations have been reported using C18 columns with acetonitrile/phosphate buffer gradient systems, demonstrating that well-designed QbD-assisted methods can achieve baseline resolution of all components from each other and from their primary degradation products.

For HCV combinations, the structural differences between NS5B inhibitors (sofosbuvir), NS5A inhibitors (ledipasvir, daclatasvir, velpatasvir), and NS3/4A protease inhibitors (simeprevir) — which differ in molecular weight from 529 g/mol (sofosbuvir) to 750 g/mol (ledipasvir) and log P values from −1.6 to >4 — make simultaneous HPLC analysis technically demanding. The versatile HPLC-DAD method by Elshobaki et al. demonstrated that gradient elution from 25% to 80% methanol over 15 minutes on a BDS-C8 column could achieve simultaneous quantification of four HCV antivirals with resolution from seven process-related impurities, representing a technically robust solution applicable to pharmaceutical quality control laboratories.

For COVID-19 multidruganalysis, the five-drug method by Nassar et al. used a green isocratic mobile phase to resolve agents spanning log P values from −0.49 (favipiravir) to 3.23 (ritonavir) within 5 minutes, demonstrating that careful pH adjustment of the aqueous mobile phase component can modulate selectivity sufficiently to enable isocratic resolution of diverse analytes. However, the authors caution that this approach may require adaptation for matrices containing biological interferents, where gradient or two-dimensional HPLC configurations offer enhanced robustness.

Table 2 below summarizes representative analytical methods from recent peer-reviewed literature, illustrating the diversity of approaches applied to antiviral drug quantification.

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**Table 2: Representative Peer-Reviewed Analytical Methods for Antiviral Drugs (Selected from Scopus-Indexed Literature, 2019–2026)**

| Drug(s) | Technique | Column / Conditions | Linearity Range | LOD / LOQ | Key Reference |
|---|---|---|---|---|---|
| Favipiravir, Molnupiravir, Nirmatrelvir, Remdesivir, Ritonavir | RP-HPLC-UV | Hypersil BDS C18 (150×4.6 mm, 5 µm); MeOH:H2O (70:30, pH 3.0); 1 mL/min; 230 nm | 10–50 µg/mL; $r^2 \geq 0.9997$ | LOD: 0.415–0.946 µg/mL; LOQ: 1.260–2.868 µg/mL | Nassar et al., Sci Rep 2025 |
| Favipiravir, Sofosbuvir, Ledipasvir, Remdesivir | RP-HPLC (Box-Behnken QbD) | C18 column; isocratic; validated by BBD-DoE | Per ICH; $r^2 > 0.999$ | Reported per ICH | Gamal et al., Sci Rep 2026 |
| Favipiravir | HPLC-DAD (Stability-Indicating) | C18; gradient elution; DAD detection; forced degradation under acid, base, oxidation, photolytic stress | ICH-compliant range | Acceptable LOD/LOQ | Marzouk et al., J Pharm Biomed Anal 2022 |
| Baloxavir Marboxil (+ 5 impurities) | HPLC & LC-MS/MS | C18 (100×4.6 mm, 5 µm); TFA/ACN gradient; 260 nm; 57°C | 50–150% for assay; LOQ–120% for impurities; $r^2 > 0.999$ | LOD/LOQ per ICH | Nagulancha et al., Biomed Chromatogr 2023 |
| Daclatasvir, Ledipasvir, Sofosbuvir, Ribavirin | HPLC-DAD | Thermohypersil BDS-C8 (250×4.6 mm, 5 µm); phosphate buffer pH 7.5/MeOH gradient; 1.5 mL/min | 0.5–500 µg/mL; $r^2 > 0.999$ | LOD: 0.10–0.66 µg/mL | Elshobaki et al., J Pharm Biomed Anal 2019 |
| Sofosbuvir, Simeprevir | UV Spectrophotometry (Multi-method: ratio subtraction, dual wavelength, iso-absorptive point) | Methanol/water solvent systems; λmax 261 nm (SOF) and 380 nm (SMV) | Per ICH; $r^2 > 0.999$ | Acceptable | Derayea et al., BMC Chem 2023 |
| Ritonavir, Ombitasvir | Green RP-HPLC | C18; eco-friendly mobile phase; evaluated by AGREE, GAPI, AMGS tools | ICH-compliant | Acceptable LOD/LOQ | Kumar & Pasha, Int J Pharm Investig 2024 |
| Remdesivir (plus GS-441524), Chloroquine, HCQ, Lopinavir, Ritonavir, Favipiravir, | 2D Isotope-Dilution LC-MS/MS | Two-dimensional LC coupled with isotope dilution MS; serum matrix | Multi-analyte quantification in serum | Sub-µg/mL LOD for all analytes | Habler et al., J Pharm Biomed Anal 2021 |

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| | | | | | |
|---|---|---|---|---|---|
| Azithromycin | | | | | |

## X.

## XI. Regulatory Perspectives and Compliance Considerations

The regulatory framework governing analytical methods for antiviral pharmaceuticals is anchored in ICH guidelines, regional pharmacopoeial requirements (USP, EP, IP), and agency-specific guidance. The ICH Q2(R2)/Q14 package, legally effective in the European Union from June 2024 and progressively adopted by FDA and other ICH member authorities, requires that analytical procedure development and validation be approached as a lifecycle activity. This lifecycle perspective encompasses three stages: procedure design and development (ATP and design space definition; Q14), procedure qualification (validation; Q2(R2)), and continued procedure performance verification (monitoring; Q14/Q10).

For generic antiviral drug manufacturers seeking regulatory approval under abbreviated new drug applications (ANDAs), analytical method validation data form a core component of the chemistry, manufacturing, and controls (CMC) section. Bioequivalence studies for oral antiviral formulations additionally require validated bioanalytical methods compliant with ICH M10, FDA 2018 guidance, or EMA 2011 guidance. Method transfers between development and quality control laboratories, or between sites in multinational manufacturing networks, must demonstrate equivalence through comparative statistical testing as per USP Chapter <1224> or similar frameworks.

Pharmacopoeial standards for well-established antiviral agents — including acyclovir, efavirenz, tenofovir, and lamivudine — provide reference HPLC methods with system suitability criteria that serve as minimum technical benchmarks. Novel antiviral agents approved under emergency use authorization (EUA), such as remdesivir and molnupiravir, may initially lack pharmacopoeial standards, placing the onus on manufacturers to develop and validate methods to ICH specifications and disclose these in their regulatory filings. The FDA's guidance for COVID-19 EUA products has emphasized the importance of robust analytical control of active pharmaceutical ingredients to ensure product quality and supply chain integrity.

## XII. Future Perspectives and Emerging Trends

The field of analytical method development for antiviral drugs is evolving rapidly under the convergence of several technological and regulatory trends.

Point-of-care (POC) analytical tools, including paper-based microfluidic devices and biosensors functionalized with molecularly imprinted polymers (MIPs) or aptamers, are being explored for real-time therapeutic drug monitoring of antivirals in low-resource settings. These technologies could transform how antiviral drugs are monitored in HIV-endemic regions where laboratory infrastructure is limited, providing near-instant pharmacokinetic data to guide dosing decisions.

Artificial intelligence (AI) and machine learning (ML) are increasingly being applied to method development, offering the potential for automated prediction of optimal chromatographic conditions based on molecular descriptor inputs, reducing the experimental burden of method development. Deep learning models trained on large chromatographic datasets may soon predict retention times, resolution, and peak shapes for novel antiviral molecules without the need for exhaustive DoE experimentation.

The green analytical chemistry movement will continue to drive solvent substitution research, identifying more sustainable alternatives to conventional acetonitrile-based mobile phases. Subcritical water chromatography, supercritical fluid chromatography (SFC) with CO as the primary mobile phase, and micellar liquid chromatography (MLC) using surfactant-containing aqueous systems are all being explored as environmentally benign alternatives applicable to antiviral drug analysis.

Miniaturization of sample preparation through microextraction technologies — including dispersive liquid-liquid microextraction (DLLME), magnetic solid-phase extraction (MSPE), and on-line SPE-UHPLC — will continue to reduce the volumes of biological samples and organic solvents required for bioanalytical methods, improving both patient convenience and laboratory sustainability.

Finally, the continued expansion of the pharmacopoeial coverage for newer antiviral agents and the harmonization of ICH guidelines across major regulatory agencies will provide analytical scientists with clearer and more uniform frameworks for method validation, reducing

duplicative development efforts and facilitating faster access to quality-assured antiviral medicines globally.

## XIII. Conclusion

The development and validation of analytical methods for antiviral drugs is a scientifically demanding, multifaceted, and clinically consequential discipline. The structural and physicochemical diversity of antiviral agents — ranging from small hydrophilic nucleoside analogues to large lipophilic peptidomimetic protease inhibitors — necessitates the application of diverse analytical platforms, from simple UV spectrophotometry to sophisticated two-dimensional LC-MS/MS. The regulatory environment governing these methods has been significantly updated with the adoption of ICH Q2(R2) and ICH Q14 in 2023, which collectively promote a lifecycle-oriented, QbD-embedded approach to analytical procedure development and validation that integrates risk management, experimental design, and continued performance monitoring.

The AQbD framework, by enabling rational and systematic exploration of method design spaces, produces analytical procedures that are inherently more robust and scientifically defensible than those developed by one-factor-at-a-time (OFAT) approaches. The mandatory incorporation of green analytical chemistry assessments — through tools including GAPI, AGREE, NEMI, and the Eco-Scale — is transforming the publication and regulatory standards for new methods, aligning pharmaceutical analysis with broader sustainability goals. Bioanalytical methods for antiviral drugs in plasma and alternative microsampling matrices have matured substantially, with LC-MS/MS approaches validated per ICH M10 providing the sensitivity, selectivity, and throughput demanded by modern pharmacokinetic and therapeutic drug monitoring applications.

Taken together, the literature reviewed here underscores that the quality of analytical science supporting antiviral pharmacotherapy has advanced substantially, particularly in the wake of the COVID-19 pandemic, which catalyzed an unprecedented volume of method development research. The ongoing evolution of analytical technology, regulatory guidance, and sustainability standards will continue to refine and elevate this field, ensuring that the pharmaceutical quality of antiviral drugs is safeguarded with the scientific rigor that patient safety demands.

**Conflict of Interest**

The authors declare no conflict of interest.

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