Optimization and Validation Methods for Assay of Acyclovir Cream Determination by High Performance Liquid Chromatography

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Abstract. The aim this study is to obtain an effective and efficient method for assay of acyclovir cream determination. Chromatographic separation on a reverse phase a YMC - Triart C\textsubscript{18} column (4.6 x 150 mm, 5 μm), the mobile phase consisted of water and acetonitrile (95: 5 v/v) at a flow rate 1.2 mL/min with UV-Vis detection at 252 nm, volume injected was 20 μL, with UV detection at 252.0 nm. The retention time of acyclovir was ± 3.5 min. Validation studies were achieved by using the limit of detection (LOD) and limit of quantification (LOQ), precision, accuracy, selectivity parameter. The LOD and LOQ values were 0.27 μg/mL and 0.83 μg/mL. The average standard deviation (RSD) value of precision is 0.35% and accuracy is 0.34%. Robustness shows that the method is resistant to variation in the mobile phase, the Acyclovir cream analysis method meets the ICH 1994 requirements, so it can be applied to the assay of Acyclovir determination in Cream dosage forms.

1. Introduction

Acyclovir is an synthetic analogue of guanine used in the treatment and prevention of infectious diseases caused by the \textit{Herpes simplex} virus or \textit{Varicella zoster}. According to WHO (World Health Organization) data, acyclovir is included in the list of essential drugs needed in basic health system. Acyclovir is an antiviral drug that was discovered in 1977 [1]. Acyclovir works specifically against the herpes virus by interfering with the viral DNA polymerase mechanism. Acyclovir is available in Tablet, intravenous injection, ointment and cream dosage forms. Acyclovir cream or ointment has specific properties without disturbing the physiology of normal cells. The side effects of Acyclovir cream to genital lesions can cause irritation or a burning feeling [2].

According to the Indonesian law No. 36 year 2009 article 105 paragraph 1 about health that drugs and medicinal ingredients must complete pharmacopoeia standards and other standard books[3]. One of the parameters of the drug complied with the standards if the assay substances contained therein complied the requirements of the Indonesian Pharmacopoeia. Requirements for assay of acyclovir cream according to the Indonesian Pharmacopoeia V edition 2014, which contains acyclovir (C\textsubscript{8}H\textsubscript{11}N\textsubscript{5}O\textsubscript{3}), not less than 95.0% and not more than 105.0% of the amount stated on the label [4]. The determination of acyclovir cream levels has been carried out in several previous studies using spectrophotometry, FTIR (fourier transforms infrared). The published assays of Acyclovir cream will
carry out optimization and complete validation of these modifications. The modification of the method carried out was optimization and validation of the determination of Acyclovir cream levels using the method of determining the levels of Acyclovir Tablets found in Indonesian Pharmacopoeia V edition 2014.

This study aims to obtain an effective and efficient method and validate the method obtained in determining the levels of acyclovir cream.

2. Experimental

Instrumentation
Chromatographic separation was performed on a UFLC (Ultra Fast Liquid Chromatography) system equipped with an LC-20AD (Shimadzu, Japan), UV-VIS Detector SPDA-20A (Shimadzu, Japan). Column YMC - Triart C18 (4.6 x 150 mm, 5 μm), LC solution was applied for data collecting and processing. UV-VIS spectrophotometer, analytical scale (Mettler Toledo), nylon membrane filters (Whatman), oven (Memmert Universal Oven UN110), hot plate (Wisestir MSH-300), micropipette (Micropipette Eppendorf Research Plus), ultrasonic (Ney® Ultrasonic 14H), and glassware.

Materials
Standardized Acyclovir raw material was obtained from Hubei Yitai - China, Acyclovir raw material was obtained from Hubei Yitai - China, placebo of acyclovir cream, a generic product of acyclovir cream was obtained from PT Kimia Farma, Tbk., sodium hydroxide pellets (analytical grade) was obtained from Emsure®, acetonitril (gradient grade) was obtained from Merck, aqua bidest was obtained from WIDA WI™ Unicap.

3. Methods

1. Determination of the maximum wavelength of acyclovir
A Acyclovir solution of concentration 0.01 mg/mL was prepared by 200.0 mg of the acyclovir cream dissolving the appropriate amount in 0.1 N NaOH solution, diluted (serial dilution of 0.1 mL to concentration 0.01 µg/mL), and Measure the absorption at a maximum absorption wavelength of approximately 255 nm.

2. Preparation of working conditions
a. Preparation of 0.1 N NaOH solution
A 0.1 N NaOH solution was prepared by dissolving 4.0 grams of sodium hydroxide pellets for analysis in 1000 mL of aquabidest.

b. Making placebo of acyclovir cream

| Table 1. Composition a placebo of acyclovir cream |
|-------------------------------------------------|
| Composition per 100 mg of placebo cream:         |
| Propylene glycol                                | 40.0% |
| Sodium Lauryl Sulfate                           | 0.8%  |
| Paraffin soft white                             | 12.5% |
| Liquid paraffin                                 | 5.0%  |
| Cetostearyl alcohol                             | 6.8%  |
| Poloxamer 407                                   | 1.0%  |
| Aqua Purificata                                 | ad 100% |

Placebo of Acyclovir cream is made by melting the oil phase (cetosteryl alcohol, soft white paraffin, liquid paraffin and poloxamer 407) and the water phase (propylene glycol, sodium lauryl
sulfate and aqua purificata) into an oven at 70 °C for 15 min. Then the oil phase and the water phase are mixed in hot conditions until it form into a creamy mass [5].

c. Standard solutions and samples preparation
   A stock solution or sample of acyclovir was prepared by dissolving the appropriate amount in 0.1 N NaOH solution in order to obtain a final concentration of 0.01 mg/mL solution of acyclovir. Working standards were freshly prepared, diluted (serial dilution of 0.1 mL to concentration 0.01 µg/mL), and used for the analysis.

d. Chromatographic Technique
   All chromatographic experiments were using YMC - Triart C18 (4.6 x 150 mm 5 µm) column. The mobile phase consisted of a mixture of water and acetonitrile (95:5% v/v). The flow rate was 1.2 mL/min and the volume injected was 20 µL. The analytes was detected using UV detection at 252.0 nm.

3. Optimization of chromatographic conditions (mobile phase, solvent and flow rate)
   At this stage an experiment was carried out by injecting a standard solution and a 20 μL of acyclovir sample at a concentration of 0.01 mg/mL into the HPLC system with a mobile phase of water and acetonitrile v/v and the optimal ratio was sought.

4. Validation
   a. System Suitability
      The standard solution was injected with 20 μL 6 replicates and the response was observed. RSD area (%) of each active substance was calculated. The chromatogram results were calculated the tailing factor, column capacity factor (K prime), the theoretical plate number (Theoretical Plate) and the resolution of each active substance.

   b. Selectivity
      The selectivity of the method was evaluated by comparing the chromatograms obtained from the samples containing acyclovir standard with those obtained from blank sample.

   c. Linearity and range
      The linierity different concentrations of standard solution were prepared to contain 8, 9, 10, 11, 12 µg/mL of acyclovir. These solutions were analysed and calculated. The calibration curves was plotted using peak area versus standard solution concentration.

   d. Limit of Detection and Limit of Quantification
      A curve is made between the concentration of each active substance and the area from the linearity test data. Then determine the regression equation, slope and intercept. Determined the limit of detection and limit of quantification using the formula:

      \[
      LOD = \frac{3.3 \sigma}{S} \quad 1) \\
      LOQ = \frac{10 \sigma}{S} \quad 2)
      \]

      Where \(\sigma\) is the standard deviation respond an \(S\) is the slope of the regression equation.

   e. Accuracy
      As much 20 μL sample solutions at 3 concentrations of 8, 10 and 12 µg/mL, were injected respectively into the HPLC instrument in the selected mobile phase and flow rate, repeated 3 replicates for each concentration. Then the accuracy (% RSD) is calculated with a value of not more than 2%.

   f. Precision
      1) Ripetitability (Repetition)
A 100% standard solution and 100% test solution were prepared and each solution was injected 6 replicates in succession. System suitability test: RSD (%) of 6 replicates the standard solution injection of ≤ 2.0%.

g. Strength (Robustness)
Performed like a precision test, but made changes in the organic composition of the mobile phase (± 2%) were measured compared to normal conditions, and carried out on the stability of the solution for 5 hours. Calculate the level and area RSD (%) of each active substance. Then compare the resulting variations.

5. Data analysis
The area of each Acyclovir chromatogram generated from the HPLC software is entered into the formula so that the percentage of acyclovir levels will be obtained. The percentage results are then processed using simple statistical methods.

4. Results and Discussion
The first step of experiments were conducted to establish the optimum experimental conditions the acyclovir cream. That is determination of the composition of the mobile phase and flow rate with to do optimization using HPLC Shimadzu LC-20AD UFLC, column YMC - Triart C18 (4.6 x 150 mm 5 µm), wavelength 252 nm, injection volume 20 µL using the mobile phase water and acetonitrile with various variations of concentration and flow rate 1,2mL/min. The analysis results selected from the optimization are the mobile phase composition of water and acetonitrile (95:5 v/v), resulting in an area 3540782, the theoretical plate number of 23375.726, a tailing factor of 1.396, with a retention time of 3.590.

Next selection of the flow rate of the mobile phase with a variation of the flow rate of 0.8, 1.0 and 1.2 mL/min. The flow rate obtained was 1.2 mL/min, with a concentration of 0.01 mg/mL resulting in an area 3540782, the theoretical plate number of 23375.726, a tailing factor of 1.396, with a retention time of 3.590.

The validation of method for assay of acyclovir cream aims to ensure the accuracy of the method. System suitability test was performed daily before running analysis to check detector response to the analyte.

The Selectivity, no compounds responded at the same time as the retention time of acyclovir or no interference around the test area (Table 2).

| No. | Sample Name | Retention Time (min.) | Compound | Area | Resolution | Effectiveness |
|-----|-------------|-----------------------|----------|------|------------|---------------|
| 1   | Mobile Phase| -                     | -        | -    | -          | -             |
| 2   | Solvent     | -                     | -        | -    | -          | -             |
| 3   | Placebo     | -                     | -        | -    | -          | -             |
| 4   | Raw Material| 3.475                 | Acyclovir| 3592417 | -          | -             |
| 5   | Sample      | 3.481                 | Acyclovir| 3568188 | -          | -             |

Requirements
No compounds responded at the same time as the retention time of acyclovir or no interference around the test area.

Resolutio n ≥ 2.0
Linearity and range. Linearity is a method for obtaining test results that are directly proportional to the concentration of the analyte in a given range. From the experiment with 5 series of sample concentrations ranging from 8-12 µg/mL, a calibration curve was obtained with the line equation \( Y = 3.48690.2 + 348690.2 \) C (µg/mL) with a correlation value (r) of 0.9990. The results are shown in Table 3, while a linearity curve in Figure 1.

| No. | Target conc. (%) | Sample weight (mg) | C theoretical (µg/mL) | Area | Sample Rate (%) | Recovery (%) |
|-----|------------------|--------------------|-----------------------|------|----------------|-------------|
| 1   | 80               | 8,01               | 8                     | 28607 43 | 79.99 | 99.99       |
| 2   | 90               | 9.01               | 9                     | 31674 89 | 88.58 | 98.42       |
| 3   | 100              | 10.00              | 10                    | 35141 79 | 98.38 | 98.38       |
| 4   | 110              | 11.01              | 11                    | 39185 51 | 109.60 | 99.64       |
| 5   | 120              | 12.01              | 12                    | 42286 63 | 118.29 | 98.57       |

**Average Recovery** 99.00  
**Standard Deviation** 0.76  
**% RSD** 0.76  
**R** 0.9990  

Requirements: \( r \geq 0.99; \) Recovery 98 - 102%

Figure 1. Linearity Curve of Assignment of Acyclovir Cream

The limits of detection (LOD) and quantification (LOQ) are calculated based on the deviation of the response and the slope of the linear regression curve. The linear regression curve is obtained from the linearity test. The result of the limit of detection is 0.27 µg/mL and the limit of quantification is 0.83 µg/mL.

Precision and accuracy results are shown in Table 4 and 5. The precision test is carried out by injecting a 100% concentration solution 6 replicates. From the results of the implementation of precision validation, it is obtained that the results meet the requirements with RSD 0.35%. For determination of accuracy, were injected in triplicate into the HPLC system (concentrations 8, 10 and 12 µg/mL) and compared to standard acyclovir solutions. The accuracy criterion is highly dependent on the concentration of the analyte in the sample matrix and on the method's accuracy (RSD). (Harmita H., 2004). The results of the accuracy test obtained that % recovery was in the range 99.56 - 100.61% with an average % recovery of 100.05% and a relative standard deviation of 0.34% (Table 5). The recovery value meets the requirements where a good range must be in the range of 98.0 – 102.0% with a relative standard deviation of ≤ 2.0%.
Table 4. Data of Precision Sample Solution

| No. | Target Conc. (% | Sample Weight (mg) | Area  | Content (%) | Recovery (%) |
|-----|-----------------|---------------------|-------|-------------|--------------|
| 1   | 100             | 200.15              | 3559096 | 99.61       | 99.61        |
| 2   | 100             | 200.17              | 3576320 | 100.09      | 100.09       |
| 3   | 100             | 200.10              | 3579626 | 100.21      | 100.21       |
| 4   | 100             | 200.11              | 3586198 | 100.39      | 100.39       |
| 5   | 100             | 200.15              | 3595223 | 100.63      | 100.63       |
| 6   | 100             | 200.12              | 3571680 | 99.98       | 99.98        |

Average Recovery: 100.15
Standard Deviation: 0.35
% RSD: 0.35
Requirements: % RSD ≤ 2.0%

Table 5. Data of Accuracy Sample Solution

| No. | Target Conc. (%) | Sample Weight (mg) | Area  | Content (%) | Recovery (%) |
|-----|-----------------|---------------------|-------|-------------|--------------|
| 1   | 80              | 160.12              | 2855400 | 79.92       | 99.90        |
| 2   | 80              | 160.10              | 2847201 | 79.70       | 99.62        |
| 3   | 80              | 160.14              | 2846127 | 79.65       | 99.56        |
| 4   | 100             | 200.12              | 3572427 | 100.00      | 100.00       |
| 5   | 100             | 200.15              | 3594637 | 100.61      | 100.61       |
| 6   | 100             | 200.10              | 3586114 | 100.40      | 100.40       |
| 7   | 120             | 240.18              | 4284882 | 119.93      | 99.94        |
| 8   | 120             | 240.20              | 4287559 | 120.26      | 100.21       |
| 9   | 120             | 240.15              | 4296036 | 120.26      | 100.21       |

Average Recovery: 100.05
Standard Deviation: 0.34
% RSD: 0.34
Requirements: % RSD ≤ 2.0%; Recovery 98 - 102%

Robustness is the durability of the analysis method is the measurement capacity of the analytical method which is not affected by small changes, but deliberately given variations in its parameters and gives an indication that the method is good as long as it is used in normal conditions. It is necessary to vary the critical parameters in testing to measure how robust the method is. Identify at least 3 analysis factors that could affect the results if they were replaced or changed [6]. In this study, a number of parameters were varied, including: variations in the mobile phase, and the stability of the sample solution was also tested for storage time of 5 hours.

The results of testing the variations in the mobile phase (Table 6) and the stability of the solution (Table 7) show good results, because the deviation results obtained meet the requirements of ≤ 2.0 %.

Table 6. Robustness Data of Mobile Phase Variation

| No. | Water: Acn Ratio | Area Std | Area Smpl | Content (%) Std | Content (%) Smpl | Savings of food (%) Std | Savings of food (%) Smpl |
|-----|------------------|----------|-----------|-----------------|-----------------|------------------------|------------------------|
| 1   | Normal (95: 5)   | 3586371  | 3571224   | 100.40          | 99.97           | -                      | -                      |
| 2   | (A) 93: 7        | 3558351  | 3555486   | 99.62           | 99.53           | 0.78                   | 0.44                   |
| 3   | (B) 97: 3        | 3537425  | 3580021   | 99.03           | 100.21          | 1.36                   | 0.25                   |

Requirements: Deviation ≤ 2.0%
Table 7. Robustness Data of Solution Stability

| No. | Time of Storage | Area Std | Area Smpl | Content (%) Std | Content (%) Smpl | Savings of food (%) Std | Savings of food (%) Smpl |
|-----|----------------|---------|-----------|----------------|----------------|-------------------------|-------------------------|
| 1   | 0 hours        | 3540353 | 3522123   | 99.11          | 98.58          | -                       | -                       |
| 2   | 1 hours        | 3533124 | 3523856   | 98.91          | 98.63          | 0.20                    | 0.05                    |
| 3   | 2 hours        | 3535472 | 3535698   | 98.98          | 98.98          | 0.14                    | 0.41                    |
| 4   | 3 hours        | 3536807 | 3525859   | 99.02          | 98.71          | 0.10                    | 0.13                    |
| 5   | 4 hours        | 3557102 | 3523154   | 99.58          | 98.63          | 0.47                    | 0.05                    |
| 6   | 5 hours        | 3529303 | 3542100   | 98.81          | 99.16          | 0.31                    | 0.59                    |

Requirements: Deviation ≤ 2.0%

5. Conclusions

Based on the research results, it was found that the chromatographic conditions acceptable, it can be concluded that the analysis of all the optimization and validation methods for assay of Acyclovir Cream determination complied all the parameters of the validation test of the analysis method and or complied the ICH 1994 requirements, so that it can be used for the determination of Acyclovir levels in the cream preparation.

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