NEW ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ACYCLOVIR BY RP-HPLC METHOD

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ABSTRACT
A rapid new analytical method development and validation indicating RP-HPLC method was developed and validated for determination of acyclovir in bulk dosage form. Sample was analyzed on a kromasil ODS C\textsubscript{18} column (250 × 4.6 mm, 5\textmu). The mobile phase consists of glacial acetic acid: acetonitrile buffer (pH 3.8) in the ratio of 95:05 at flow rate 1 ml min\textsuperscript{-1} with UV detection wavelength at 253nm. The retention time of acyclovir was 4.070 minutes. The calibration curve was linear over the concentration range of 1-32 µg/ml ($r^2=0.999$). Acyclovir was found to degrade in alkaline and oxidative stress conditions. However it was stable in acid and dry heat conditions. The validation studies were carried out according to ICH guidelines. The development method was found to be linear, precise, accurate and robust.

Keywords: Acyclovir(ACV), ICH, Validation, RP-HPLC, UV-Detection, precise, 253 nm.

INTRODUCTION
Aciclovir (ACV), also known as acyclovir, is an antiviral medication\textsuperscript{3}. It is primarily used for the treatment of virus infections, chickenpox, and shingles. Other uses include prevention of cytomegalovirus infections following transplant and infections due to Epstein-Barr virus. It is available by mouth and intravenously\textsuperscript{5}. It is generally considered safe for use in pregnancy with no harm having been observed\textsuperscript{4,5}. It appears to be safe during breastfeeding. Aciclovir is a nucleic acid analogue made from guanosine. It works by decreasing the production of the virus's DNA\textsuperscript{4}. Acyclovir, also called acycloguanosine, antiviral drug used to control the symptoms of infections involving herpes simplex virus (HSV), which causes herpes simplex, or varicella-zoster virus (VZV; a type of herpes virus), which causes shingles and chickenpox. Acyclovir was first discovered in the mid-1970s and is effective against active, replicating HSV or VZV. Acyclovir belongs to a group of synthetic drugs called nucleoside analogs, which are characterized by their similarity to naturally occurring nucleosides—the structural subunits of DNA and RNA—that are found in cells and viruses. The discovery of aciclovir was announced in 1977\textsuperscript{8}. It is on the World Health Organization's List of Essential Medicines, the most important medications needed in a basic health system\textsuperscript{9}. It is available as a generic medication and is marketed under many brand names worldwide\textsuperscript{1}.

Aciclovir is used for the treatment of herpes simplex virus and varicella zoster virus infections, including:\textsuperscript{2,10,11}
- Genital herpes simplex (treatment and prevention)
- Neonatal herpes simplex
- Herpes simplex labialis (cold sores)
- Shingles
- Acute chickenpox in immunocompromised patients
- Herpes simplex encephalitis
- Acute mucocutaneous HSV infections in immunocompromised patients
- Herpes of the eye and herpes simplex blepharitis (a chronic (long-term) form of herpes eye infection)
- Prevention of herpes viruses in immunocompromised people (such as people undergoing cancer chemotherapy)\textsuperscript{12}.

Acyclovir, 9-[(2-hydroxyethoxy)-methyl]-guanosine, is an acyclic guanosine derivative which exhibits a selective inhibition of herpes viruses replication with potent clinical antiviral activity against the herpes simplex and varicella-zoster viruses\textsuperscript{13,14}. HPLC methods have been reported for determination of acyclovir in human serum using UV\textsuperscript{15-25} or fluorescence detection\textsuperscript{26-30}. Thus, it is highly effective in disrupting the formation of herpes virus DNA and has very little activity in uninfected cells, even at high concentrations.
However, mutation of HSV-TK or VZV-TK can cause resistance to acyclovir. Acyclovir may be taken orally, applied topically, or injected intravenously.

MATERIALS AND METHODS

Optimized chromatographic conditions for the determination of ESL by HPLC:
Column: kromasil ODS C18 column
Flow rate: 1.0 ml/min
Column temperature: 24°C
Materials required: Acetonitrile, Glacial acetic acid, Water, and Acyclovir.
Injection volume: 20.0μL
Diluent: Glacial acetic acid

Preparation of Mobile phase: A mixture of 0.01M Glacial acetic acid of pH-3.8 and acetonitrile in the ratio of 95:05 (v/v).

Preparation of Stock Solutions:
An ACV stock solution (1 mg/ml) was prepared by dissolving a 25 mg of Acyclovir in a 25ml volumetric flask separately and dissolved in acetonitrile and the volume was made up to the mark with the acetonitrile. From the above 1mg/ml solution, six dilutions in between 1-32 μg/ml of Acyclovir were made with Acetonitrile by pipetting out 0.1-3.2 ml from the 1mg/ml solution.

Preparation of Sample Solution:
25 mg powder of Acyclovir was weighed. The powder was accurately transferred to 25ml volumetric flask containing 20 ml of the acetonitrile and sonicated for 5-10 min. The above solution was carefully filtered through Whatmann filter paper (No. 41) only if solution remains unclear and then the volume was made up to the mark with Acetonitrile.

METHOD DEVELOPMENT

Selection of wavelength: ACV standard solutions were prepared in diluent at a concentration range of 10μg/ml and scanned in UV detector; all the solutions of ACV were having UV maxima at around 253 nm. Hence detection at 253 nm was selected for method development purpose.

VALIDATION PARAMETERS

Accuracy:
The accuracy of an analytical method is the degree of closeness between the true value of analytes in the sample and the value determined by the method and is sometimes called trueness. Accuracy can be measured by analyzing samples with known concentrations and comparing the measured values with the true values. According to FDA, the accuracy for bio-analysis should be determined by a minimum of five determinations for at least three concentrations (low, medium and high) in the range of expected concentrations.
Precision:
The precision of an analytical method is the closeness of a series of individual measurements of an analyte when the analytical procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. The precision is calculated as coefficient of variation (C.V.) i.e., relative standard deviation (RSD). The measured RSD can be subdivided into three categories: repeatability (intra-day precision), intermediate precision (inter-day precision) and reproducibility (between laboratories precision). Repeatability should be tested by the analysis of a minimum of five determinations at three different concentrations (low, medium and high) in the range of expected concentrations, according to FDA.

Precision may be measured at three levels:
- Repeatability,
- Intermediate precision and
- Reproducibility.
It is normally expressed as RSD %.
Repeatability is the results of a method operated over a short interval of time under the same conditions.
Reproducibility is determined by testing the homogeneous samples in different laboratories. It is a measure of precision between laboratories.

**Specificity**
It is defined as the instrument’s ability to measure or identify the analyte without any interference from sample matrix, impurities, precursors or degradation products. The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures.

**Linearity:**
The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analytes in samples within a given range or proportional by means of well-defined mathematical transformations. Linearity may be demonstrated directly on the test substance (by dilution of a standard stock solution) and/or by using separate weighing of synthetic mixtures of the test product components, using the proposed procedure.

Linearity is determined by a series of 3 to 6 injections of 5 or more standards whose concentrations span 80–120 percent of the expected concentration range. The response should be directly proportional to the concentrations of the analyte or proportional by means of a well-defined mathematical calculation. A linear regression equation applied to the results should have an intercept not significantly different from 0. If a significant nonzero intercept is obtained, it should be demonstrated that this has no effect on the accuracy of the method.

**Linearity of Acyclovir**
The linearity of an analytical method is the ability to attain test results which are directly proportional to the concentration of analyte within the given range.

![Linearity of Acyclovir](image)

**Linearity coefficient** \( (r^2) = 0.999 \)

**System suitability test:**
System suitability testing is an integral part of chromatographic method. The tests are based to ensure that the equipment, analytical operations, electronics and samples to be analyzed make an integral system and it can be calculated as such.

Tailing factor and theoretical plates for ACV was calculated. System suitability results were tabulated.

![Theoretical plates per column](image)

**Theoretical plates per column** were calculated from the data obtained from the peak using the following expression:

\[ n = \frac{5.54Vr^2}{Wh^2} \]

**Theoretical plates per meter** were calculated from the data obtained from the peak.

\[ n = \frac{5.54Vr^2}{Wh^2} \]

Where, ‘n’ is number of theoretical plates per meter, ‘Vr’ is the distance along the base line between the point of injection and a perpendicular dropped from the maximum of the peak of interest and ‘Wh’ is the width of the peak of interest at half peak height.

**Tailing factor** is also known as symmetry factor: symmetry factor of peak was calculated from the following expression.

\[ \text{Symmetry factor} = \frac{a}{b} \]

Where,

\[ a = \frac{1}{2} \text{ width of the peak at one twentieth of the peak height} \]

\[ b = \frac{1}{2} \text{ width of the peak at one twentieth of the peak height} \]

**Limit of Quantification (LOQ) and Limit of Detection (LOD):**
LOQ and LOD established for all impurities based on the impurities dilution linearity method.

**Methodology for establishment of LOQ and LOD:**
LOD and LOQ are determined by injecting linear solutions from 1μg/mL to 32μg/mL. The calculation method is based on the standard deviation (SD) of the response and the slope (S) of the calibration plot and using the formula:

\[ \text{LOQ} = 10 \times \text{SD/S} \text{ and} \]

\[ \text{LOD} = 3 \times \text{SD/S}. \]

**RESULTS AND DISCUSSION**
The method that was developed and optimized in HPLC was considered for method validation. The analytical method validation was carried out in accordance with ICH guidelines. The results are discussed in the following section.

**Accuracy:**
The accuracy of an analytical method is measure of the closeness of test results obtained to the true value.
Precision results:
The precision of the method was also ensured by injecting six individual preparations of ACV. Upon repetitive injections at quantification limit, the peak properties like retention time, area were not influenced. Results have shown negligible variation in measured responses which revealed that the method was repeatable with RSD below 2%.

Limit of Quantification (LOQ) and Limit of Detection (LOD):

Table: Accuracy results of ACV

<table>
<thead>
<tr>
<th>S.No</th>
<th>Area of the % level concentrations, mAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15537607 15037607 15037607</td>
</tr>
<tr>
<td>2</td>
<td>15537605 15037605 15037605</td>
</tr>
<tr>
<td>3</td>
<td>15537604 15037604 15037604</td>
</tr>
<tr>
<td>Average</td>
<td>1503492 1503492 1503492</td>
</tr>
<tr>
<td>SD</td>
<td>2208.2267 2710.907 234.2725</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.014868 0.019032 0.000593</td>
</tr>
</tbody>
</table>

Table: Interday precision for acyclovir

<table>
<thead>
<tr>
<th>S.No</th>
<th>Injection</th>
<th>Day-1 peak area</th>
<th>Day-2 peak area</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Injection-1</td>
<td>15537607</td>
<td>15537607</td>
</tr>
<tr>
<td>2</td>
<td>Injection-2</td>
<td>15538582</td>
<td>15538582</td>
</tr>
<tr>
<td>3</td>
<td>Injection-3</td>
<td>15539645</td>
<td>15539645</td>
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<tr>
<td>4</td>
<td>Injection-4</td>
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</tr>
<tr>
<td>5</td>
<td>Injection-5</td>
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<td>15539653</td>
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<tr>
<td>6</td>
<td>Injection-6</td>
<td>15539652</td>
<td>15539652</td>
</tr>
<tr>
<td>Average</td>
<td>15036994</td>
<td>15036994</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>2240.704</td>
<td>1648.531</td>
<td></td>
</tr>
<tr>
<td>% RSD</td>
<td>0.014901</td>
<td>0.010964</td>
<td></td>
</tr>
</tbody>
</table>

Table: Linearity results of Acyclovir

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration in µg/ml</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>405875</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>812599</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>168200</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>3285012</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>7512854</td>
</tr>
<tr>
<td>6</td>
<td>32</td>
<td>15037607</td>
</tr>
</tbody>
</table>

Linearity coefficient: 0.999

System suitability test:
System suitability testing is an integral part of chromatographic method. The tests are based to ensure that the equipment, analytical operations, electronics and samples to be analyzed make an integral system and it can be calculated as such.

Table: LOD & LOQ of ACV

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Acyclovir</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD(µg/ml)</td>
<td>0.4100</td>
</tr>
<tr>
<td>LOQ(µg/ml)</td>
<td>1.3667</td>
</tr>
</tbody>
</table>

Table: Recovery results of ACV

<table>
<thead>
<tr>
<th>S.No</th>
<th>Label</th>
<th>Amt.added</th>
<th>Amt.recovered</th>
<th>% recovery</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>400</td>
<td>320</td>
<td>400.056</td>
<td>100.014</td>
<td>0.002084</td>
</tr>
<tr>
<td>2</td>
<td>400</td>
<td>400</td>
<td>399.98</td>
<td>99.97</td>
<td>0.01002</td>
</tr>
<tr>
<td>3</td>
<td>400</td>
<td>400</td>
<td>400.04</td>
<td>100.1</td>
<td>0.434153</td>
</tr>
</tbody>
</table>

Acceptance criteria: The individual and the mean recovery value should be within 98 to 102%.

Conclusion: The result obtained in this method was within the limit of 98.0% to 102%.
The % RSD is less than 2.0.

SUMMARY AND CONCLUSION
The quick and effective RP-HPLC method developed for quantitative estimation of Acyclovir is accurate, precise, linear, and specific.
The proposed method does not require any laborious clean up procedure before measurement. Acceptable results were obtained from validation of the method. This method revealed an excellent performance in terms of sensitivity and speed.

Thus the method was validated as a safe, precise, fast, and much specific approach and accurate.

REFERENCES

17. Poirier JM, Radembino N, and Jaillon P. Determination of acyclovir in plasma by solid-phase extraction and column liquid chromatography. Therapeutic Drug Monitoring, 1999; 21(1): 129–133.


