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A new validated ultra performance liquid chromatographic method for determination of acyclovir

S. Hasan[a]*, P. Chander[b], J. Ali[a], S. Babootа[a] and M. Ali[a]

Recently, ultra performance liquid chromatography (UPLC) has proven to be one of the most promising developments in the area of fast chromatographic separations, as it's been true to the objective of reducing analysis time and maintaining good efficiency. The present report describes development and validation of a new, rapid, and sensitive UPLC method with UV detection to quantify acyclovir in bulk sample using a Waters Acquity HSS T-3 (100 × 2.1 mm, 1.8 μm) column maintained at a temperature of 50 °C with a gradient elution consisting of 1.0% triethyl amine in water and acetonitrile at a flow rate of 0.4 mL/min and detected at 254 nm. Method was found to be selective, linear, accurate, and precise as per ICH guidelines. Detection and quantitation limits of drug were 3 and 8 ng/mL, respectively.

Keywords: acyclovir; method development; UPLC; validation

Introduction

With the advent of technology with time, chromatographic separation and analysis techniques are under continual process of improvisation of analytical methodologies. This process of constant improvisation persuades academics, scientists, and analysts to strive to develop the best possible analytical method with a focus on cutting separation time. Setbacks were often because of the intricacies in analysis of some of the samples. Improvement in speed of analysis with higher efficiency can be achieved by various approaches; one opportunity being reducing the particle size, as it improves the rate of mass transfer and reduces the eddy diffusion effect, resulting in smaller plate height and higher optimum linear velocity. On sub-2 μ particles, due to the narrow peaks obtained, sensitivity and separation are improved at the cost of pressure. It was proven that the analysis time could be reduced to a 1 to 2 min interval without the loss of resolution and sensitivity.[1] Thus, running samples at high linear velocities through short columns gives high sample throughput, which in turn means increasing the number of samples analyzed in a given period of time. The conventional high performance liquid chromatography (HPLC) systems offer about 400 bar of pressure at maximum performance. As flow resistance is inversely proportional to the square of particle size, in the majority of cases the pressure threshold of 400 bar is not enough to accomplish a high-speed analysis.[2] The new hardware was able to work up to 1000 bar (15 000 psi) and the system was called ultra performance liquid chromatography (UPLC). UPLC is an ingenious separation technique which exploits entrenched principles of liquid chromatography, with sub-2 μ particles for stationary phase and elevated linear velocities for mobile phase, thus operating at very high pressure leading to radical improvement of resolution, sensitivity, and speed analysis.[3,4] Apart from high speed and sensitivity, this technique is gaining substantial attention in a relatively short period of time for pharmaceutical and biomedical analysis, where HPLC analysis encounters problem due to analytical complexities.

In the present report, this technology has been applied to a new method development and validation study for the determination of acyclovir in bulk drug. Acyclovir (9-[2-hydroxyethoxymethyl]-9H-guanine, ACV) is an acyclic analog of the natural nucleoside 29-deoxyguanosine. It has been described as a potent and selective inhibitor of the replication of herpes simplex virus (HSV) and Varicella zoster virus (VZV) via phosphorylation of acyclovir to acyclovir triphosphate into infected cells, which is a substrate for and preferential inhibitor of viral DNA polymerase. It has extremely low toxicity towards uninfected host cells, making it the drug of choice for the treatment of HSV and VZV infections.[5,6] Though radioimmunoassay [7,8] and enzyme- linked immunoabsorbent assays [9] are extremely sensitive, the large number of slow steps, high cost and the necessity of antiserum or monoclonal antibodies proves unfavourable to such methods. [10] Some analytical methods have been proposed for the analysis of ACV in plasma through HPLC with UV detection,[11–15] fluorescence detection,[16–18] and tandem mass spectrometry.[19] Loregian and his group reviewed all other analytical methods reported for determination for acyclovir.[20] So far, no analytical method allows measurement of ACV through UPLC.

Here, we describe a simple but efficient method using UPLC with UV detection for the determination of ACV in bulk sample. Furthermore, with the proposed UPLC method, ACV demonstrated simplicity, high recovery, less retention time, and good validation results on all parameters.

* *Correspondence to: S. Hasan, Department of Pharmaceutics, Faculty of Pharmacy, Hamdard University, Hamdard Nagar, New Delhi 110062, India. E-mail: dr.sohailhasan@gmail.com*

a *Department of Pharmaceutics, Jamia Hamdard, Hamdard University, New Delhi, India*

b *Water India Pvt Ltd, CS-08/09, 7th Floor, Lobe 2, Tower A, The Corenthum, Plot No. A-41, Sector 62, Noida 201301, U.P., India*

Experimental

Materials and reagents

Acyclovir was generously gifted by Dr Reddy's Laboratories Ltd. (Hyderabad, India) for research purpose. Acetonitrile of HPLC grade was obtained from Qualigens Fine Chemicals (Mumbai, India) and HPLC water was produced in the laboratory by a Milli-Q purification system (Millipore, Billerica, MA, USA). Triethyl amine (TEA) was procured from Merck Ltd. (Mumbai, India). All other reagents used were of analytical grade.

Chromatographic system

UPLC analysis was performed on a Waters Acquity UPLC system (Milford, MA, USA) equipped with a binary solvent manager fitted with an auto sampler, a column manager composed of a column oven, a precolumn heater, and a PDA detector. Three µL of final analytical solution was injected into a Waters HSS T-3 C18 (100 × 2.1 mm, 1.8 µm) UPLC column and the chromatographic separation was performed by gradient elution. The mobile phase consisting of a mixture of 0.1% TEA in water (pH 3.5) and acetonitrile (95 : 5, v/v) with a flow rate of 0.45 mL/min was employed. The column was kept at a temperature of 50 °C and the analysis was performed at 254 nm wavelength with total run time of 3 min. Data acquisition, data handling and instrument control were performed by Empower Software v1.0 installed on a Microsoft Windows® based system.

Sample preparation and calibration

An appropriate amount of pure drug was dissolved in 100 mL of methanol and sonicated for 10 min to prepare stock solution (10.0 µg/mL). Further dilutions were prepared from stock solution which was followed by filtration through a 0.22 µ nylon filter and 3 µL of the sample was directly injected into the UPLC system. Calibration standards were processed according to sample preparation procedure and were analyzed by UPLC method.

Validation procedure

The newly developed UPLC method was validated in terms of selectivity, linearity, quantitation limit (QL) and detection limit (DL), precision, accuracy, recovery, and robustness according to International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines.[21]

Results and Discussions

UPLC method development

Nucleoside analogues such as ACV exist as both weak acids and weak bases. These ionic forms of ACV have significant difference in their apparent hydrophobicity and thus tend to migrate through the column with different velocities resulting in poor peak shape, tailing, and decreased sensitivity during HPLC method development.[22] In order to achieve high column efficiency and sensitivity, studies with fluorescent or mass detections have been reported. In this study, an attempt was made to overcome these shortcoming by using HSS T-3 C18 column packed with small-sized (1.8 µm) particles and employing ultra-high pressures. The small-sized particles reduce plate height and consequently allow the number of theoretical plates to be increased. They also favour faster linear velocities and allow reduction of analysis time and improve peak shape. The entire UPLC chromatogram run lasted 3 min, which permitted the analysis of a large number of samples in a short period.

Nucleoside analogues are polar compounds, so the addition of ion pairing agents or organic modifiers in the mobile phase could increase their retention in reverse phase chromatography.[23] Moreover, as they are both weak acids and weak bases, it is feasible to separate nucleoside analogues as ion-pairs with a strong base as the counter-ion. TEA is commonly used as an ion pairing agent,[24] and a pH modifier in the mobile phase. To obtain the best chromatographic separation and sensitivity in a short time, different ratios of aqueous TEA and acetonitrile were systematically investigated. The best separation was achieved with a mixture of 0.1% aqueous TEA and acetonitrile (95 : 5%, *v*/*v*) as the mobile phase. Using the chromatographic conditions described, rapid separation of ACV was achieved at 1.3 min (Figure 1). GCV, also a nucleoside analogue, was selected as internal standard/related substance due to its physicochemical and structural similarity with ACV for assessing selectivity parameter during validation study for the new method. The simplicity of the method is also considered as a significant aspect of the method, as previous methods reported for HPLC use buffer systems in mobile phase, which had a negative influence on column life. Thus, this new method not only simplifies the method by eliminating the buffer system from the mobile phase, but also prolongs column longevity. Furthermore, with the proposed UPLC method, ACV demonstrated high recovery with significant reduction in retention time, which thus enhances productivity by increasing the number of samples analyzed per unit time.

UPLC method validation

Validation study was performed to confirm the method suitability for its intended purpose of routine analysis. The assay value of ACV was found to be in the range of 80–120%.

System suitability

System suitability test was performed to validate that the anticipated method was adept at producing good resolution between the peaks of interest with high reproducibility. System suitability is analyzed in terms of tailing factor (must be <1.5), theoretical plate counts (should be $>20\,000$), retention time, etc. The result for the proposed UPLC method was in accordance with the defined parameters and fulfilled these requirements within the accepted limits.

Linearity

Linear calibration plots for the related substance method were obtained over the calibration range of 0.1–1.0 µg/mL at five concentration levels in triplicate. The correlation coefficient (r^2) for calibration curve was greater than 0.999; where, the regression equation for the calibration curve was $y = (9.873 \pm 0.042)x + (1832.48 \pm 13.35)$; where, x is the concentration of ACV, and y is the peak area of ACV. The results showed excellent correlation between the peak area and concentration of impurities.

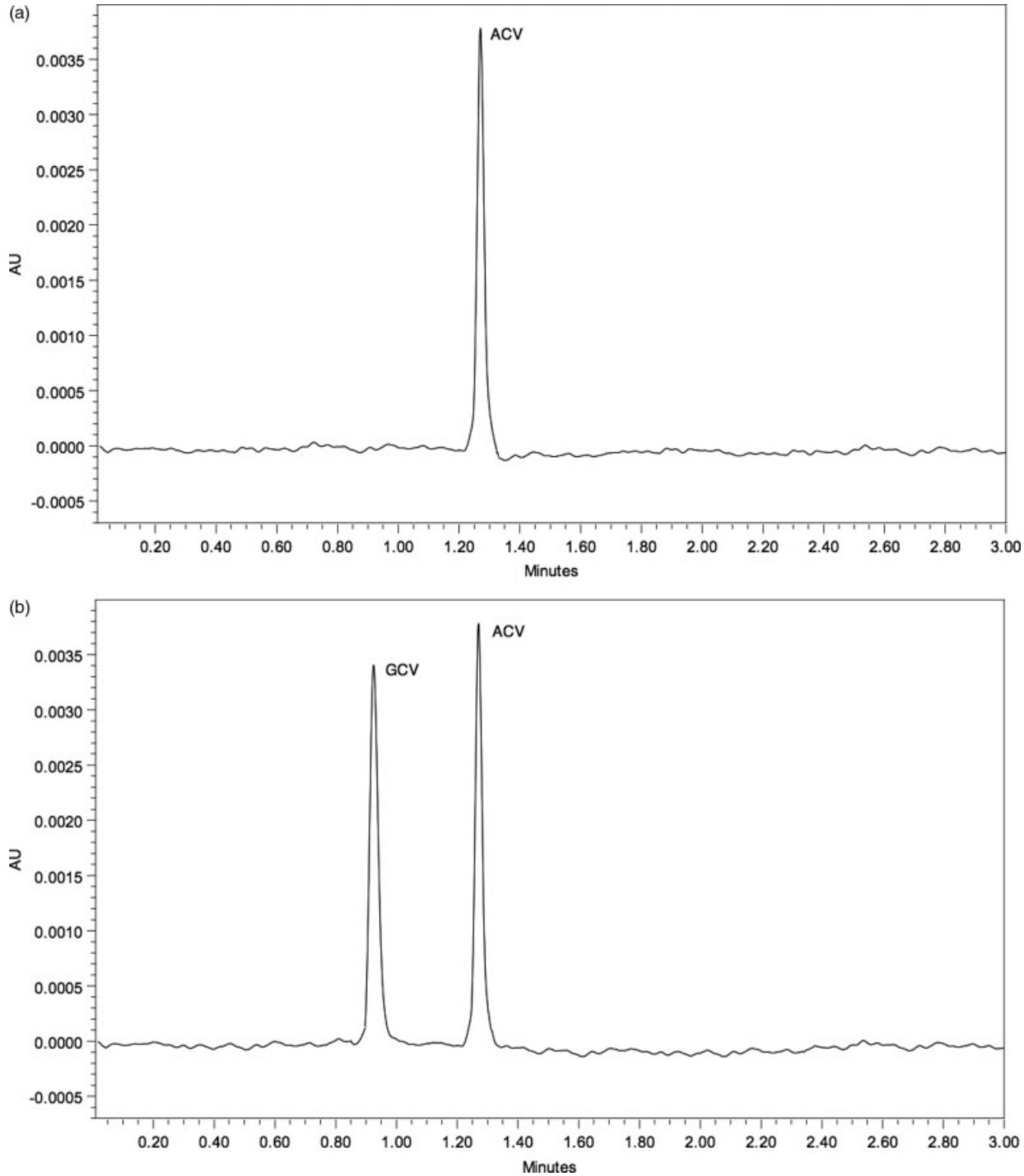

Figure 1. UPLC chromatogram (A) ACV sample; (B) ACV sample spiked with GCV.

Selectivity

Selectivity was demonstrated by the ability to assess unequivocally the analyte in the presence of some related substance or impurity. UPLC chromatograms of ACV alone (Figure 1A) and ACV sample spiked with GCV (Figure 1B) were compared to show the selectivity of the proposed procedure. The retention time of ACV was 1.3 min and of GCV was 0.928 min and no interference was observed near the retention time, demonstrating method selectivity.

Detection limit (DL) and quantitation limit (QL)

The DL and QL were determined based on signal-to-noise ratios using an analytical response of three and ten times the background noise, respectively.[25] The DL and QL were found to be 3.0 ng/ml and 8.0 ng/ml, respectively. The DL of 30 ng/ml has been reported but using fluorescence detection.[26] Our method enabled the QL for ACV to be 8.0 ng/ml using UV detection, which was more sensitive than the results from the previous studies.

Table 1. Precision and accuracy data for ACV (n = 3 days, triplicate per day)

ACV spiked (μg/mL)	Mean concentration found (μg/mL)	Precision[a] (CV, %)	Accuracy[b] (% recovery)
Intra-day reproducibility			
100	97.6	4.098	97.60
500	493.8	1.214	98.76
1000	989.2	0.505	98.92
Inter-day reproducibility			
100	94.8	5.274	94.8
500	489.3	1.778	97.86
1000	983.9	0.823	98.39

[a] Precision as CV (%) = standard deviation divided by mean concentration found × 100.
[b] Accuracy = (mean concentration found/concentration spiked) × 100.

Precision and accuracy

Precision and accuracy were determined by triplicate analysis of QC samples at three different concentrations followed by their comparison with the calibration curves prepared on the same day and on three different days. Table 1 summarizes the intra- and inter-day precision and accuracy of ACV analysis by the proposed method. The intra-day and inter-day precisions determined as coefficient of variation (CV) ranged between 0.505–4.098 and 0.823–5.274%, respectively. Intra- and inter-day accuracy calculated as percent recovery was in the range of 94.8–98.92%. Both precision and accuracy were found to be suitable and did not depend on the concentration assayed or on the day of the assay.

Robustness

The low values of % RSD (≤1.9) and SE (<2) obtained after introducing small deliberate changes in the developed UPLC method indicated the robustness of the method.

Conclusion

The newly developed UPLC method for the determination of acyclovir was found to be simple, sensitive and capable of giving faster retention times and maintaining excellent resolution, thus enabling rapid sample analysis; more than that can be achieved with conventional HPLC. The method was completely validated showing satisfactory data for all the parameters tested. This method exhibited an excellent performance in terms of sensitivity and speed.

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