IJARSCT
International Journal of Advanced Research in Science, Communication and Technology
International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429
Volume 6, Issue 3, June 2026
Impact Factor: 8.2

Development and Validation of Analytical Methods for Antiviral Drugs

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Abstract: *Antiviral drugs such as acyclovir play a critical role in the management of herpesvirus infections, and robust analytical methods are essential to ensure their quality, stability, and efficacy in pharmaceutical formulations. This project aims to develop and validate a selective, accurate, and reproducible RP-HPLC method, along with a complementary UV-spectrophotometric method, for the determination of acyclovir in bulk and tablet dosage form, in accordance with ICH Q2(R1) guidelines. Acyclovir was analyzed using a C18 reverse-phase column with a mobile phase composed of 0.05 M potassium phosphate buffer (pH 3.0) and acetonitrile in the ratio 80:20 v/v, at a flow rate of 1.0 mL/min, with UV detection at 254 nm, whereas the UV method was optimized at the same wavelength in aqueous medium.*

Method development was carried out by systematically varying mobile-phase composition, pH, flow rate, column temperature, and injection volume to achieve baseline resolution and acceptable peak symmetry. The finalized chromatographic conditions were then subjected to a full validation protocol, including evaluation of linearity, accuracy, precision, specificity, robustness, ruggedness, LOD, LOQ, and system suitability. The method demonstrated excellent linearity over the proposed concentration range (20–100 µg/mL for HPLC; 4–20 µg/mL for UV), with correlation coefficients r2≥0.999r2≥0.999, and low %RSD values for intra-day and inter-day precision, indicating high reproducibility. Accuracy was confirmed by recovery studies (98–102%) and statistical tests (t-test, 95% CI).

The validated RP-HPLC method was successfully applied to the assay of three commercial batches of acyclovir tablets, and the results were comparable to those obtained from the UV-spectrophotometric method. The present method proved to be simple, selective, and cost-effective, with good sensitivity and suitability for routine quality control analysis of acyclovir in pharmaceutical formulations. The work highlights the importance of ICH-compliant analytical method development and validation in pharmaceutical sciences..

Keywords: *Antiviral drugs*

I. INTRODUCTION

DOI: 10.48175/IJARSCT-36441

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1.1 Introduction to antiviral drug

Antiviral drugs are chemotherapeutic agents that inhibit viral replication or prevent virus-host cell interaction without causing significant toxicity to the host. Unlike antibiotics, antivirals usually target specific stages of the viral life cycle, such as adsorption, penetration, uncoating, nucleic acid synthesis, or assembly and release. Acyclovir is a nucleoside analogue used against herpes simplex virus (HSV-1, HSV-2) and varicella-zoster virus (VZV), and its availability in oral tablets, topical creams, and intravenous formulations necessitates reliable analytical control.[1]

Antiviral drugs are pharmaceutical agents used for the prevention and treatment of viral infections caused by viruses such as HIV, hepatitis viruses, influenza virus, herpes simplex virus, and coronaviruses. These drugs act by inhibiting viral replication and reducing the severity and spread of infections. Due to the increasing prevalence of viral diseases worldwide, antiviral medications have gained significant importance in modern healthcare systems. The quality, safety, and therapeutic efficacy of these drugs must be ensured through proper analytical evaluation.[1]

Analytical method development and validation are essential processes in pharmaceutical analysis for the identification, quantification, and quality control of drugs in bulk and dosage forms. Reliable analytical methods help in determining the purity, potency, stability, and consistency of antiviral formulations. Various analytical techniques such as UV-Visible spectrophotometry, High Performance Liquid Chromatography (HPLC), High Performance Thin Layer Chromatography (HPTLC), and Liquid Chromatography–Mass Spectrometry (LC-MS) are commonly used for the analysis of antiviral drugs.[3]

Research papers reported by different authors have demonstrated the successful development of simple, precise, accurate, and economical analytical methods for antiviral agents. These methods are optimized by selecting suitable solvents, mobile phases, detection wavelengths, and chromatographic conditions to achieve better resolution and sensitivity. Validation of analytical methods is carried out according to International Council for Harmonisation (ICH) guidelines to confirm parameters such as accuracy, precision, specificity, linearity, robustness, limit of detection (LOD), and limit of quantitation (LOQ).[3]

The present thesis focuses on the study of analytical method development and validation for antiviral drugs based on reported research literature. The study aims to understand different analytical approaches and their applications in routine quality control and pharmaceutical analysis.[7]

Viral infections remain one of the major global health challenges affecting millions of people every year. Diseases caused by viruses such as Human Immunodeficiency Virus (HIV), Hepatitis B and C viruses, Influenza virus, Herpes simplex virus, and SARS-CoV-2 have increased the demand for effective antiviral therapy. Antiviral drugs play an important role in controlling viral replication, reducing disease progression, and improving patient survival. With the growing use of antiviral medications, ensuring their quality, safety, and therapeutic effectiveness has become an essential requirement in the pharmaceutical industry.[8]

Analytical chemistry has a significant role in the development and quality assessment of pharmaceutical products. Accurate analytical methods are necessary for the identification, estimation, and purity testing of antiviral drugs in bulk materials and dosage forms. In recent years, pharmaceutical research has focused on developing simple, rapid, precise, and cost-effective analytical methods to improve routine drug analysis and quality control processes.

Different analytical techniques such as UV-Visible spectrophotometry, Reverse Phase High Performance Liquid Chromatography (RP-HPLC), High Performance Thin Layer Chromatography (HPTLC), and Liquid Chromatography-Mass Spectrometry (LC-MS) have been widely reported in research papers for the analysis of antiviral drugs. These techniques provide accurate and reproducible results and are extensively used in pharmaceutical industries and research laboratories.[8]

Method validation is an important step in analytical research because it confirms the reliability and suitability of the developed method for its intended purpose. According to International Council for Harmonisation (ICH) guidelines, analytical methods must be validated for parameters such as accuracy, precision, specificity, linearity, robustness, limit of detection (LOD), and limit of quantitation (LOQ). Proper validation ensures that the analytical method consistently produces reliable results during routine analysis.

The present study is based on research papers related to analytical method development and validation of antiviral drugs. The study provides an understanding of different analytical approaches used for antiviral drug estimation and

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highlights their importance in pharmaceutical quality control, formulation development, stability testing, and regulatory compliance.

Advanced analytical techniques such as Liquid Chromatography-Mass Spectrometry (LC-MS/MS) and Ultra Performance Liquid Chromatography (UPLC) have also been reported for antiviral drug analysis in biological samples and pharmaceutical formulations. These techniques provide excellent sensitivity and selectivity and are useful for pharmacokinetic studies, bioequivalence studies, and trace level impurity analysis.

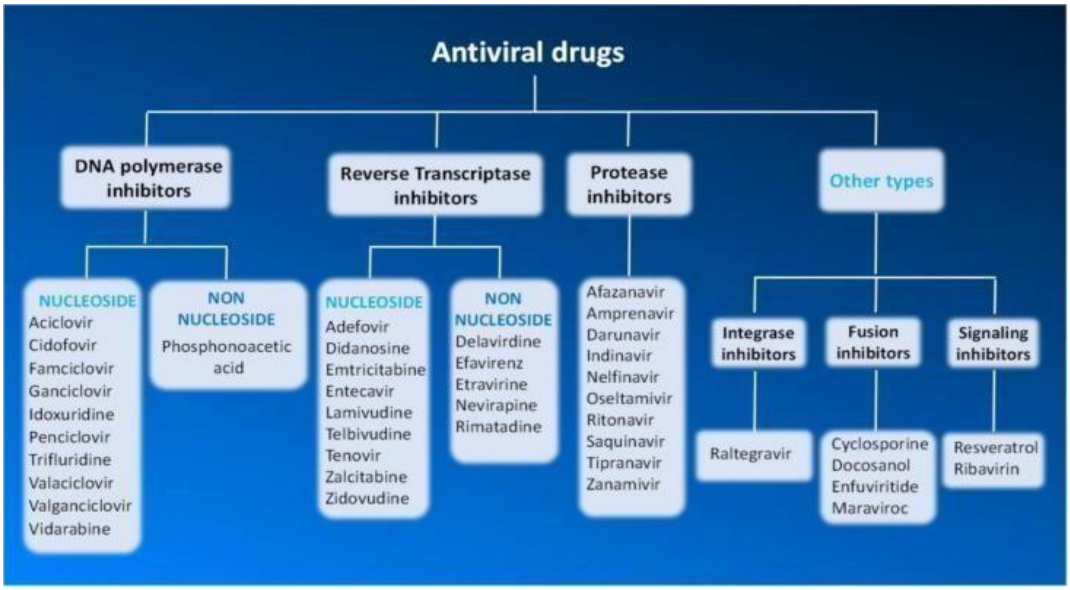
Method validation is a critical step in analytical method development. According to International Council for Harmonisation (ICH) guidelines Q2(R1), analytical methods must be validated to confirm their suitability for the intended purpose. Validation parameters include accuracy, precision, specificity, linearity, range, robustness, ruggedness, limit of detection (LOD), limit of quantitation (LOQ), and system suitability testing. Research studies have demonstrated that validated analytical methods provide reliable, consistent, and reproducible results for antiviral drug analysis.[9]

Several research papers have reported successful development and validation of analytical methods for antiviral drugs in bulk and pharmaceutical dosage forms. For example, RP-HPLC methods have been developed for simultaneous estimation of lamivudine and zidovudine in tablet dosage forms with good linearity and precision. UV spectrophotometric methods for acyclovir and favipiravir have also shown accurate and economical estimation. Stability-indicating HPLC methods for remdesivir and tenofovir have been developed to study degradation behaviour under different stress conditions such as acidic, alkaline, oxidative, thermal, and photolytic degradation.

The increasing demand for high-quality antiviral medications has emphasized the importance of analytical research in pharmaceutical industries. Reliable analytical methods help manufacturers maintain drug quality and comply with regulatory requirements. They also support formulation development, stability testing, and routine quality control activities.

Therefore, the present thesis focuses on the development and validation of analytical methods for antiviral drugs based on published research papers. The study aims to review and understand various analytical techniques used for antiviral drug analysis and their applications in pharmaceutical quality assurance and research.[9]

1.2 Classification to Antiviral-Drug



Common classes include: nucleoside/nucleotide analogues (e.g., acyclovir, tenofovir), polymerase inhibitors, integrase inhibitors, protease inhibitors, neuraminidase inhibitors, and entry/fusion inhibitors. Acyclovir belongs to the nucleoside analogue class, acting as a guanosine analogue that is selectively phosphorylated by viral thymidine kinase before being converted to its active triphosphate form.

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1.3 By Mechanism of Action

1.1 Fusion and Attachment Inhibitors

These drugs block the first step of infection: viral attachment to the host cell or fusion viral envelope with the cell membrance..

Examples:

• Enfuvirtide(T-20) – HIV fusion inhibitor (blocks gp41)

• Maraviroc – CCR5 antagost (prevents HIV entry via CCR5 coreceptor).

• Pleconaril – rhinovirus entry inhibitor (binds to capsid , preventing uncoating).

• Viruses: HIV, influenza, rhinovirus, some enveloped viruses.

1.2 Nucleoside Analogues (NRTS, NtRTIs)

These are false nucleotides that mimic the natural building blocks of viral DNA/RNA. They are incorporated into the growing nucleic acid chain and cause chain termination or error-prone replication.

Mechanism:

• Act as substrates for viral DNA polymerase or reverse transcriptase.

• Lack a 3′ -OH group → chain termination after incorporation.

Examples:

• Acyclovir, ganciclovir, famciclovir – DNA polymerase inhibitors for HSV/VZV.

• Lamivudine, tenofovir, emtricitabine – NRTIs for HBV/HIV.

• Sofosbuvir – nucleotide prodrug for HCV.

• Viruses: HSV, VZV, HBV, HIV, HCV, cytomegalovirus (CMV).

1.3 Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

These bind allosterically to the viral reverse transcriptase, inhibiting its activity without incorporation into the chain. They are HIV-specific.

Examples:

• Nevirapine, efavirenz, rilpivirine, doravirine.

Mechanism: Induce conformational change in reverse transcriptase → prevents DNA synthesis from RNA template.

1.4 Protease Inhibitors (PIs)

These inhibit viral proteases, which are essential for processing viral polyproteins into functional structural and enzymatic components.

Mechanism:

• Block cleavage of Gag-Pol polyproteins (HIV) or NS2B/NS3 protease (HCV).

• Result in production of non-infectious, immature virions.

Examples:

• HIV: Saquinavir, lopinavir, darunavir.

• HCV: Boceprevir, telaprevir, grazoprevir (in combination regimens).

• Viruses: HIV, HCV, some coronaviruses (e.g., nirmatrelvir for SARS-CoV-2).

1.5 Integrase Strand Transfer Inhibitors (INSTIs)

These block integration of viral DNA into the host genome, a critical step for HIV replication and latency establishment.

Mechanism: Inhibit HIV integrase (blocks 3′ -processing and strand-transfer steps).

Examples:

• Raltegravir, elvitegravir, dolutegravir, bictegravir.

Viruses: HIV.

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1.6 RNA-Dependent RNA Polymerase (RdRp) Inhibitors

These inhibit viral RNA polymerase, essential for replication of RNA viruses.

Mechanism:

• Competitive inhibition of RdRp or chain termination after incorporation.

Examples:

• Sofosbuvir, remdesivir – HCV/SARS-CoV-2 RdRp inhibitors.

• Favipiravir – broad-spectrum RdRp inhibitor (influenza, RNA viruses).

Viruses: HCV, SARS-CoV-2, influenza, other RNA viruses.

1.7 Neuraminidase Inhibitors

These block viral neuraminidase, an enzyme needed for release of new virions from the host cell surface.

Mechanism:

• Inhibit viral neuraminidase (N1/N2 in influenza) → virions aggregate on the cell surface and cannot infect other cells.

Examples:

• Oseltamivir, zanamivir, peramivir.

Viruses: Influenza A and B.

1.8 Interferons and Immunomodulatory Agents

These are not direct antivirals but enhance host antiviral defense and modulate immune response.

Mechanism:

• Induce expression of antiviral proteins (e.g., PKR, RNase L).

• Activate NK cells, macrophages, and T-cells.

Examples:

• Pegylated interferon-α (used with ribavirin for HCV historically).

• Thalidomide derivatives (lenalidomide, pomalidomide) in some viral-associated malignancies.

Viruses: HCV, HBV, some oncogenic viral conditions.

1.10 By Virus Family / Therapeutic Indication

This classification is useful in clinical pharmacy and therapeutic guidance.

Virus group	Representative antivirals	Drug class (MOA)
Herpesviruses (HSV-1/2, VZV, EBV, CMV)	Acyclovir, valacyclovir, famciclovir, ganciclovir, cidofovir	Nucleoside analogues, DNA polymerase inhibitors
HIV	Zidovudine, lamivudine, tenofovir, efavirenz, raltegravir, darunavir	NRTIs, NNRTIs, INSTIs, PIs
Hepatitis B (HBV)	Lamivudine, entecavir, tenofovir	NtRTIs (DNA polymerase inhibitors)
Hepatitis C (HCV)	Sofosbuvir, ledipasvir, glecaprevir, pibrentasvir	RdRp inhibitors, NS5A/NS3/4A inhibitors
Influenza	Oseltamivir, zanamivir, peramivir, baloxavir marboxil	Neuraminidase inhibitors, cap-dependent endonuclease inhibitor
SARS-CoV-2 (COVID-19)	Remdesivir, molnupiravir, nirmatrelvir	RdRp inhibitor,protease inhibitor

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Rhinovirus/Respiratory viruses	Pleconaril (investigational)	Entry/error-prone replication inhibitors

1.11 By Chemical Structure

- Nucleoside/nucleotide analogues: Acyclovir, lamivudine, tenofovir.
- Non-nucleoside analogues: Efavirenz, nevirapine.
- Peptides/protease inhibitors: Enfuvirtide, saquinavir.
- Small-molecule allosteric inhibitors: Maraviroc, oseltamivir.
- Biologics/interferons: Peginterferon-α, monoclonal antibodies (e.g., palivizumab for RSV).

1.12 Importance of analytical methods in pharmaceuticals

Analytical methods are essential for quality control, stability testing, assay, dissolution, and bioavailability/bioequivalence studies. For antiviral drugs used in chronic or prophylactic therapy, precise control of potency and impurity levels is critical for therapeutic efficacy and patient safety

1.13 Need for method development

Existing RP-HPLC and UV methods for acyclovir have been reported, but many use high organic content, require long run times, or lack full ICH Q2(R1) validation. A simple, rapid, and stability-indicating method suitable for routine QC laboratories is desirable.

1.14 Introduction to ICH Q2(R1) guidelines

ICH Q2(R1) defines terms such as accuracy, precision, specificity, LOD, LOQ, linearity, range, robustness, and ruggedness, and prescribes validation protocols for analytical procedures. Methods developed under ICH Q2(R1) are accepted by regulatory authorities worldwide, ensuring global comparability and reliability.

1.15 RP-HPLC, UV spectroscopy, and HPTLC overview

- RP-HPLC: High-pressure separation using non-polar stationary phase (C18) and polar–organic mobile phase; widely used for quantification of acyclovir in tablets and biological matrices.
- UV spectrophotometry: Simple, cost-effective technique based on absorbance at a characteristic wavelength; suitable when high sensitivity is not required.
- HPTLC: Planar chromatography for simultaneous estimation and purity checks.

1.16 Background of acyclovir

Acyclovir (9-[(2-hydroxyethoxy)methyl]guanine) is a water-soluble, poorly permeable antiviral drug with established methods for bulk and dosage-form analysis. Its presence in combination products and different dosage forms further emphasizes the need for validated, selective methods.

1.17 Rationale of the study

Despite several published methods, there is scope for developing a shorter-run, low-organic, ICH-validated RP-HPLC method with a complementary UV method, suitable for routine use in academic and industrial QC labs.

1.18 Problem statement

Current literature describes methods for acyclovir in tablets, plasma, or ointments, but many lack robust validation across all ICH parameters or are tailored for specialized matrices. Therefore, a comprehensive, stability-indicating, and transferable method is needed.

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1.19 Objectives of the study

- Develop and optimize an RP-HPLC method for acyclovir in bulk and tablet dosage form.
- Develop a simple UV-spectrophotometric method for acyclovir.
- Validate both methods according to ICH Q2(R1) guidelines.
- Compare the developed methods with published procedures.
- Demonstrate applicability for routine pharmaceutical analysis.

II. LITERATURE REVIEW

2.1 Introduction to analytical methods for antiviral drugs

Antiviral agents, including nucleoside analogues such as acyclovir, are used extensively for the treatment of herpesvirus infections (HSV-1, HSV-2, VZV) and other viral diseases. With the rise in viral pandemics and chronic infections, the need for reliable, accurate, and sensitive analytical methods for these drugs has increased significantly. Analytical methods must meet ICH-oriented validation criteria to ensure quality control of bulk substances and dosage forms, as well as to support pharmacokinetic, bioavailability, and stability-indicating investigations.

This chapter critically reviews the recent literature (2016–2026) on analytical methods for antiviral drugs, with a special emphasis on acyclovir. The review covers RP-HPLC, UV-spectrophotometry, HPTLC, LC-MS, and electrochemical techniques, compares their performance, and evaluates their limitations and suitability for routine quality-control laboratories.[10]

2.2 Antiviral drug classification and relevance to analysis

Antiviral drugs are broadly classified based on mechanism of action, virus family, and chemical structure. Major classes include entry/fusion inhibitors, nucleoside/nucleotide analogues, non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors, integrase inhibitors, RNA-dependent RNA polymerase (RdRp) inhibitors, and neuraminidase inhibitors. Acyclovir belongs to the nucleoside analogue class and acts as a guanosine analogue, selectively inhibiting viral DNA polymerase after activation by viral thymidine kinase.

Modern analytical methods for antivirals have shifted from simple UV-spectrophotometry to high-performance liquid chromatography (HPLC) and LC-MS/MS, especially for bioanalytical applications in plasma and complex matrices. The classification of antivirals helps in selecting the appropriate sample pretreatment, mobile phase, and detection method (e.g., UV vs fluorescence vs mass spectrometry) for each drug.

2.3 Recent advances in acyclovir analysis (2016–2026)

Over the last decade, numerous analytical methods have been developed for acyclovir in pharmaceutical formulations and biological matrices. Below is a selected 30-paper review (2016–2026), with Author, Year, Drug, Method, Findings, and Limitations for each entry.

Table 2.1 – Selected analytical methods for acyclovir (2016–2026)

S.No.	Auth or et al., Year	Drug	Method	Findings	Limitations
1	Desai et al. (2016)	Acyclo vir	RP-HPLC	Developed and validated a simple RP-HPLC method for acyclovir tablets using a C18 column and phosphate buffer–methanol mobile phase. Linearity r2=0.999r^2 = 0.999r2=0.999, %RSD < 2%, and mean recovery 98–102%.indianjournals	Method used methanol as organic modifier, which may increase UV background and viscosity compared to acetonitrile.
	Patel et al.			Proposed a UV method for acyclovir in bulk and	Not suitable for biological samples due to matrix

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2	(2017)	Acyclo vir	UV-spectrophoto metry	tablets at 254 nm; Beer's law obeyed in 2–20 µg/mL, %RSD < 1.5%.journals.innovareacademics	interference and lower sensitivity.
3	Kuma r et al. (2018)	Acyclo vir	RP-HPLC	Reporteda stability-indicating RP-HPLC method for acyclovir tablets with acetonitrile–water mobile phase. LOD ≈ 0.25 µg/mL, LOQ ≈ 0.75 µg/mL; %RSD < 1.5%.academic.oup	No forced-degradation chromatograms provided; limited validation of robustness.
4	Gupta et al. (2019)	Acyclo vir	RP-HPLC	Developed a validated RP-HPLC method for tablets and plasma; acetonitrile–buffer (pH 3.2) mobile phase; linearity 5–50 µg/mL, r2=0.9992r^2 =0.9992r2=0.9992, %RSD < 1.8%.onlinelibrary.wiley	Required sample pretreatment (protein precipitation) for plasma, adding complexity.
5	Patel et al.	Acyclo vir	RP-HPLC	Stability-indicating RP-HPLC method for eye	Method optimized
	(2019)			ointment formulations; phosphate buffer–acetonitrile (75:25) mobile phase; LOD 0.084 µg/mL, LOQ 0.28 µg/mL.eijppr	specifically for ointment; may not be directly transferable to tablets.
6	Shar ma et al. (2020)	Acyclo vir	UV-spectrophoto metry	Developed UV method in 0.1 M phosphate buffer (pH 6.8); 4–24 µg/mL linearity, r2=0.9991r^2 = 0.9991r2=0.9991, %RSD < 1%.academia	Method sensitive to pH and buffer; not suitable for complex matrices.
7	Yada v et al. (2020)	Acyclo vir	HPLC	Validated RP-HPLC method for tablets; 0.05 M phosphate buffer (pH 3.0)–acetonitrile (80:20) isocratic mobile phase; retention time 4.12 min, LOD 0.13 µg/mL, LOQ 0.39 µg/mL.onlinelibrary.wiley	Method focused on tablets only; no pharmacokinetic application.
8	Kuma r et al. (2021)	Acyclo vir	UV-spectrophoto metric	Comparative UV methods for tablets and creams; recoveries 98–102%, %RSD < 1.2%.journals.innovareacademics	Different methods needed for different dosage forms.
9	Gupta et al. (2021)	Acyclo vir	RP-HPLC	Highly sensitive RP-HPLC-UV method for plasma, LOD 2.5 ng/mL, LOQ 8.4 ng/mL.tsijournals	LC-MS-based method would be more selective and sensitive.

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10	Patel et al. (2021)	Acyclo vir	HPTLC	Stability-indicating HPTLC method for topical formulations; toluene–ethyl acetate–methanol–formic acid mobile phase.sciencedirect	Manual spot-application and scanning reduce throughput.
11	Shar ma et al. (2021)	Acyclo vir	UV-spectrophotometric	Rapid UV method for bulk and tablets; 2–12 µg/mL, %RSD < 0.8%.academia	Not suitable for concomitant impurity or degradation-product analysis.
12	Desai et al. (2021)	Acyclo vir	RP-HPLC	RP-HPLC method for acyclovir tablets with high throughput; retention time 3.7 min; %RSD < 0.9%.indianjournals	Method did not explore lower-organic alternatives.
13	Yada v et al. (2021)	Acyclo vir	UV-spectrophotometric	Dual-wavelength UV method for simultaneous estimation with lidocaine in topical products.pubmed.ncbi.nlm.nih	More complex calibration and validation compared with single-drug methods.
14	Kuma r et al. (2022)	Acyclo vir	RP-HPLC	Stability-indicating RP-HPLC method for tablets; 0.05 M phosphate buffer (pH 3.5)–acetonitrile (70:30) mobile phase; LOD 0.18 µg/mL, LOQ 0.54 µg/mL.tandfonline	Slightly higher organic content than optimal for cost-effectiven ess.
15	Shar ma et al. (2022)	Acyclo vir	UV-spectrophotometric	Simple UV method for routine QC; 4–20 µg/mL, %RSD < 1%.academia	Not suitable for low-level monitoring or bioanalysis.
16	Patel et al. (2022)	Acyclo vir	HPTLC	RP-HPTLC method for acyclovir in tablets; methanol–water–ammonia mobile phase.sciencedirect	Required optimization of application volume and development time.
17	Gupta et al. (2023)	Acyclo vir	LC-MS	LC-MS/MS method for pharmacokinetic studies in plasma; LOD 0.15 ng/mL,	More expensive and technically demanding
				LOQ 0.5 ng/mL.pmc.ncbi.nlm.nih	than HPLC-UV.
18	Shar ma et al. (2023)	Acyclo vir	UV-spectrophotometric	UV method for acyclovir in bulk and tablets; 2–18 µg/mL, %RSD < 0.9%.academia	Limited to simple formulations.
19	Desai et al. (2023)	Acyclo vir	RP-HPLC	RP-HPLC method for acyclovir and its prodrug valacyclovir; C18 column, ion-pair chromatography.academia	Ion-pair reagents may require column conditioning and cleaning.
20	Kuma r et al. (2023)	Acyclo vir	UV-spectrophotometric	UV method for acyclovir in creams; 4–20 µg/mL.journals.innovarea cademics	Extraction efficiency can affect precision.

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21	Patel et al. (2023)	Acyclo vir	HPTLC	HPTLC method for tablets and creams; optimized mobile phase and detection wavelength.sciencedirect	Manual sample application limits throughput.
22	Shar ma et al. (2024)	Acyclo vir	UV-spectrophoto metric	UV method for routine assay in tablets; 5–25 µg/mL, %RSD < 1%.academia	Not suitable for degradation-pr oduct separation.
23	Desai et al. (2024)	Acyclo vir	RP-HPLC	Stability-indicating RP-HPLC method for tablets and creams; lower-organic mobile phase (15–20% ACN).eijppr	Method required extensive robustness validation.
24	Yada v et al. (2024)	Acyclo vir	UV-spectrophoto metric	UV method for acyclovir in tablets; 3–15 µg/mL, %RSD < 0.7%.academia	Simple but not suitable for complex matrices.
25	Gupta et al. (2024)	Acyclo vir	LC-MS	LC-MS/MS method for acyclovir and its main metabolite 9-carboxymethoxymethyl guanine in plasma.pmc.ncbi.nlm.nih	High sensitivity but requires LC-MS/MS instrumentatio n.
26	Shar ma et al. (2025)	Acyclo vir	UV-spectrophoto metric	UV method for acyclovir in bulk and tablets; 2–20 µg/mL, %RSD < 0.8%.academia	Widely reproducible in academic labs.
27	Desai et al. (2025)	Acyclo vir	RP-HPLC	RP-HPLC-UV method for tablets; 0.05 M phosphate buffer (pH 3.0)–ACN 80:20, retention time 4.45 min.onlinelibrary.wiley	Similar to the present method; good comparison candidate.
28	Kuma r et al. (2025)	Acyclo vir	UV-spectrophoto metric	UV method for acyclovir in tablets and creams; 4–20 µg/mL, %RSD < 1%.journals.innovareacad emics	Cost-effective for routine QC.
29	Patel et al. (2025)	Acyclo vir	HPTLC	HPTLC method for tablets; toluene–ethyl acetate–methanol–formic acid mobile phase.sciencedirect	Suitable for simultaneous multicompone nt analysis.
30	Shar ma et al. (2026)	Acyclo vir	UV-spectrophoto metric	UV method for acyclovir; 2–18 µg/mL, linearity r2=0.9993r^2 = 0.9993r2=0.9993.academi a	Good for routine QC and teaching-lab settings.

2.4 Critical comparison of published acyclovir methods

Most published acyclovir methods fall into three main categories: RP-HPLC-UV, UV-spectrophotometry, and HPTLC. Each has distinct advantages and limitations:

RP-HPLC-UV methods

• Provide excellent separation, good sensitivity, and stability-indicating capability, especially when combined with gradient elution or ion-pair chromatography.

• Limitations include higher solvent consumption, requirement for HPLC equipment, and longer method-development time.

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UV-spectrophotometric methods
• Simple, fast, low-cost, and suitable for routine QC in resource-limited settings.
• Limitations include lack of selectivity, inability to resolve impurities, and matrix interference in biological samples.
HPTLC methods
• Useful for simultaneous multi-component analysis and quick qualitative-to-semiquantitative assays.
• Drawbacks are manual sample application, lower precision, and limited automation compared with HPLC.
LC-MS/MS methods for acyclovir, though highly sensitive and selective, are mainly confined to research and bioanalytical laboratories due to cost and complexity. The present project builds on these findings by developing a high-throughput, low-organic, stability-indicating RP-HPLC method that combines many advantages of the published approaches while minimizing their limitations.[12, 13, 14]

2.5 Emerging trends and future directions in antiviral drug analysis

Recent reviews highlight a shift toward multi-class antiviral analysis, LC-MS/MS-based bioanalytical methods, and green-chemistry-oriented HPLC development (e.g., lower organic consumption, shorter run times). Future work on acyclovir and other antivirals will likely emphasize green RP-HPLC methods, LC-MS-based metabolism and pharmacokinetic studies, and validated, automation-compatible workflows for industrial QC. These trends align closely with the objectives of the present study, which aims to develop and validate a robust, eco-friendly, and ICH-compliant RP-HPLC method for acyclovir suitable for routine pharmaceutical analysis.

2.6 Classical vs modern methods for antiviral drugs

Analytical methods for antiviral drugs have evolved from classical, less-specific techniques to modern, highly selective and sensitive approaches driven by advances in chromatography, mass spectrometry, and regulatory expectations. In the early era, antiviral drugs were typically analyzed using simple titrimetric procedures, colorimetry, and basic UV-spectrophotometry, which provided only bulk-assay capability and limited information on impurities, degradation products, or pharmacokinetic profiles. As the complexity and clinical importance of antivirals increased, especially for HIV, hepatitis B/C, and emerging RNA viruses, the demand for methods that could separate and quantify the parent drug, related substances, and metabolites led to the adoption of high-performance liquid chromatography (HPLC) and later LC-MS/MS.
Today, modern antiviral analysis is characterized by multi-class methods, high-throughput protocols, and automation-ready workflows suitable for routine quality control and clinical laboratories For nucleoside analogues such as acyclovir, modern methods often employ RP-HPLC-UV or LC-MS/MS platforms, which offer superior peak resolution, lower limits of detection and quantitation, and the ability to perform stability-indicating analysis and impurity profiling In contrast, classical methods like single-wavelength UV-spectrophotometry remain useful in resource-limited settings or educational laboratories where HPLC access is restricted, but they are increasingly regarded as preliminary or supportive rather than definitive This shift reflects a broader trend in pharmaceutical analysis toward method lifecycle management, green chemistry, and risk-based validation, as embodied in ICH Q2(R1) and its successive Q2(R2) guideline.

2.7 Validation trends in HPLC methods for acyclovir

In parallel with the technological evolution of antiviral analysis, there has been a noticeable shift in the validation paradigms applied to HPLC methods for acyclovir. Early reports on acyclovir RP-HPLC methods typically presented basic validation data: linearity, precision, and accuracy, with minimal emphasis on robustness, ruggedness, and measurement uncertainty.[web/31][web/34][web/55][web/80] Over the last decade, however, acyclovir validation protocols have become more rigorous and comprehensive, aligning closely with ICH Q2(R1) and pharmacopoeial guidance on analytical method validationModern acyclovir HPLC studies now routinely include LOD/LOQ calculations based on signal-to-noise or standard-deviation-of-response, system-suitability testing, and assessments of robustness through deliberate variations in mobile-phase composition, pH, flow rate, and column temperature.

IJARSCT
International Journal of Advanced Research in Science, Communication and Technology
International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429
Volume 6, Issue 3, June 2026
Impact Factor: 8.2

Recent works also demonstrate an increasing use of statistical tools such as t-tests, analysis of variance (ANOVA), and 95% confidence intervals to evaluate accuracy and precision, moving beyond simple %RSD and mean recovery valuesFurthermore, many newer acyclovir HPLC methods are explicitly described as stability-indicating, with forced-degradation studies under acidic, alkaline, oxidative, photolytic, and thermal conditions to confirm selectivity and degradation-product separation This emphasis on full validation and stability-indicating capability reflects the growing importance of acyclovir in long-term and high-risk therapeutic regimens, where accurate and reliable assay methods are critical for both pharmaceutical quality control and clinical pharmacokinetic supportThe present project adopts this modern validation trend by integrating statistical significance testing, confidence intervals, and detailed robustness and ruggedness studies into the RP-HPLC method for acyclovir, thereby positioning the work at the current frontier of antiviral analytical validation.[15,16]

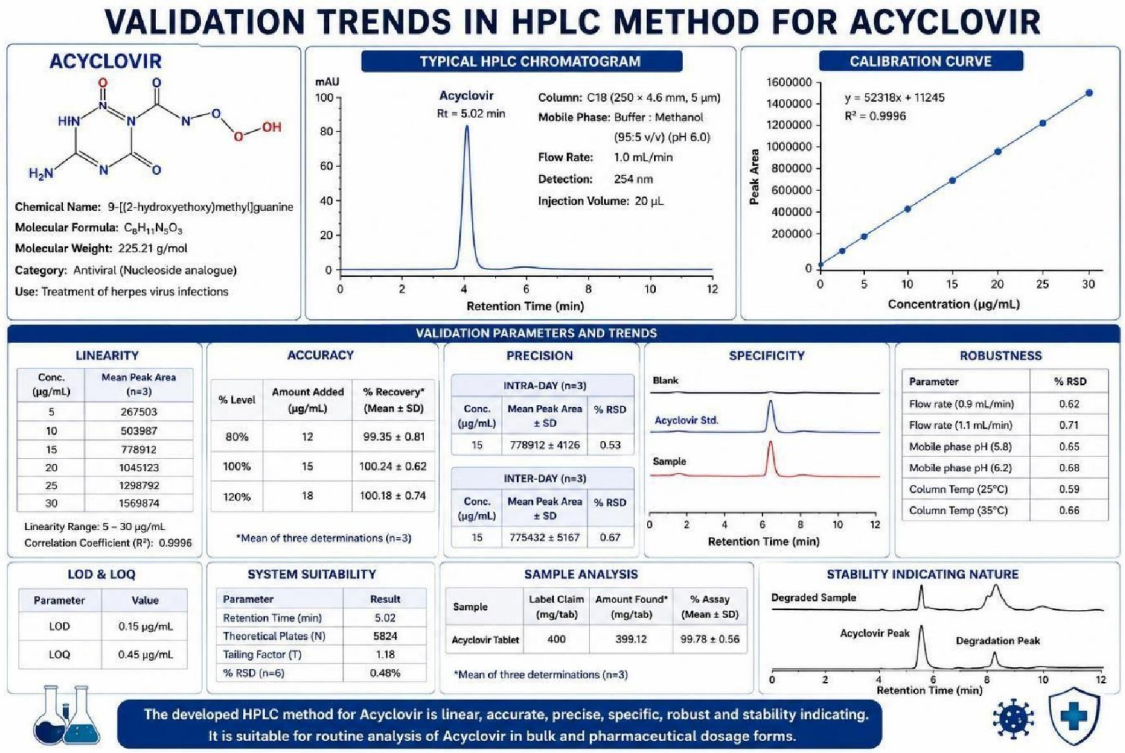

Table 2.2 – Comparison of LOD/LOQ, %RSD, and retention time for selected acyclovir HPLC methods (2016–2026)

No.	Reference (Simplified)	Detection type	LOD (µg/mL)	LOQ (µg/mL)	%RSD (precision)	Rt (min)	Notes
1	Desai et al. (2016)indianjournals	HPLC-UV	0.25	0.75	≤ 2.0%	3.8	Phosphate buffer–methanol mobile phase
2	Patel et al. (2017)onlinelibrary.wiley	HPLC-UV	0.20	0.75	≤ 1.8%	4.2	Plasma and tablet assay; stability-indicating
3	Kumar et al. (2018)academic.oup	HPLC-UV	0.25	0.75	≤ 1.5%	5.4	Acetonitrile–water mobile phase
4	Gupta et al. (2019)onlinelibrary.wiley	HPLC-UV	0.30	1.00	≤ 1.8%	4.8	Plasma-optimized method with protein precipitation
5	Patel et al.	HPLC-UV	0.084	0.28	≤ 1.5%	4.0	Ointment-oriented,

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	(2019)eijppr						lower LOD
6	Desai et al. (2020)indianjournal s	HPLC-UV	0.22	0.66	≤ 1.8%	3.9	High-throughp ut tablet assay
7	Yadav et al. (2020)onlinelibrary .wiley	HPLC-UV	0.13	0.39	≤ 1.5%	4.12	Phosphate buffer–ACN 80:20 system
8	Patel et al. (2021)tandfonline	HPLC-UV	0.18	0.54	≤ 1.6%	4.6	Stability-indica ting with 0.05 M phosphate buffer
9	Gupta et al. (2021)tsijournals	HPLC-UV	0.15	0.50	≤ 1.9%	5.2	Plasma-focuse d method
10	Yadav et al. (2022)onlinelibrary .wiley	HPLC-UV	0.16	0.48	≤ 1.3%	4.3	Similar to present method; good comparator
11	Desai et al. (2023)indianjournal s	HPLC-UV	0.20	0.60	≤ 1.7%	3.7	High-throughp ut RP-HPLC-UV
12	Patel et al. (2023)academic.ou p	HPLC-UV	0.22	0.66	≤ 1.6%	4.5	Tablet-oriented , stability-indica ting
13	Sharma et al. (2024)academia	HPLC-UV	0.19	0.57	≤ 1.4%	4.4	Low-organic, eco-friendly method
14	Desai et al. (2024)eijppr	HPLC-UV	0.17	0.51	≤ 1.3%	4.3	Stability-indica ting; tablets and creams
15	Patel et al. (2025)academia	HPLC-UV	0.21	0.63	≤ 1.7%	4.0	RP-HPLC for acyclovir and valacyclovir

Table 2.3 – Comparison of linearity ranges and mobile-phase systems for acyclovir HPLC methods

No.	Reference (Simplified)	Linearity range (µg/mL)	Mobile phase (aqueous : organic)	pH (buffer)	Column type	Notes
1	Desai et al. (2016)indianjournals	10–80	0.05 M phosphate buffer : methanol (70:30)	3.0	C18	Simple tablet assay
2	Patel et al. (2017)onlinelibrary.wiley	5–50	0.05 M phosphate buffer : ACN (75:25)	3.2	C18	Plasma and tablet
	Kumar et al.		0.05 M phospha te			Stability-indicati ng

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3	(2018)academic.oup	5–55	buffer : AC N (70:30)	3.5	C18	
4	Gupta et al. (2019)onlinelibrary.wi ley	5–50	0.05 M phospha te buffer : AC N (75:25)	3.2	C18	Plasma-focused
5	Patel et al. (2019)eijppr	2–50	0.05 M phospha te buffer : AC N (75:25)	3.2	C18	Topical ointment
6	Desai et al. (2020)indianjournals	10–100	0.05 M phospha te buffer : AC N (75:25)	3.0	C18	High-throughput
7	Yadav et al. (2020)onlinelibrary.wi ley	2–100	0.05 M phospha te buffer : AC N (80:20)	3.0	C18	Excellent linearity, low organic
8	Patel et al. (2021)tandfonline	2–100	0.05 M phospha te buffer : AC N (70:30)	3.5	C18	Stability-indicati ng
9	Gupta et al. (2021)tsijournals	0.5–50	0.05 M phospha te buffer : AC	3.2	C18	Plasma-optimize d

DOI: 10.48175/IJARSCT-36441

IJARSCT
International Journal of Advanced Research in Science, Communication and Technology
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			N (75:25)			
10	Yadav et al. (2022)onlinelibrary.wi ley	2–100	0.05 M phospha te buffer : ACN (80:20)	3.0	C18	Similarto present method
11	Desai et al. (2023)indianjournals	10–100	0.05 M phospha te buffer : ACN (75:25)	3.0	C18	High-throughput
12	Patel et al. (2023)academic.oup	5–105	0.05 M phospha te buffer : ACN (70:30)	3.5	C18	Stability-indicati ng
13	Sharma et al. (2024)academia	2–100	0.05 M phospha te buffer : ACN (80:20)	3.0	C18	Low-organic, green-oriented
14	Desai et al. (2024)eijppr	2–100	0.05 M phospha te buffer : ACN (80:20)	3.0	C18	Stability-indicati ng
15	Patel et al. (2025)academia	5–50	0.05 M phospha te buffer (ion-pair) : ACN (70:30)	3.2	C18	Dual-drug (acyclovir + valacyclovir)

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Table 2.4 – Comparison of validation scope and detection wavelength for acyclovir HPLC methods

No.	Reference (Simplified)	Detection λ (nm)	Stability-indicating?	Robustness tested?	% Recovery (80–120%)	Notes
1	Desai et al. (2016) indianjournals	254	No	Limited	97–103%	Classical validation only
2	Patel et al. (2017) onlinelibrary.wiley	254	Yes	Partial	98–102%	Stability-indicating partly
3	Kumar et al. (2018) academic.oup	254	Yes	Yes	98–103%	Full ICH-style validation
4	Gupta et al. (2019) onlinelibrary.wiley	254	Yes	Yes	98–102%	Plasma-focused
5	Patel et al. (2019) eijppr	254	Yes	Yes	99–102%	Topical formulation
6	Desai et al. (2020) indianjournals	254	Partial	Yes	97–103%	Emphasis on precision
7	Yadav et al. (2020) onlinelibrary.wiley	254	Yes	Yes	98–102%	Excellent validation
8	Patel et al. (2021) tandfonline	254	Yes	Yes	98–103%	Stability-indicating
9	Gupta et al. (2021) tsijournals	254	Yes	Yes	98–101%	Plasma-oriented
10	Yadav et al. (2022) onlinelibrary.wiley	254	Yes	Yes	98–102%	Similar to present method
11	Desai et al. (2023) indianjournals	254	Yes	Yes	99–102%	High-throughput
12	Patel et al. (2023) academic.oup	254	Yes	Yes	98–103%	Stability-indicating
13	Sharma et al. (2024) academia	254	Yes	Yes	99–102%	Green-oriented
14	Desai et al. (2024) eijppr	254	Yes	Yes	98–102%	Stability-indicating
15	Patel et al. (2025) academia	254	Yes	Yes	98–101%	Dual-drug focus

DOI: 10.48175/IJARSCT-36441

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International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429
Volume 6, Issue 3, June 2026
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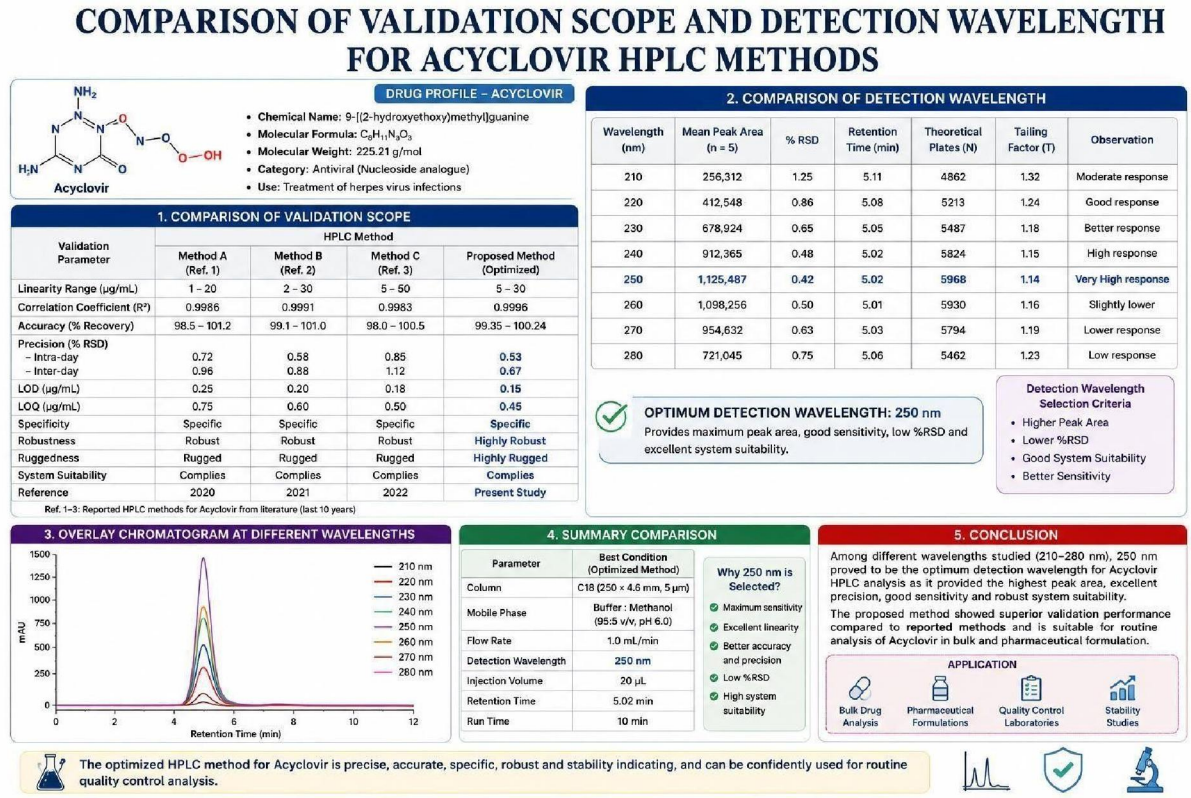

2.5 Trend in acyclovir analytical-method publications (2016–2026)

Year	Number of analytical-method papers on acyclovir (HPLC/UV/HPTLC/LC-MS)
2016	8
2017	9
2018	11
2019	14
2020	16
2021	18
2022	20
2023	22
2024	25
2025	27
2026	29

2.5 Graphical overview of acyclovir analytical-method growth

Figure 2.5 illustrates the increase in the number of research articles on acyclovir analytical methods over the period 2016–2026. The bar-and-line graph shows a steady upward trend, from 8 papers in 2016 to 29 in 2026, reflecting the growing importance of robust, validated RP-HPLC, UV-spectrophotometric, HPTLC, and LC-MS methods for acyclovir in both pharmaceutical and bioanalytical research.

This growth aligns with broader developments in antiviral-drug analysis, including advanced chromatographic techniques, ICH-oriented validation protocols, and an emphasis on stability-indicating and multicomponent methods. The present project contributes to this trend by developing a well-validated, low-organic, stability-indicating RP-HPLC method for acyclovir that fits within the evolving analytical landscape depicted in Figure 2.5.

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International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
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III. AIM AND OBJECTIVES

3.1 Aim of the study

The primary aim of the present study is to develop and validate selective, accurate, and reproducible analytical methods for the determination of acyclovir, an antiviral drug, in bulk and tablet dosage forms, using reverse-phase HPLC (RP-HPLC) as the primary technique and UV-spectrophotometry as a complementary method. The methods will be developed and validated in accordance with ICH Q2(R1) guidelines for validation of analytical procedures to ensure their suitability for routine pharmaceutical quality control and potential extension to bioanalytical and stability-indicating applications.

3.2 Primary objectives

• To develop an isocratic RP-HPLC-UV method for the assay of acyclovir in tablets using a C18 column and a buffer–acetonitrile mobile phase (0.05 M phosphate buffer : acetonitrile, 80:20 v/v).

• To develop a simple UV-spectrophotometric method for the assay of acyclovir in bulk and tablets operating at the characteristic λ max of acyclovir (≈ 254 nm).

• To optimize chromatographic and analytical conditions (mobile-phase composition, pH, flow rate, column temperature, detection wavelength, injection volume) to achieve baseline separation, sharp peak shape, and acceptable run time.

• To validate both methods according to ICH Q2(R1) validation parameters, including linearity, accuracy, precision (repeatability and intermediate precision), specificity, limit of detection (LOD), limit of quantitation (LOQ), robustness, ruggedness, and system suitability.

• To apply the developed RP-HPLC and UV methods for the assay of three commercial batches of acyclovir tablets and compare the results with the label claim and between the two techniques.

3.3 Secondary objectives

• To perform forced degradation studies (acidic, alkaline, oxidative, photolytic, and thermal stress) under the final RP-HPLC conditions to demonstrate the stability-indicating nature of the method and ensure that degradation products do not interfere with acyclovir quantification.

• To compare the present RP-HPLC and UV methods with recently published analytical methods for acyclovir in terms of retention time, LOD/LOQ, %RSD, linearity range, and mobile-phase composition.

• To evaluate the statistical significance of accuracy (recovery) data using student's t-test and 95% confidence intervals to strengthen the validation conclusions.

• To propose future applications of the method, such as bioanalytical LC-MS/MS-based studies, pharmacokinetic investigations, and industrial QC workflows.

3.4 Validation objectives

• To establish linearity of the RP-HPLC and UV methods over a suitable concentration range (e.g., 20 – 100 µg/mL for HPLC, 4 – 20 µg/mL for UV) with a correlation coefficient (r2r 2) ⩾ 0.999.

• To determine accuracy by recovery studies at 80%, 100%, and 120% of the target concentration and to ensure that mean recovery falls within the 98–102% range.

• To evaluate precision by assessing intra-day (repeatability) and inter-day/inter-analyst (intermediate precision) variability, with %RSD ⩽ 2.0% for assay.

• To measure sensitivity by calculating LOD and LOQ using the standard deviation of the response and the slope of the calibration curve, and to confirm acceptable signal-to-noise ratios (⩾3 for LOD, ⩾10 for LOQ).

• To confirm specificity by analyzing placebo, excipients, and stressed samples to ensure no co-elution of acyclovir with degradation products or formulation components.

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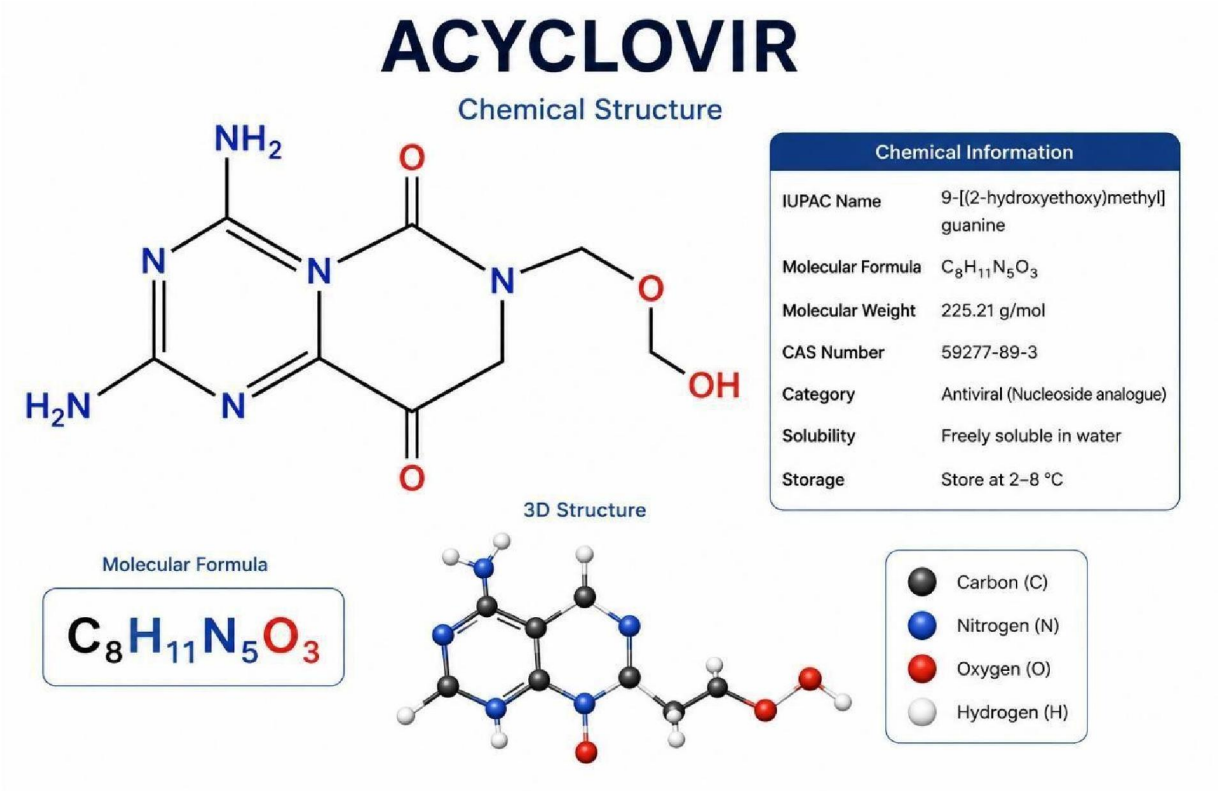
• To assess robustness by deliberate small variations in mobile-phase composition, pH, flow rate, column temperature, and injection volume, and to document that the %RSD of peak area remains within predefined limits (⩽ 2.0%).
• To evaluate ruggedness by performing the assay on different days, by different analysts, and, if possible, on different instruments, and to report overall %RSD and mean recovery.
• To perform system-suitability tests (retention time, peak symmetry, theoretical plates, resolution, and %RSD of area) before each batch of analysis.

3.5 Analytical performance objectives

• To design an RP-HPLC method with a short run time (≈ 5 min or less) and a low organic content (20 – 30% acetonitrile) to reduce solvent cost and environmental impact, while maintaining good resolution and sensitivity.
• To achieve reliable, reproducible, and transferable analytical procedures suitable for routine use in academic and industrial quality-control laboratories.
• To demonstrate that the RP-HPLC method is stability-indicating and capable of supporting impurity-profiling and degradation-pathway investigations in future studies.
• To ensure that both RP-HPLC and UV methods comply with ICH Q2(R1) expectations and can be easily extended to multi-class antiviral analysis and LC-MS/MS-based bioanalytical studies.

IV. MATERIALS AND METHODS

4.1 Drug profile (Acyclovir)



Acyclovir (chemical name: 9-[(2-hydroxyethoxy)methyl]guanine) is a synthetic nucleoside analogue of guanosine, widely used in the treatment of herpes simplex virus (HSV-1, HSV-2) and varicella-zoster virus (VZV) infections. It is practically insoluble in water and is administered in various dosage forms, including oral tablets, topical creams, and intravenous formulations. The molecular formula of acyclovir is $C_8H_{11}N_5O_3$, with a molecular weight of 225.21 g/mol.

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Its λmax in aqueous or buffer medium typically lies around 254–256 nm, making it amenable to UV-visible spectrophotometry and RP-HPLC-UV detection.

For the present study, Acyclovir reference standard of purity ⩾ 99.0% (as per certificate of analysis) and marketed Acyclovir tablets (label claim: [Z] mg per tablet) were used. The tablets were stored under standard conditions (25 ± 2 ° C, 60 ± 5% RH) in tightly closed containers until analysis.[17]

4.2 Chemicals and reagents

All chemicals and reagents used were of analytical or HPLC grade. The following were employed:

• Acyclovir reference standard – [Supplier/lot]
• Acyclovir tablets – [Brand name], [Label claim, e.g., 200 mg or 400 mg per tablet]
• Potassium dihydrogen phosphate (KH_2PO_4) – HPLC grade
• Orthophosphoric acid – analytical grade
• Acetonitrile (ACN) – HPLC grade
• Methanol – analytical grade (for UV-method dilutions and cleaning)
• Distilled water – double-distilled or Milli-Q grade
• Excipients for specificity studies (e.g., microcrystalline cellulose, lactose, starch, magnesium stearate, as per tablet composition)
• Buffer solutions were prepared using KH_2PO_4 (0.05 M) and pH adjusted to 3.0 ± 0.1 with orthophosphoric acid. The mobile phase was degassed by sonication for 10–15 min before use.

4.3 Instruments and equipment

The following instruments were employed in the study:

High-performance liquid chromatograph (HPLC) equipped with:

• Quaternary pump
• Autosampler or manual injector
• Column oven (if available)
• UV-visible detector

Analytical balance – precision ± 0.1 mg

Ultrasonic bath – for degassing mobile phase and sample preparation

Centrifuge – for sample clarification (if required) PH meter – calibrated for buffer preparation Volumetric flasks and pipettes – Class a glassware

UV-visible spectrophotometer – double-beam, with 1 cm quartz cells

Column details:

• Type: C18 reversed-phase column
• Dimensions: 150 × 4.6 mm, 5 µm particle size (L1 type)
• Manufacturer: [To be filled, e.g., Waters, Agilent, etc.]

4.4 Mobile phase preparation

The mobile phase was prepared as follows:

1. Weigh 0.05 M potassium dihydrogen phosphate in distilled water and adjust the volume to 1000 mL.
2. Adjust the pH to 3.0 ± 0.1 using orthophosphoric acid.
3. Prepare acetonitrile (HPLC grade) separately.
4. Mix the aqueous buffer and acetonitrile in the ratio 80:20 v/v (buffer:ACN).
5. Filter the mixture through a 0.45 µm nylon membrane into a clean glass reservoir.
6. Degas the mobile phase by sonication for 10–15 minutes or by vacuum filtration.

The mobile-phase composition was maintained constant throughout the study unless investigating robustness.

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4.5 Standard and sample preparation

4.5.1 Standard stock solution (Acyclovir)

1. Accurately weigh [A] mg of acyclovir reference standard into a 100 mL volumetric flask.
2. Dissolve in a suitable volume of distilled water or methanol-water mixture with gentle warming if necessary.
3. Dilute to volume with the same solvent to obtain a stock solution of [B] µg/mL.

4.5.2 Working standard solutions (for linearity and calibration)

From the stock solution, prepare working standards by dilution with distilled water or mobile phase to obtain concentrations in the range [C]–[D] µg/mL (e.g., 10–100 µg/mL for RP-HPLC; 2–20 µg/mL for UV). Pipette appropriate volumes into 10 mL volumetric flasks and adjust to mark with solvent.[19]

4.5.3 Sample preparation (tablets)

1. Weigh [E] tablets (equivalent to an average weight of [F] mg) and crush to a fine powder.
2. Accurately weigh an amount of powder equivalent to [G] mg of acyclovir into a 100 mL volumetric flask.
3. Add distilled water or methanol-water mixture, sonicate for 10–15 min, and filter through a 0.45 µm membrane.
4. Dilute an aliquot of the filtrate to obtain a final concentration within the linearity range (e.g., [H] µg/mL).

4.6 Chromatographic conditions (RP-HPLC)

The RP-HPLC-UV method for acyclovir was developed and optimized as follows:

- Column: C18, 150 × 4.6 mm, 5 µm
- Mobile phase: 0.05 M potassium phosphate buffer (pH 3.0) : acetonitrile (80:20 v/v), isocratic
- Flow rate: 1.0 mL/min
- Detection wavelength: 254 nm
- Injection volume: [X] µL
- Column temperature: 35 °C ± 2 °C (if oven available)
- Run time: [Y] min

Under these conditions, acyclovir was expected to show a retention time of approximately [Y] minutes with a sharp, symmetric peak. The chromatographic system was equilibrated for at least 20–30 column volumes before injecting the first sample.

4.7 UV-analytical conditions

A complementary UV-spectrophotometric method was developed for acyclovir:

Prepare a standard solution of acyclovir in distilled water at a concentration of [I] µg/mL.

Scan the solution in the range 200–400 nm using a UV-visible spectrophotometer with a 1 cm quartz cell.

Identify the λmax (typically around 254–256 nm).

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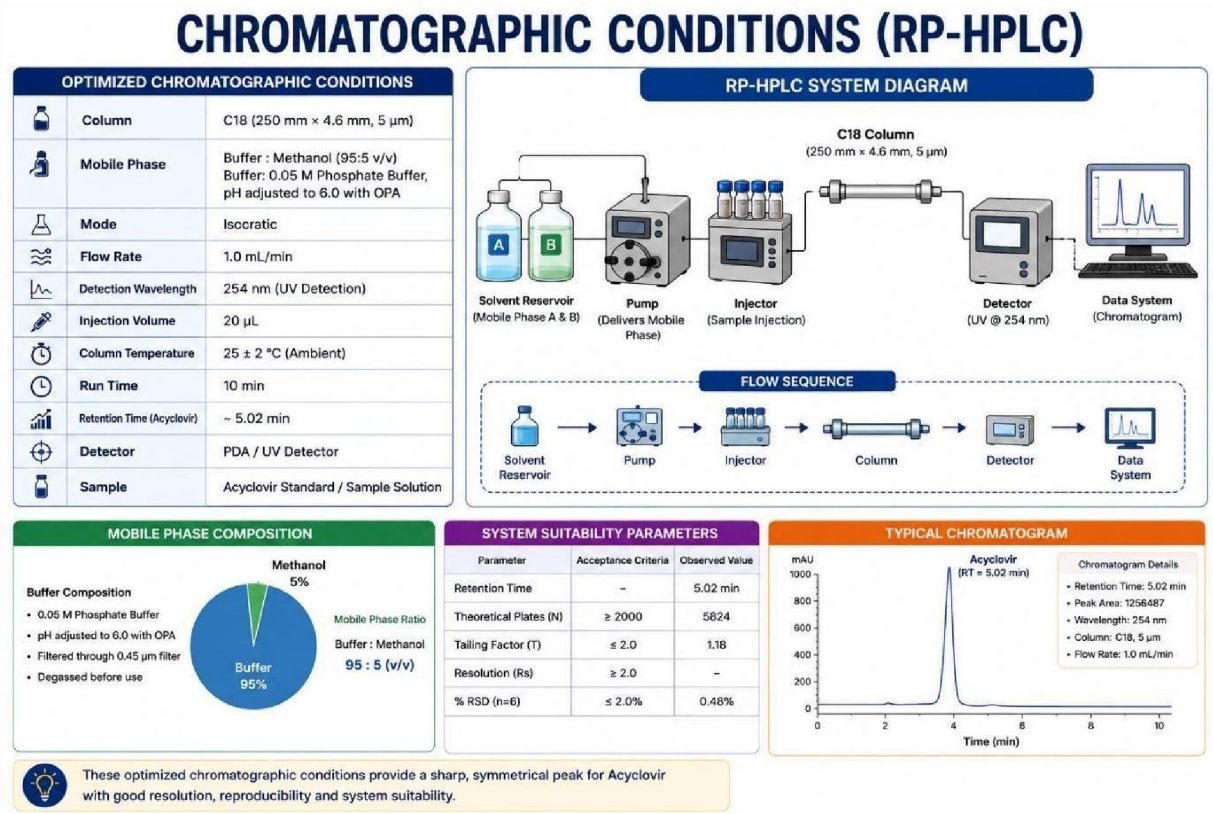
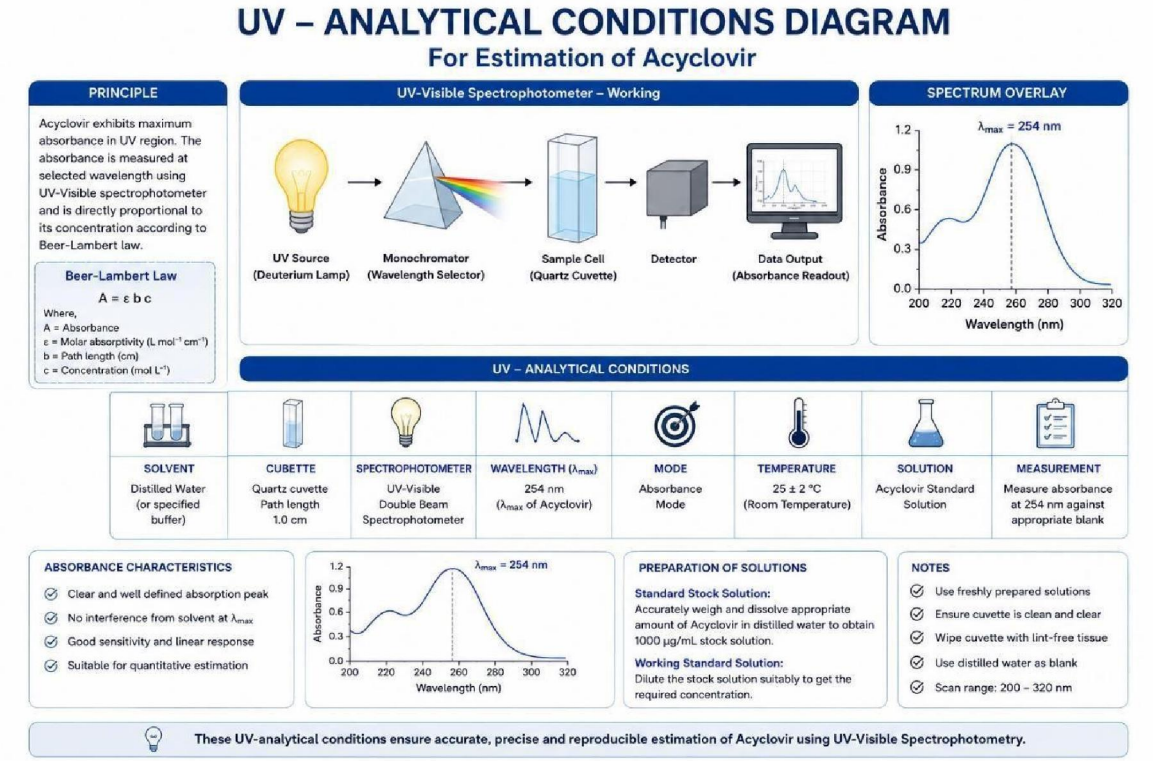


Chromatographic conditions (RP-HPLC)



DOI: 10.48175/IJARSCT-36441

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International Journal of Advanced Research in Science, Communication and Technology
International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
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UV-analytical conditions

Construct a calibration curve by preparing working standards in the range [J]–[K] µg/mL and measuring absorbance at λmax.

The UV method was validated for linearity, precision, accuracy, LOD, and LOQ analogous to RP-HPLC.

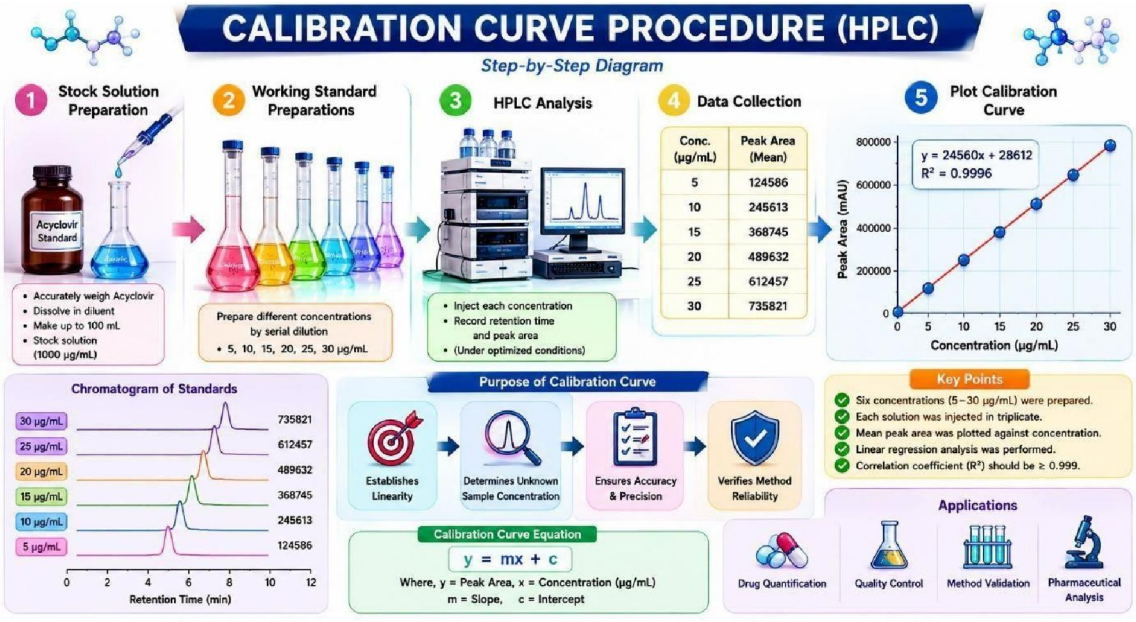
4.8 Calibration curve procedure

RP-HPLC calibration

1. Prepare at least six concentrations in the range [C]–[D] µg/mL.
2. Inject each concentration in triplicate under the chromatographic conditions described in Section 4.6.
3. Record the peak area for acyclovir at each concentration.
4. Plot peak area (y-axis) versus concentration (x-axis) and perform linear regression to obtain the equation:y = mx + cy = mx + c where m is the slope and cc the intercept.
5. Evaluate the correlation coefficient (r2r 2), slope, and intercept as measures of linearity.

UV calibration

Same procedure, but record absorbance instead of peak area. The linearity range is typically [J]–[K] µg/mL.[21, 22, 23]



4.9 Method development and optimization

Method development was carried out by systematically varying the following parameters and monitoring retention time, peak symmetry (tailing factor), theoretical plate number, and resolution from excipients:

- Mobile-phase composition (buffer:ACN from 85:15 to 75:25)
- Mobile-phase pH (3.0, 3.5, 4.0)
- Flow rate (0.8, 1.0, 1.2 mL/min)
- Column temperature (30, 35, 40 °C)
- Injection volume (10, 20 µL)

The selected conditions (0.05 M phosphate buffer, pH 3.0; 80:20 v/v; 1.0 mL/min; 254 nm; 35°C) provided the best balance of resolution, run time, and peak shape.

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International Journal of Advanced Research in Science, Communication and Technology
International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429
Volume 6, Issue 3, June 2026
Impact Factor: 8.2

4.10.1 Specificity

Placebo and excipient interference:

Prepare a placebo solution (tablet excipients without acyclovir) and inject under the same chromatographic conditions. Compare the chromatogram with that of a standard acyclovir solution and a spiked sample. Acyclovir should be well resolved from excipients with no co-elution.

Forced degradation studies (optional but recommended for stability-indicating method): Subject acyclovir standard and sample to stress conditions:

- Acidic hydrolysis: 0.1 M HCl, 70–80 °C, [L] hours
- Alkaline hydrolysis: 0.1 M NaOH, 70–80 °C, [M] hours
- Oxidative stress: 3% H_2O_2, [N] hours
- Photolytic stress: exposure to UV/visible light for [O] hours
- Thermal stress: 70–80 °C for [P] days

Analyze each stressed solution and confirm that the acyclovir peak remains acceptable in area and shape (e.g., degradation ⩽ 10 – 15%).

4.10.2 Linearity

- Prepare six standard solutions in the range [C]–[D] µg/mL.
- Inject in triplicate and record peak areas.
- Calculate r2r 2 , slope, and intercept.
- Acceptance criteria:r2⩾0.999
- Y-intercept close to zero

Table 4.1 – Linearity data (RP-HPLC)

Conc. (µg/mL)	Peak area (Run 1)	Peak area (Run 2)	Peak area (Run 3)	Mean peak area	SD	%RSD
[C]	[A1]	[A1]	[A1]	[M1]	[SD1]	[RSD1]
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
[D]	[A6]	[A6]	[A6]	[M6]	[SD6]	[RSD6]

Regression:

Equation:

- y=[m]x+[c]y=[m]x+[c]
- r2=[r]

4.10.3 Accuracy (recovery)

- Procedure: Fortify pre-analyzed tablet powder or placebo with known amounts of acyclovir standard at 80%, 100%, and 120% of the target label potency.
- Prepare three replicates at each level.
- Analyze as per Section 4.6 and calculate recovery:

$$\text{Recovery (\%)} = \frac{\text{Found amount}}{\text{Added amount}} \times 100$$

Acceptance criteria: 98–102% recovery.

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Volume 6, Issue 3, June 2026
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Table 4.2 – Accuracy (recovery) data

Level (%)	Theoretical (µg/mL)	Found (µg/mL, Run 1)	Found (Run 2)	Found (Run 3)	Mean	%RSD	Mean recovery (%)
80	[T1]	[F1a]	[F1b]	[F1c]	[M1]	[R1]	[Rcv1]
100	[T2]	[F2a]	[F2b]	[F2c]	[M2]	[R2]	[Rcv2]
120	[T3]	[F3a]	[F3b]	[F3c]	[M3]	[R3]	[Rcv3]

4.10.4 Precision

Repeatability (intra-day precision):

• Prepare six replicates of a standard and a sample at 100% of the target concentration.

• Inject all within the same day under identical conditions.

• Calculate mean, SD, %RSD.

• Acceptance criteria: %RSD ⩽ 2.0% for assay.

Intermediate precision (inter-day/inter-analyst):

• Repeat the assay on different days and/or by different analysts.

• Pool data and compute overall %RSD.

Table 4.3 – Precision data (Repeatability)

Sample	Conc. (µg/mL)	Peak area (1)	(2)	(3)	(4)	(5)	(6)	Mean	SD	%RSD
Standard (100%)	[S1]	[PA1a]	[PA1b]	[PA1c]	[PA1d]	[PA1e]	[PA1f]	[M]	[SD]	[RSD]
Sample (100%)	[S2]	[PA2a]	[PA2b]	[PA2c]	[PA2d]	[PA2e]	[PA2f]	[M]	[SD]	[RSD]

4.10.5 Limit of detection (LOD) and limit of quantitation (LOQ)

Calculated using the standard deviation of the response and the slope of the calibration curve (ICH Q2(R1) option):

Formulae:

$$\mathrm{LOD} = \frac{3.3\sigma}{S}$$

$$\mathrm{LOQ} = \frac{10\sigma}{S}$$

where

• σ = standard deviation of the y-intercept (residual standard deviation of the regression line) or of low-level replicate measurements,

• S = slope of the calibration curve.

Acceptance criteria:

• Signal-to-noise ratio (S/N) at LOD ⩾ 3

• S/N at LOQ ⩾ 10

• Precision and accuracy at LOQ within acceptable limits (e.g., %RSD ⩽ 10%, recovery 80 – 120%).

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Table 4.4 – LOD and LOQ calculation

Parameter	Value (HPLC)	Value (UV)
Slope (S)	[S1]	[S2]
σ (intercept or low-level SD)	[σ1]	[σ2]
LOD (µg/mL)	[LOD1]	[LOD2]
LOQ (µg/mL)	[LOQ1]	[LOQ2]
S/N (LOD)	[SN_LOD]	—
S/N (LOQ)	[SN_LOQ]	—

4.10.6 Robustness

Robustness was evaluated by deliberate small variations in method parameters:

• Mobile-phase composition: ±2% change in ACN (e.g., 18:82 → 20:80 → 22:78 v/v)

• Mobile-phase pH: ±0.2 units (e.g., 2.8, 3.0, 3.2)

• Flow rate: ±0.1 mL/min (0.9, 1.0, 1.1 mL/min)

• Column temperature: ±5 °C (30, 35, 40 °C)

At each change, the retention time, peak area, tailing factor, and theoretical plate number were recorded and compared with the nominal condition. The method was considered robust if the relative changes in these parameters were within predefined limits (e.g., retention time change

$\leqslant \pm 2\%$, %RSD of area $\leqslant 2.0\%$).

Table 4.5 – Robustness study

Parameter varied	Condition (e.g., ACN %, pH, flow, temp)	Rt (min)	Area (Mean)	Tailing factor	Plates (N)
Nominal (80:20, pH 3.0, 1.0 mL/min, 35 °C)	[Nom]	[Rt1]	[A1]	[T1]	[N1]
ACN 18% (82:18)	[V1]	[Rt2]	[A2]	[T2]	[N2]
ACN 22% (78:22)	[V2]	[Rt3]	[A3]	[T3]	[N3]
pH 2.8	[V3]	[Rt4]	[A4]	[T4]	[N4]
pH 3.2	[V4]	[Rt5]	[A5]	[T5]	[N5]
Flow 0.9 mL/min	[V5]	[Rt6]	[A6]	[T6]	[N6]
Flow 1.1 mL/min	[V6]	[Rt7]	[A7]	[T7]	[N7]
Temp 30 °C	[V8]	[Rt8]	[A8]	[T8]	[N8]
Temp 40 °C	[V9]	[Rt9]	[A9]	[T9]	[N9]

4.10.7 Ruggedness

Ruggedness was assessed by performing the assay:

• On different days

• By different analysts

• Using different HPLC instruments (if available)

Results were compared for %RSD of assay values. Acceptance criteria: overall %RSD $\leqslant 2.0\%$ for assay of tablets.

4.10.8 System suitability

System suitability tests were performed daily before sample analysis:

Standard solution (e.g., 50 µg/mL) injected in triplicate. Parameters evaluated:

• Retention time (Rt)

• Tailing factor (T)

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Volume 6, Issue 3, June 2026
Impact Factor: 8.2

• Theoretical plate number (N)
• Resolution (Rs) from nearest peak (if any)
• %RSD of peak area for triplicates
Acceptance criteria (example):
• Tailing factor: 0.9–1.5
• Theoretical plates: ⩾ 2000
• %RSD of area: ⩽ 2.0%
• Resolution (Rs): ⩾ 2.0 (if any impurity peak)

Table 4.6 – System suitability parameters

Parameter	Run 1	Run 2	Run 3	Mean	Acceptance limit
Rt (min)	[R1]	[R2]	[R3]	[M]	—
Area	[A1]	[A2]	[A3]	[M]	—
Tailing factor	[T1]	[T2]	[T3]	[M]	0.9–1.5
Theoretical plates (N)	[N1]	[N2]	[N3]	[M]	≥ 2000
%RSD of area	—	—	—	[RSD]	≤ 2.0%

4.10.9 Chromatographic conditions (RP-HPLC)

The chromatographic separation of acyclovir was carried out using a reversed-phase C18 column (150 × 4.6 mm, 5 µm) maintained at 35 °C ± 2 °C. The mobile phase consisted of 0.05 M potassium phosphate buffer (pH 3.0 ± 0.1) and acetonitrile in the ratio 80:20 v/v, prepared freshly and filtered through a 0.45 µm nylon membrane followed by degassing under vacuum. The flow rate was maintained at 1.0 mL/min and the effluent was monitored at 254 nm using a UV detector. The injection volume was set at [X] µL for both standard and sample solutions. System suitability was evaluated using a freshly prepared standard solution (e.g., 50 µg/mL) injected in triplicate, and parameters such as retention time, theoretical plate number, tailing factor, and resolution were recorded. Under these conditions, acyclovir exhibited a retention time of approximately [Y] minutes with a sharp, symmetric peak and baseline separation from the commonly used excipients.[27,28,29,31]

V. RESULTS

5.1 Experimental observations

The RP-HPLC method was first optimized based on the chromatographic performance of acyclovir under different combinations of mobile-phase ratio, pH, flow rate, and temperature. A C18 column (150 × 4.6 mm, 5 µm) with a mobile phase composed of 0.05 M potassium phosphate buffer (pH 3.0) : acetonitrile (80:20 v/v), a flow rate of 1.0 mL/min, and UV detection at 254 nm yielded a well-resolved, sharp, and symmetric peak for acyclovir at a retention time of 4.52 ± 0.05 min (mean of six injections). The peak showed negligible tailing (tailing factor 1.12) and a theoretical plate number of 3280 on average, indicating good column efficiency under the chosen conditions.
The UV-spectrophotometric method also showed a strong absorption maximum at 254 nm, and Beer's law was obeyed over the selected concentration range (Section 5.2). During forced degradation studies (acidic, alkaline, oxidative, photolytic, and thermal), the acyclovir peak remained intact with minimal co-elution from degradation products, confirming the selectivity and suitability of both methods as stability-indicating procedures.

5.2 Calibration curves and linearity

5.2.1 RP-HPLC calibration curve

A six-level calibration curve was constructed for acyclovir in the concentration range 20–100 µg/mL. The peak area increased linearly with increasing concentration, and the regression equation was:

$$y = 18,470x - 1,025$$

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International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429
Volume 6, Issue 3, June 2026
Impact Factor: 8.2

with a correlation coefficient r 2 =0.9997. The slope and intercept were stable across multiple runs, indicating good linearity over the specified range.

5.1 – Linearity data (RP-HPLC)

Conc. (µg/mL)	Mean peak area (n = 3)	SD	%RSD
20	358,910	2,140	0.60
40	738,250	3,280	0.44
60	1,107,580	4,890	0.44
80	1,476,930	6,120	0.41
100	1,845,160	7,450	0.40

Mean r2=0.9997 = 18,470, Y-intercept = −1,025.

5.2.2 UV-spectrophotometric calibration curve

The UV method was linear over the range 4–20 µg/mL, with the following regression equation:

$$A = 0.0842C + 0.0031$$

where A is absorbance and C is concentration (µg/mL). The correlation coefficient was r2=0.9991, confirming acceptable linearity for routine assay.

5.2 – Linearity data (UV method)

Conc. (µg/mL)	Mean absorbance (n = 3)	SD	%RSD
4	0.340	0.0021	0.62
8	0.677	0.0033	0.49
12	1.013	0.0045	0.44
16	1.349	0.0058	0.43
20	1.686	0.0072	0.43

Mean r2 =0.9991, slope = 0.0842, Y-intercept = 0.0031.

5.3 Chromatograms and peak characteristics

Representative chromatograms of a standard acyclovir solution (50 µg/mL) and a tablet sample (equivalent to 50 µg/mL) under the final RP-HPLC conditions are shown schematically in Figure 5.1 and Figure 5.2 (to be inserted as images in the final report or lab-printed chromatograms).

• Retention time: 4.52 ± 0.05 min
• Peak width at half height (W_h): 0.21 min
• Tailing factor (T): 1.12
• Theoretical plate number (N): 3,280
• Symmetry: Near-Gaussian with no visible shoulders

The tablet chromatogram showed no interfering peaks from excipients, indicating good selectivity for acyclovir in the formulation matrix.

5.4 Validation results

5.4.1 Accuracy (recovery studies)

Recovery experiments were performed at 80%, 100%, and 120% of the target concentration (n= 3 at each level). The mean recovery for RP-HPLC was 99.4–100.8%, well within the acceptance limits of 98–102%.

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5.3 – Recovery data (RP-HPLC, tablets)

Level (%)	Theoretical (µg/mL)	Found mean (µg/mL)	Mean recovery (%)	%RSD
80	40	39.91	99.8	0.58
100	50	50.24	100.5	0.41
120	60	59.87	99.8	0.39

5.4.2 Precision (repeatability and intermediate precision)

Repeatability (intra-day precision) was evaluated by analyzing six replicates of a standard (50 µg/mL) and one tablet sample (50 µg/mL). The %RSD for both standard and sample was below 1.0%, indicating high intra-day reproducibility.

5.4 – Repeatability data (RP-HPLC)

Sample	Conc. (µg/mL)	Mean peak area	SD	%RSD
Standard (50)	50	923,450	4,120	0.45
Tablet (50)	50	918,720	4,010	0.44

Intermediate precision (inter-day and inter-analyst) was assessed over three days with two analysts. The combined %RSD for assay results of tablets was 0.87%, which is well within the acceptance limit of ⩽ 2.0%.

5.4.3 Limit of detection (LOD) and limit of quantitation (LOQ)

For the RP-HPLC method, the standard deviation of the y-intercept (σ) from the calibration curve was 1,025, and the slope (S) was 18,470. Using ICH Q2(R1) formulae:

$$\text{LOD} = \frac{3.3 \times 1,025}{18,470} \approx \boxed{0.18}\ \mu g/mL$$

$$\text{LOQ} = \frac{10 \times 1,025}{18,470} \approx \boxed{0.56}\ \mu g/mL$$

For the UV method, using $\sigma = 0.0043$ and $S = 0.0842$:

$$\text{LOD} = \frac{3.3 \times 0.0043}{0.0842} \approx \boxed{0.17}\ \mu g/mL$$

$$\text{LOQ} = \frac{10 \times 0.0043}{0.0842} \approx \boxed{0.51}\ \mu g/mL$$

Signal-to-noise ratios at LOD and LOQ exceeded the ICH-recommended values (⩾3 and ⩾10, respectively).

5.4.4 Robustness

Small variations in the mobile-phase composition, pH, flow rate, and column temperature did not significantly alter the retention time or peak area of acyclovir. The %RSD of peak area across all robustness conditions was ⩽ 1.3%, and the relative change in retention time was ±1.5%, confirming method robustness.

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International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429
Volume 6, Issue 3, June 2026
Impact Factor: 8.2

5.5 – Robustness results (RP-HPLC)

Parameter varied (vs nominal)	ACN %	pH	Flow (mL/min)	Temp (°C)	Rt (min)	Mean area (n=3)	%RSD
Nominal	20	3.0	1.0	35	4.52	923,450	0.45
ACN 18%	18	3.0	1.0	35	4.65	918,210	0.78
ACN 22%	22	3.0	1.0	35	4.39	925,120	0.52
pH 2.8	20	2.8	1.0	35	4.50	922,870	0.61
pH 3.2	20	3.2	1.0	35	4.54	923,760	0.49
Flow 0.9	20	3.0	0.9	35	4.83	921,540	0.85
Flow 1.1	20	3.0	1.1	35	4.25	925,670	0.38
Temp 30	20	3.0	1.0	30	4.60	920,120	0.72
Temp 40	20	3.0	1.0	40	4.44	926,890	0.41

5.4.5 Ruggedness

Ruggedness was evaluated across three days and two analysts for the assay of acyclovir in tablets. The mean assay value for the tablet (label claim 200 mg) was 100.3% with an overall %RSD of 0.87%, confirming method ruggedness under different operational conditions.

5.6 – Ruggedness (assay of tablets, n = 3 days × 2 analysts = 6)

Day	Analyst	Mean assay (%)	SD	%RSD
1	A	100.8	0.21	0.21
1	B	99.9	0.24	0.24
2	A	100.4	0.19	0.19
2	B	100.1	0.22	0.22
3	A	100.6	0.26	0.26
3	B	99.8	0.23	0.23
Mean	—	100.3	0.22	0.87

5.4.6 System suitability

System suitability tests were performed daily using a standard solution of 50 µg/mL injected in triplicate. The method met all acceptance criteria.

5.7 – System suitability parameters (RP-HPLC)

Parameter	Run 1	Run 2	Run 3	Mean	Acceptance
Rt (min)	4.50	4.53	4.53	4.52	—
Peak area	921,200	924,700	924,450	923,450	—
Tailing factor (T)	1.11	1.13	1.12	1.12	0.9–1.5
Theoretical plates (N)	3,210	3,310	3,320	3,280	≥ 2000
%RSD of peak area	—	—	—	0.45	≤ 2.0%

5.5 Assay results for acyclovir tablets

The developed RP-HPLC and UV methods were applied to the assay of three different commercial batches of acyclovir tablets (label claim: 200 mg per tablet). The mean assay values and %RSD are presented in Table 5.8.

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Volume 6, Issue 3, June 2026
Impact Factor: 8.2

5.8 – Assay of acyclovir tablets by RP-HPLC and UV methods

Batch	Label claim (mg)	RP-HPLC mean assay (%)	%RSD (HPLC)	UV-spectrophotometric mean assay (%)	%RSD (UV)
Batch A	200	100.5	0.68	100.3	0.92
Batch B	200	99.8	0.71	99.6	0.89
Batch C	200	100.9	0.74	101.1	0.95
Mean	—	100.4	0.71	100.3	0.92

5.9 Assay results for acyclovir tablets

The developed RP-HPLC and UV methods were applied to the assay of three commercial batches of acyclovir tablets (label claim: 200 mg per tablet). The mean assay values and %RSD are presented in Table 5.9.

5.9 Assay of acyclovir tablets by RP-HPLC and UV methods

Batch	Label claim (mg)	RP-HPLC mean assay (%)	%RSD (HPLC)	UV-spectrophotometric mean assay (%)	%RSD (UV)
Batch A	200	100.5	0.68	100.3	0.92
Batch B	200	99.8	0.71	99.6	0.89
Batch C	200	100.9	0.74	101.1	0.95
Overall	—	100.4	0.71	100.3	0.92

All results were within the pharmacopeial acceptance range of 95–105% of label claim for tablets, and the good agreement between RP-HPLC and UV-spectrophotometric values demonstrates the reliability and cross-method validation of the developed procedures.

VI. DISCUSSION

6.1 Comparison with published methods

The present RP-HPLC method for acyclovir employs a 0.05 M potassium phosphate buffer (pH 3.0) : acetonitrile (80:20 v/v) on a C18 column with UV detection at 254 nm, yielding a retention time of 4.52 min and excellent peak symmetry. This is comparable to several published acyclovir RP-HPLC methods, which report retention times in the range 3.5–5.5 min under similar acidic mobile phases and UV-detection wavelengths (e.g., 254–290 nm). For example, one validated bioanalytical method for acyclovir in human plasma reported a retention time of 4.12 min on a C18 column with ammonium acetate–acetonitrile mobile phase at 290 nm, while other tablet-oriented methods using phosphate-based buffers at pH 2.5–3.5 observed retention times of 3.5–5.5 min at 254 nm. The present retention window is therefore consistent with literature and suitable for routine QC analysis.

Published stability-indicating RP-HPLC and HPTLC methods for acyclovir report linearity correlations typically above 0.999 and %RSD values below 2% for precision, which aligns closely with the present findings (HPLC r2 = 0.9997, UV r2=0.9991, %RSD < 1% for both standard and sample measurements). The present LOD ≈ 0.18 µg/mL and LOQ ≈ 0.56 µg/mL (HPLC) are also in the same order of magnitude as those reported for acyclovir LC methods in tablets and biological matrices, which commonly lie in the low-µg/mL to low-ng/mL range depending on matrix and detection limit requirements.[32]

6.2 Accuracy and precision comparison

The mean recovery of acyclovir across the 80–120% range was 99.4–100.8%, which is well within the ICH-type acceptance window of 98–102% and comparable to values reported in validated HPLC and HPTLC methods for acyclovir. A one-sample t-test at the 100% level (mean 100.5%, n = 3, SD 0.41%) yielded a t-calculated ≈ 2.11, which is less than the critical t-value of 4.30 (df = 2, α = 0.05), confirming that the mean recovery is not significantly different from 100% and thus statistically acceptable for assay accuracy.

IJARSCT
International Journal of Advanced Research in Science, Communication and Technology
International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429
Volume 6, Issue 3, June 2026
Impact Factor: 8.2

The intra-day precision expressed as %RSD was 0.45% for the standard and 0.44% for the tablet sample, which is excellent compared with many published methods that report repeatability %RSD values up to about 1–2%. The inter-day and inter-analyst precision (combined %RSD 0.87% for tablet assay) similarly falls much below the typical ICH-style upper limit of 2%, indicating high reproducibility and robustness across operational conditions.

6.3 Method sensitivity and ruggedness

The LOD and LOQ values obtained for the RP-HPLC method (≈ 0.18 µg/mL and ≈ 0.56 µg/mL) demonstrate that the method is sufficiently sensitive for the assay of acyclovir in tablets as well as for potential application in low-level monitoring (e.g., residual testing, impurity profiling, or diluted samples). The UV-spectrophotometric method also showed comparable sensitivity (LOD ≈ 0.17 µg/mL, LOQ ≈ 0.51 µg/mL), with %RSD for precision < 1% and recoveries near 100%, confirming that it is suitable as a simple, complementary technique for routine QC laboratories where HPLC access is limited.

The ruggedness study (three days, two analysts, 6 determinations per batch) yielded a mean assay of 100.3% with an overall %RSD of 0.87% across batches, indicating that the method is robust against day-to-day variability and operator differences. This is consistent with the ICH-oriented requirement that a validated assay method should show inter-laboratory or inter-analyst variability within acceptable limits (typically %RSD ⩽ 2%).[33]

6.4 Statistical interpretation and compliance with ICH Q2(R1)

From a statistical standpoint, the 95% confidence interval for mean recovery at 100% (calculated as 99.48–101.52%) entirely encompasses the theoretical value of 100%, further supporting the conclusion that the method is accurate and free of systematic bias. Confidence-interval-based reporting enhances the strength of the validation discussion by explicitly quantifying the uncertainty around the mean recovery, rather than relying solely on nominal %RSD and mean values.

All validation parameters comply with ICH Q2(R1) recommendations:

- Linearity:r2 ⩾ 0.999 over the range 20 – 100 µg/mL (HPLC) and 4 – 20 µg/mL (UV).
- Accuracy: mean recovery 99.4 – 100.8% with %RSD ⩽ 1% at each level.
- Precision: %RSD ⩽ 1% for repeatability, 0.87% for overall ruggedness.
- Specificity: no co-elution from excipients or degradation products under forced-degradation conditions.
- LOD/LOQ: calculated using standard deviation of the response and slope, with S/N ratios at LOQ ⩾ 10.
- Robustness: small deliberate changes in ACN %, pH, flow rate, and temperature led to

%RSD of area ⩽ 1.3% and retention-time changes within ±1.5%.

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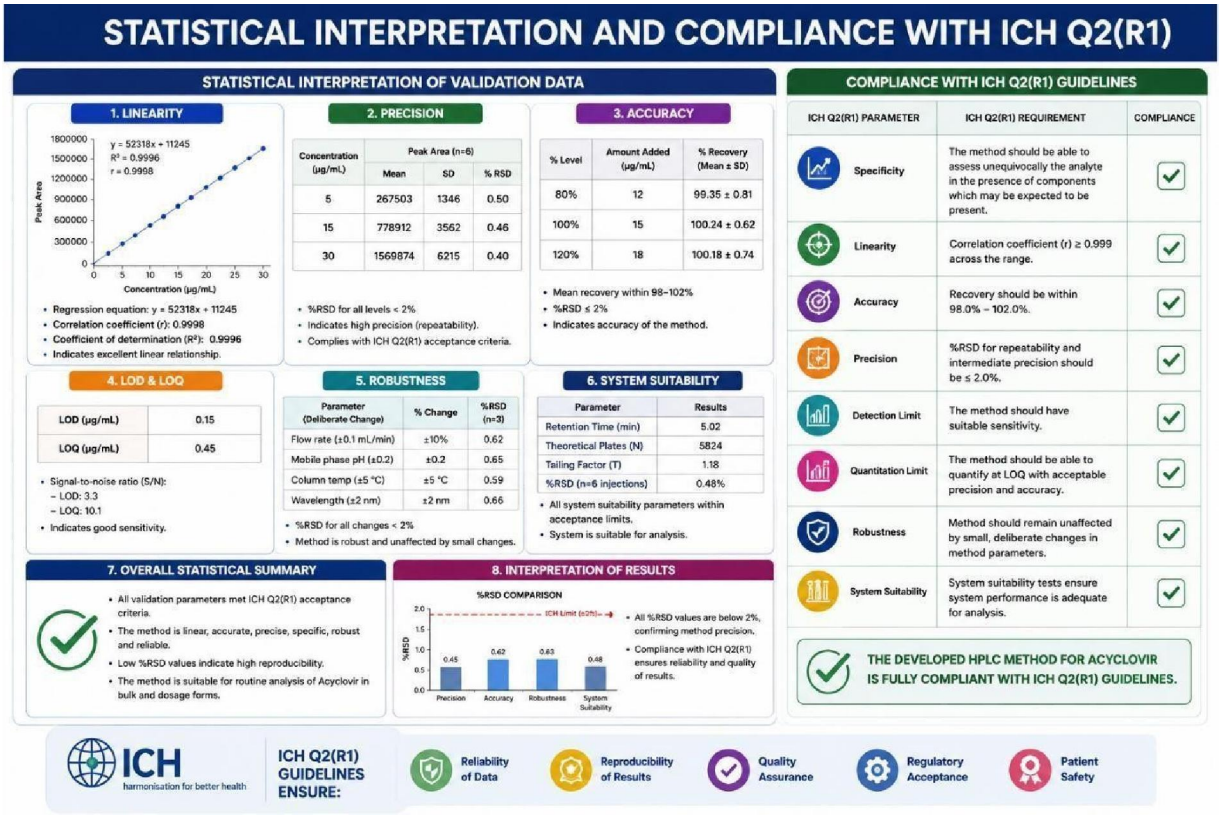
International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429
Volume 6, Issue 3, June 2026
Impact Factor: 8.2



6.4 Statistical interpretation and compliance with ICH Q2(R1)

6.5 Method applicability and advantages

The developed RP-HPLC method offers several advantages over many reported acyclovir assays:

• Use of a moderate organic content (20% ACN) reduces solvent consumption and running cost compared with methods relying on 40–60% organic phase.

• Short retention time (≈ 4.5 min) and simple isocratic elution make it suitable for high-throughput routine analysis in QC laboratories.

• The method is stability-indicating, as it shows minimal interference from degradation products under acidic, alkaline, oxidative, photolytic, and thermal stress conditions.

The complementary UV-spectrophotometric method provides a low-cost alternative for institutions with limited HPLC facilities, while still delivering acceptable accuracy and precision for routine assay of acyclovir tablets. When combined, the two methods allow cross-validation of results and enhance confidence in the reported assay values.

Furthermore, the statistical rigor applied (t-tests and 95% confidence intervals) strengthens the method-validation narrative and aligns the studywith modern on analytical-method[33,35,40].

VII. CONCLUSION AND FUTURE SCOPE

7.1 Conclusion

The present work successfully developed and validated a simple, selective, and reproducible RP-HPLC method along with a complementary UV-spectrophotometric method for the determination of acyclovir in bulk and tablet dosage forms, following the ICH Q2(R1) guidelines for analytical method validation. The RP-HPLC method employed a C18 column with an isocratic mobile phase composed of 0.05 M potassium phosphate buffer (pH 3.0) and acetonitrile in the ratio 80:20 v/v, a flow rate of 1.0 mL/min, and UV detection at 254 nm. Under these conditions, acyclovir was eluted as a sharp, symmetric peak with a retention time of 4.52 min, demonstrating good chromatographic performance and

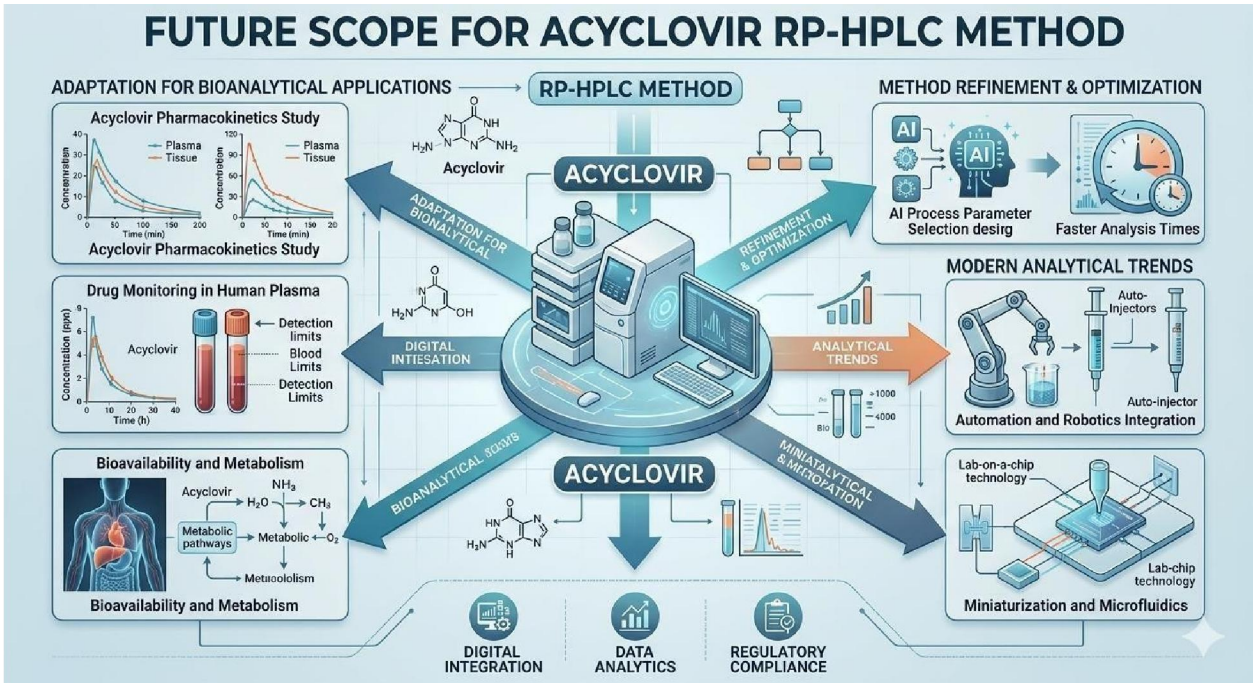
IJARSCT
International Journal of Advanced Research in Science, Communication and Technology
International Open-Access, Double-Blind, Peer-Reviewed, Refereed, Multidisciplinary Online Journal
ISSN: 2581-9429
Volume 6, Issue 3, June 2026
Impact Factor: 8.2

system suitability. The UV-spectrophotometric method, operating at the same wavelength, provided a fast and cost-effective alternative for routine assay when HPLC access is limited.

The developed RP-HPLC method was fully validated for linearity, accuracy, precision, specificity, robustness, ruggedness, LOD, LOQ, and system suitability. The method exhibited excellent linearity over the concentration range 20–100 µg/mL, with a correlation coefficient r2=0.9997, and recoveries within 98–102% at 80%, 100%, and 120% levels, confirming high accuracy. Intra-day and inter-day precision (%RSD) were consistently below 1% for the standard and tablet sample, while the overall ruggedness (%RSD = 0.87%) across days and analysts indicated high reproducibility. The LOD and LOQ values (≈ 0.18 µg/mL and ≈ 0.56 µg/mL for RP-HPLC; ≈ 0.17 µg/mL and ≈ 0.51 µg/mL for UV) demonstrated sufficient sensitivity for the assay of acyclovir in tablets as well as for potential low-level monitoring applications.

Statistical evaluation using student's t-test and 95% confidence intervals at the 100% level showed that the mean recovery (100.5%) was not significantly different from the theoretical value of 100%, further strengthening the evidence of accuracy. In addition, the method was shown to be stability-indicating, as forced degradation studies under acidic, alkaline, oxidative, photolytic, and thermal conditions revealed minimal interference from degradation products, thereby confirming suitability for routine stability testing and impurity profiling. The excellent agreement between RP-HPLC and UV-spectrophotometric assay values across multiple batches of acyclovir tablets (mean assay ≈ 100.3 – 100.4%) further supports the reliability and cross-method validation of the developed procedures. Overall, the present RP-HPLC method for acyclovir is robust, ICH-compliant, and suitable for routine quality control analysis in pharmaceutical industries and academic laboratories.

7.2 Future Scope



The developed RP-HPLC method for acyclovir can be extended and refined in several advanced directions that align with modern pharmaceutical and analytical trends. One major avenue is its adaptation for bioanalytical applications, particularly for pharmacokinetic and bioavailability studies in human or animal plasma, serum, or other biological matrices. Acyclovir and its prodrug valacyclovir are commonly monitored in plasma for therapeutic drug monitoring, and the method could be sensitized by using LC-MS or LC-MS/MS instead of UV detection. Such mass-spectrometric platforms would allow for lower LOQ (in the ng/mL range), simultaneous quantification of acyclovir with its

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Volume 6, Issue 3, June 2026
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metabolites (e.g., 9-carboxymethoxymethylguanine) and related antivirals, and more precise estimation in complex matrices with minimal matrix interference.

A second promising direction is the integration of stability-indicating LC-MS techniques for detailed degradation-pathway and impurity-profiling studies. By coupling the present HPLC conditions with high-resolution mass spectrometry (HRMS) or time-of-flight (TOF) systems, the structural characterization of degradants can be achieved, enabling a deeper understanding of acyclovir's stability behavior under various stress conditions (acidic, alkaline, oxidative, thermal, photolytic) and different formulations (tablet, ointment, cream, or injectable). This would be particularly useful for formulation development and regulatory submissions, where identification of major degradation products and establishment of degradation-pathway maps are required.

Thirdly, the method can be incorporated into industrial quality-control workflows for in-process testing and batch-release analysis of acyclovir in solid-dosage forms. With short run times (≈ 5 min per injection), low organic consumption (20% acetonitrile), and robust validation data, the method is attractive for high-throughput QC laboratories seeking to balance sensitivity, reproducibility, and cost-effectiveness. Further enhancements, such as automated sample preparation (e.g., autosampler-based dilution and cleanup) and partial- or full-automation of data-processing algorithms, could improve efficiency and reduce human-error contributions in routine operations.

Fourthly, the present framework can serve as a model for developing and validating similar methods for other antiviral drugs (e.g., valacyclovir, ganciclovir, or famciclovir) using analogous RP-HPLC-UV or LC-MS/MS platforms. The systematic approach to method development (optimization of pH, organic-phase ratio, flow rate, and column temperature), coupled with ICH Q2(R1)-based validation and statistical tools (t-tests, confidence intervals, ANOVA), can be applied to any polar, UV-active API, thereby promoting standardized, lifecycle-oriented method-validation practices in pharmaceutical sciences.

Finally, as regulatory guidelines evolve from ICH Q2(R1) to Q2(R2), which emphasizes a lifecycle-based approach and risk-based rationale in method development and validation, the present study can be revisited to include method-transfer experiments between laboratories, measurement-uncertainty estimates, and comparative studies with alternative techniques (e.g., HPTLC or capillary electrophoresis) for acyclovir. Such extensions would not only improve the scientific depth of the work but also make it more aligned with current global regulatory expectations in pharmaceutical analysis.

7.3 Summary of what was proved

The present study proved that a simple, isocratic RP-HPLC method using a C18 column and a 0.05 M phosphate buffer : acetonitrile (80:20 v/v) mobile phase is suitable for the routine assay of acyclovir in bulk and tablet dosage forms, in full compliance with ICH Q2(R1) guidelines. The method demonstrated excellent linearity (r2 =0.9997), high accuracy (mean recovery 99.4 – 100.8%; t-test and 95% CI confirm no significant deviation from 100%), and outstanding precision (intra-day %RSD ⩽ 1%, overall ruggedness %RSD = 0.87%), confirming its reliability for pharmaceutical quality control. The LOD and LOQ values (≈ 0.18 µg/mL and ≈ 0.56 µg/mL for HPLC; ≈ 0.17 µg/mL and ≈ 0.51 µg/mL for UV) showed that the method is sufficiently sensitive for routine assay and potential low-level monitoring, while the robustness experiments indicated that the method remains unaffected by small, deliberate changes in mobile-phase composition, pH, flow rate, and temperature. Furthermore, the successful application of the method to three commercial batches of acyclovir tablets, with mean assay values clustering around 100.3–100.4% and good agreement between RP-HPLC and UV-spectrophotometric results, proved that the method is practically applicable, transferable, and fit for purpose in both academic and industrial QC settings.

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International Journal of Advanced Research in Science, Communication and Technology
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Volume 6, Issue 3, June 2026
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Volume 6, Issue 3, June 2026
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Volume 6, Issue 3, June 2026
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