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Research Article

Development and Validation of Acyclovir HPLC External Standard Method in Human Plasma: Application to Pharmacokinetic Studies

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A simple, rapid, and selective RP-HPLC method was developed for the estimation of acyclovir in human plasma. The method involves a simple protein precipitation technique. Chromatographic separation was carried out on a reverse phase C_{18} column using mixture of 5 mM ammonium acetate (pH 4.0) and acetonitrile (40 : 60, v/v) at a flow rate of 1.0 mL/min with UV detection at 290 nm. The retention time of acyclovir was 4.12 minutes. The method was validated and found to be linear in the range of 25.0–150.0 ng/mL. Validation studies were achieved by using the fundamental parameters, including accuracy, precision, selectivity, sensitivity, linearity and range, stability studies, limit of detection (LOD), and limit of quantitation (LOQ). It shows recovery at 91.0% which is more precise and accurate compared to the other method. These results indicated that the bioanalytical method was linear, precise, and accurate. The new bioanalytical method was successfully applied to a pharmacokinetic linearity study in human plasma.

1. Introduction

Acyclovir, 9-[(2-hydroxyethoxy)-methyl]-guanosine, is an acyclic guanosine derivative which exhibits a selective inhibition of herpesviruses replication with potent clinical antiviral activity against the herpes simplex and varicella-zoster viruses [1, 2]. There are many works published for the determination of acyclovir in biological fluids of different species. For a laboratory, to develop a method is sometimes a compromise between cost, time consumption, and purpose of study. Some of the reported methods about acyclovir quantification in human plasma supposed to be expensive sample extraction method by using liquid-liquid extraction. Several HPLC methods have been reported for determination of acyclovir in human serum using UV [3–13] or fluorescence detection [14–18]. We present herein for the first time, a sensitive and selective HPLC method for the acyclovir assay in human plasma. This paper describes a new sensitive bioanalytical method for acyclovir using RP-HPLC method. By this method, chromatographic conditions have been optimized and validated in accordance with FDA guidelines. This results in a more sensitive, less time consuming, and easier method of quantification compared to the other existing methods and it gives better recovery from the human plasma, which is 91.0%.

The present method was found reliable and applicable for the bioequivalence studies.

2. Reagent and Materials

Gift sample of acyclovir was obtained from Ranbaxy Pharmaceuticals, Sungai Petani, Malaysia. Acetonitrile (HPLC grade) was obtained from Merck, Germany. Ammonium acetate (molecular biology reagent grade) was obtained from Systerm Malaysia. Methanol obtained from QREC and HPLC grade water was used throughout.

2.1. Instrumentation. HPLC chromatographic separation was performed on a Shimadzu liquid chromatographic system

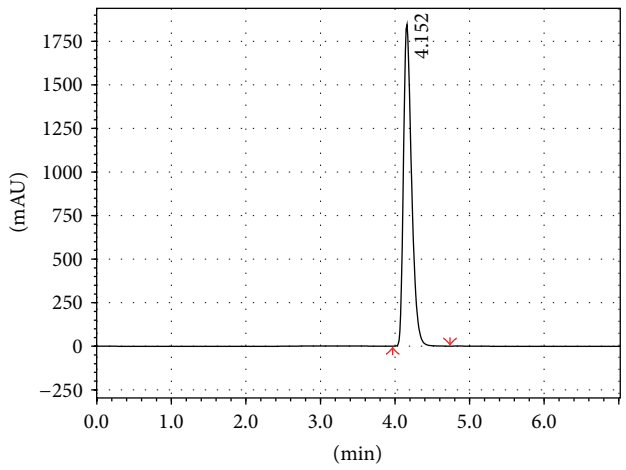

Figure 1: Typical chromatogram of standard solution.

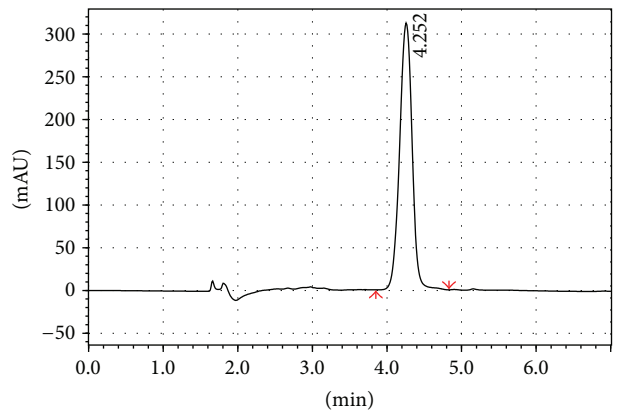

Figure 2: Typical chromatogram of sample solution.

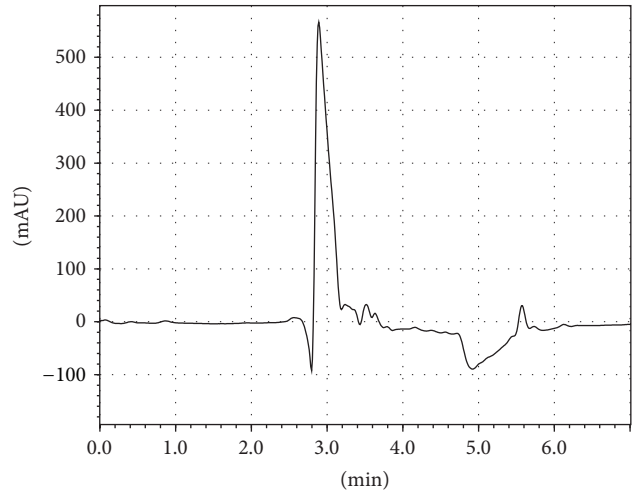

Figure 3: Typical chromatogram of blank plasma.

equipped with an LC-20AD solvent delivery system (pump), SPD-20A photo diode array detector, and SIL-20ACHT injector. LC solution version 1.25 was applied for data collecting and processing (Shimadzu, Japan).

2.2. Standard Solutions. A stock solution of acyclovir was prepared by dissolving the appropriate amount in acetonitrile and water (1 : 1) in order to obtain a final concentration of 1.0 mg/mL solution of acyclovir and stored at 8°C. Working standards were freshly prepared, diluted (serial dilution of 25 to 150 ng/mL), and used for the analysis.

2.3. Chromatographic Technique. All chromatographic experiments were carried out in the isocratic mode using Thermo C_{18} (250 × 4.6 mm i.d., 5 μ) column. The mobile phase consisted of a mixture of 5 mM ammonium acetate (pH 4.0) and acetonitrile (40 : 60% v/v). The flow rate was 1 mL/min and the volume injected was 50 μL using autoinjector. The analytes was detected using UV detection at 290 nm. The standard chromatogram is presented in Figure 1.

2.4. Sample Preparation. At the time of validation, the samples were removed from the deep freezer and kept in the room temperature and allowed to thaw. A volume of 0.5 mL of sample was pipetted into 2.0 mL centrifuge tube and 0.5 mL of precipitating agent (10% perchloric acid) was added. The resulting solution was vortexed for 2 minutes and centrifuged at 4000 RPM for 7 min. Supernatants from the above solutions were evaporated and dryness to the residue was reconstituted with mobile phase and used for the analysis. The sample chromatogram is presented in Figure 2.

2.5. Validation

2.5.1. System Suitability. The column efficiency, resolution, and peak asymmetry were calculated for the standard solutions. The values obtained demonstrated the suitability of the system for the analysis of this drug combination and the system suitability parameters fall within ±3% standard deviation range during routine performance of the method.

2.5.2. Selectivity. The selectivity of the method was evaluated by comparing the chromatograms obtained from the samples containing acyclovir standard with those obtained from blank samples (Figure 3).

2.5.3. Sensitivity. Determine sensitivity in terms of "lower limit of quantification" where the response of lower limit of quantification should be at least five times greater than the response of interference in blank matrix at the retention time of the analyte.

2.5.4. Plasma Extraction Method. Three methods have been tried such as protein precipitation (PPT), solid phase extraction, and liquid-liquid extraction. In these three methods PPT method achieved more % recovery compared to other methods. The SPE and LLE methods of plasma interferences eluted near to drug peak retention time it affected the drug peak.

2.5.5. Linearity Range. The linearity different concentrations of standard solutions were prepared to contain 25, 50, 75, 100, 125, and 150 ng/mL of acyclovir. These solutions were

Table 1: Sensitivity.

Nominal concentration ng/mL	25.0
Mean	24.69
SD	0.03
RSD (%)	4.85
Nominal (%)	98.62
N	5

analysed and the peak areas were calculated. The calibration curve was plotted using peak area versus concentration of the standard solutions. The standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighing and statistical tests for goodness of fit.

2.5.6. Precision and Accuracy. The precision of the method was determined by intraday precision and interday precision. The intraday precision was evaluated by analysis of blank plasma sample containing acyclovir three different concentrations of LQC, MQC, and HQC using five replicate determinations for three occasions. The interday precision was similarly evaluated over two-week period.

2.5.7. Standard Stock Solution Stability. Standard stock solutions of 1.0 mg/mL acyclovir were prepared separately using a mixture of water and acetonitrile (1 : 1 v/v), from the standard stock solution used for the further analysis.

2.5.8. Room Temperature Stock Solution Stability. Room temperature stock solution stability was carried out at 0, 3, and 8 hours by injecting four replicates of prepared stock dilutions of acyclovir equivalent to middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. Comparison of the mean area response of acyclovir and internal standard at 3 and 8 hours was carried out against the zero hour value.

2.5.9. Refrigerated Stock Solution Stability. Refrigerated stock solution stability was carried out at 7, 14, and 27 days by injecting four replicates of prepared stock dilutions of the analyte equivalent to the middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration.

2.5.10. Freeze-Thaw Stability and Short-Term Stability. The stability studies of plasma samples spiked with acyclovir were subjected to three freeze-thaw cycles, short-term stability at room temperature for 3 hrs, and long-term stability at −70°C over four weeks. In addition, stability of standard solutions was performed at room temperature for 6 hrs and freeze condition for four weeks. The stability of triplicate spiked human plasma samples following three freeze-thaw cycles was analysed. The mean concentrations of the stability samples were compared to the theoretical concentrations. The stability of triplicate ($n = 5$) short-term samples spiked with acyclovir was kept at room temperature for 1.00 to 3.00 hours before extraction.

Table 2: Intraday precision and accuracy.

Nominal concentration ng/mL	25.0	100	150
Mean	24.56	99.02	149.84
SD	0.05	0.06	0.04
RSD (%)	7.65	9.05	6.87
Nominal (%)	98.62	99.01	98.94
N	5	5	5

Table 3: Interday precision and accuracy.

Nominal concentration ng/mL	25.0	100	150
Mean	24.92	98.74	149.91
SD	0.02	0.04	0.01
RSD (%)	9.15	7.82	6.10
Nominal (%)	98.93	98.21	99.16
N	5	5	5

Table 4: Stock solution stability of acyclovir.

Room temperature		Below 12°C	
Number of hours	Initial (%)	Number of days	Initial (%)
0	100.00	0	101.25
3	104.49	7	106.14
8	110.10	14	102.39
Mean	104.86		103.26
SD	4.89		5.15
%RSD	6.01		4.89

3. Result and Discussions

System suitability test was performed daily before the run of analytical batch to check detector response to the analyte.

3.1. Selectivity. No interfering endogenous compound peak was observed at the retention time of drug and internal standard.

3.2. Recovery Studies. The sample was prepared by adding 0.5 mL of plasma containing drug and followed by adding 100 μL of 10% perchloric acid, which was vortexed followed by centrifugation at 4000 RPM for 7 min. The supernatant solution was separated and injected. There is no endogenous source of interference was observed at the retention times of the analytes (Figure 2). The precision and accuracy for acyclovir ranged from 4.85% to 98.62%, respectively (Table 1).

3.3. Linearity. The linearity of each calibration curve was determined by plotting the peak area ratio of drug versus nominal concentration of acyclovir. For linearity study six different concentrations of acyclovir were analyzed (25.0, 50.0, 75.0, 100.0, 125.0, and 150.0 ng/mL). The peak area response was linear over the concentration range studied. Each experiment at all concentrations was repeated three times on three separate days to obtain the calibration data. The coefficient of correlation r was found to be 0.999. The limit of quantification and limit of detection were 23.0 and

Table 5: Stability studies.

Nominal concentration ng/mL ($N = 5$)	Concentration found ng/mL	Precision (%)	Accuracy (%)
Short-term stability for 3 h in plasma			
25.0	24.5612	2.01	98.43
150.0	148.9627	2.54	94.59
Long-term stability for 3 h in plasma			
25.0	23.6984	2.57	93.52
150.0	146.9013	3.18	92.04
Freeze-thaw cycles (−70°C)			
25.0	23.98	3.21	95.52
150.0	147.74	8.54	92.47

Table 6: System suitability studies.

Serial number	Parameters	Acyclovir
1	Number of plates/meter	4867
2	Asymmetric factor	0.91
3	LOD (ng/mL)	8
4	LOQ (ng/mL)	24

7.0 ng/mL, respectively. The mean extraction recoveries of acyclovir determined over the concentration of 25.0, 100.0, and 150.0 ng/mL were 96.98 ± 3.17, 98.91 ± 1.72, and 98.42 ± 0.79%.

3.4. Accuracy and Precision Studies. Inter- and intraday precision and accuracy results are shown in Tables 2 and 3. Intraday precision for acyclovir ranged from 6.87% to 9.05% and the intraday accuracy for acyclovir ranged from 98.62% to 99.01%. Interday precision for acyclovir ranged from 6.10% to 9.15% and the interday accuracy for acyclovir ranged from 98.21% to 99.16%. In the stock solution stability, freeze-thaw stability, short-term stability, and long-term stability studies, no tendency of degradation result is shown in Table 4.

3.5. Stability Studies. The stability studies of plasma samples spiked with Acyclovir were subjected to three freeze-thaw cycles, short-term stability at room temperature for 3 h and long-term stability at −70°C over four weeks. In addition, stability of standard solutions was performed at room temperature over 6 h and after freezing for four weeks. The stability of triplicate ($n = 5$) spiked human plasma samples following three freeze-thaw cycles was analysed. The mean concentrations of the stability samples were compared to the theoretical concentrations. The stability of triplicate short-term samples spiked with acyclovir was investigated at room temperature for 1.00 to 3.00 h before extraction. The plasma samples for long-term stability were stored in the freezer at −70°C until the time of analysis. The stability studies are shown in Table 5. System suitability test was performed daily before the run of analytical batch to check detector response to the analyte (Table 6).

4. Conclusion

In summary, HPLC method for the quantitation of acyclovir in human plasma was developed and fully validated as per FDA guidelines [19]. This method offers significant advantages over those previously reported, in terms of improved sensitivity and selectivity, faster run time (5 min) and lower sample requirements. Hence, this method may be useful for single and multiple ascending dose studies in human subjects. The current method has shown acceptable precision and adequate sensitivity for the quantification of acyclovir in human plasma. The developed method has excellent sensitivity, reproducibility, and specificity. The method has been successfully used to provide the bioequivalent study of acyclovir in human plasma. The developed assay showed acceptable precision, accuracy, linearity, stability, and specificity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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