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# DEVELOPMENT AND VALIDATION OF RP- HPLC METHOD FOR THE DETERMINATION OF ACYCLOVIR IN HUMAN PLASMA

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## ABSTRACT

A rapid, selective and sensitive high performance liquid chromatographic method for the estimation of acyclovir in human plasma has been developed. Chromatography was carried out on a Hypersil BDS $C_{18}$ column using a mixture of phosphate buffer (pH 2.5) and methanol in a ratio of 95 : 5 v/v as the mobile phase. The eluents were monitored at 254 nm. Lamivudine was used as an internal standard for this study. The retention times for acyclovir and lamivudine were found to be 5.5 and 8.0 min, respectively. The method was found to be linear in the concentration range of 0.201 to 13.545 $\mu gmL^{-1.}$ The method validated as per FDA guidelines and was found to be suitable for bioequivalence and pharmacokinetic studies.

**Key Words:** Acyclovir, Determination, Plasma, HPLC.

## INTRODUCTION

Acyclovir, (9-[(2-hydroxyethoxy) methyl] guanine) is a purine nucleoside analogue, which is active against herpes simplex (type 1 and 2) and viricella zoster viruses[1, 2]. It inhibits thymidine kinase and interferes with DNA synthesis. The drug is official in USP and BP. Its empirical formula is $C_{14}H_{15}NOS$. The structure of acyclovir is shown in Fig. 1. A literature survey reveals the reports of a few methods for the determination of acyclovir in various biological fluids, in human beings and animals and in dosage forms by LC-MS/ MS[3-5], HPLC[6-8], spectrophotometry[9,10] and capillary electrophoresis[11]. The authors now propose a sensitive, accurate and precise RP-HPLC method for the determination of acyclovir in human plasma.

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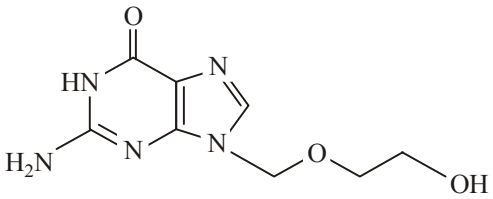


**Fig. 1 Structure of acyclovir**

## EXPERIMENTAL

### Chemicals and solvents

HPLC grade acetonitrile and methanol used in the study were obtained from Qualigens, India. HPLC grade water (milli Q) was prepared from Millipore (USA) equipment. The mixed phosphate buffer was prepared by dissolving 1.36 g of potassium dihydrogen phosphate and 1.15 g of ammonium dihydrogen phosphate in 1000 mL of milli-Q water. The pH of the solution was adjusted to 2.5 with ortho phosphoric acid. The reference samples of acyclovir and lamivudine were obtained from Hereto Drugs Ltd. (Hyderabad).

### Chromatographic conditions

A Shimadzu HPLC equipment comprising of two LC-10AT VP pumps, a VP CTO-10AS VP column oven, a Hypersil BDS $C_{18}$ (4.6 ID x 250 mm, 5µ particle size) column and an SPD-10A variable-wavelength programmable UV-Visible detector was used for chromatographic separation. The detection of the compounds was monitored at 254 nm. A mobile phase consisting of a mixture of mixed phosphate buffer (pH 2.5) and methanol in a ratio of 95 : 5 v/v was pumped at a rate of 1.2 mL/min. The data was acquired and processed with Class VP Software.

### The working standard solutions and the calibration curve

The stock solutions of the analyte and the internal standard were prepared in a mixture of acetonitrile and water (70 : 30 v/v) at a free base concentration of 1 mg $mL^{-1}$. The working standard solutions were prepared from the stock solution by using the same diluent. These diluted working standard solutions were used to prepare the calibration curve standards and quality control samples. The solutions for obtaining the eight point standard calibration curve were prepared by spiking the screened blank plasma with appropriate amounts of acyclovir dilutions. The calibration curve was linear in the range of 0.201-13.545 µg $mL^{-1}$ ($r \geq 0.9900$). The calibration plot was drawn with a weighing factor of $1/X^2$. The

quality control samples were prepared at four concentration levels of 0.203 μg $mL^{-1}$ (LLOQ QC), 0.602 μg $mL^{-1}$ (LQC), 6.542 μg $mL^{-1}$ (MQC) and 10.374 μg $mL^{-1}$ (HQC). The results are presented in Tables 1 and 2.

**Table 1: Summary of calibration parameters**

| Calibration curve | Slope | Intercept | Correlation coefficient |
|---|---|---|---|
| 1 | 0.018393 | 0.00502 | 0.9961 |
| 2 | 0.019218 | 0.00247 | 0.9990 |
| 3 | 0.021002 | -0.00325 | 0.9927 |
| 4 | 0.018209 | 0.00357 | 0.9994 |
| 5 | 0.019419 | -0.00016 | 0.9997 |
| 6 | 0.020885 | -0.00279 | 0.9894 |

**Table 2: Precision of plasma calibration curve standards (n = 6)**

| Nominal concentration (μg $mL^{-1}$) | Mean calculated concentration (μg $mL^{-1}$) | % Coefficient variation | % Relative error |
|---|---|---|---|
| 0.201 | 0.205 | 8.2 | -1.8 |
| 0.600 | 0.619 | 6.3 | -3.2 |
| 0.985 | 0.952 | 10.0 | 3.4 |
| 1.905 | 1.972 | 5.2 | -3.5 |
| 3.809 | 3.888 | 2.4 | -2.1 |
| 7.619 | 7.396 | 9.0 | 2.9 |
| 10.158 | 9.559 | 4.1 | 5.9 |
| 13.545 | 13.601 | 10.0 | -0.4 |

## Sample extraction procedure

Five hundred micro liters of the spiked plasma calibration curve standards and the

quality control samples were transferred to pre-labeled polypropylene tubes containing 50 µL of the internal standard, lamivudine (50 µg $mL^{-1}$ dilution). The tubes were vortexed for ten seconds. Each of the MCX 30 mg/1CC cartridges was conditioned with 1 mL of methanol followed by equilibrating with 1 mL of 0.5% ortho phosphoric acid in water on the solid phase extraction chamber. The above samples were loaded on to the cartridges and the cartridges were again washed with 1 mL of 0.5 % ortho phosphoric acid in water followed by 1 mL of 10% methanol in water. The cartridges were dried for about one minute and eluted with 1 mL of 1% ammonia in methanol. The eluents were evaporated in a stream of nitrogen for 20 minutes at 40°C and the residues in the dried tubes were reconstituted with 0.4 mL of the mobile phase. The contents of the tubes were vortexed and transferred into auto-sampler vials and then analyzed with HPLC unit by injecting 50 µL of sample volume.

## Validation

The proposed method has been validated[12] for selectivity, sensitivity, linearity, precision, accuracy, recovery, stability and dilution integrity. Selectivity was determined by testing the different blank plasma samples (from different donors) for interference at the retention times of the analyte and the internal standard. Sensitivity was determined by analyzing six replicates of blank human plasma and the plasma spiked with the analyte at the lowest value in the calibration curve. The intra run and inter run accuracy was determined by replicate analysis (n = 6) of the quality control samples at each level and at the limit of quantification value (LOQ) that was derived from the sample batch. Inter run precision and accuracy of the calibration standards were computed from the six calibration curves used for assay validation. The results were presented in Tables 3, 4 and 5.

**Table 3: Precision and accuracy**

| QC ID | Nominal concentration (µg/mL) | Intra batch | | | |
|---|---|---|---|---|---|
| | | n | Mean concentration observed (µg/mL) | % CV | % RE |
| HQC | 10.374 | 6 | 9.900 | 3.3 | 0.33 |
| MQC | 6.542 | 6 | 6.492 | 4.8 | 0.31 |
| LQC | 0.602 | 6 | 0.585 | 6.8 | 0.04 |
| LLOQ QC | 0.203 | 6 | 0.213 | 5.1 | 0.01 |

**Table 4: Precision and accuracy**

| QC ID | Nominal concentration (µg/mL) | Intra batch | | | |
|---|---|---|---|---|---|
| | | n | Mean concentration observed (µg/mL) | % CV | % RE |
| HQC | 10.374 | 36 | 10.119 | 4.9 | 2.80 |
| MQC | 6.542 | 36 | 6.452 | 6.4 | 1.25 |
| LQC | 0.602 | 36 | 0.605 | 7.2 | 0.17 |
| LLOQ QC | 0.203 | 36 | 0.202 | 7.8 | 1.08 |

**Table 5: Stability data**

| Details of stability experiment | n | Spiked concentration µg $mL^{-1}$ | Mean calculated comparison sample concentration | Mean calculated stability sample concentration | Mean percent change |
|---|---|---|---|---|---|
| Bench top stability HQC | 6 | 10.374 | 10.301 | 9.664 | -6.2 |
| Bench top stability LQC | 6 | 0.602 | 0.605 | 0.619 | 2.4 |
| Auto-sampler or Wet extract stability HQC | 6 | 10.374 | 9.887 | 10.539 | 6.6 |
| Auto-sampler or Wet extract stability LQC | 6 | 0.602 | 0.627 | 0.575 | -8.3 |
| Freeze-thaw stability HQC | 6 | 10.374 | 9.811 | 9.664 | -1.5 |
| Freeze-thaw stability LQC | 6 | 0.602 | 0.621 | 0.619 | -0.3 |
| Dry extract stability HQC | 6 | 10.374 | 10.560 | 10.539 | -0.2 |
| Dry extract stability LQC | 6 | 0.602 | 0.638 | 0.575 | -9.8 |
| Long term stability HQC | 6 | 10.374 | 10.560 | 10.474 | -0.8 |
| Long term stability LQC | 6 | 0.602 | 0.624 | 0.619 | -0.7 |

Accuracy is defined as the percent relative error (% RE) and is calculated using the formula % RE = (E - T) x (100/T) were E is the experimentally determined concentration and T is the theoretical concentration of the drug. Assay precision was calculated by using the formula % RSD = (SD/M) x (100) where M is the mean of the experimentally determined concentration and SD is standard deviation of M. Dilution integrity was performed with samples falling above the upper concentration limits of the calibration curve. For this, a concentration of double the uppermost calibration standard was diluted two fold and four fold with blank plasma. Six replicates each of the diluted samples were processed and analyzed for accuracy and precision.

The auto – sampler or wet extracted stability of the processed sample was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were injected after keeping in auto-sampler at $10^0$C for 24.0 hrs. The stability of spiked human plasma samples stored at room temperature (Bench top stability) was evaluated for a period of eight hours and compared with that of the freshly prepared samples. The freeze-thaw stability was assessed by comparing the stability of the samples that had been frozen and thawed three times, with that of the freshly spiked quality control samples. The stability of spiked human plasma stored at -$70^0$C (long – term stability) was evaluated by analyzing the quality control samples that were stored at -$70^0$C for 8 days together with the freshly spiked calibration standards and the quality control samples. All stability evaluations were based on back-calculated concentrations. Analytes were considered stable, if the deviations of the mean test responses were within 15% of the freshly prepared or comparison samples.

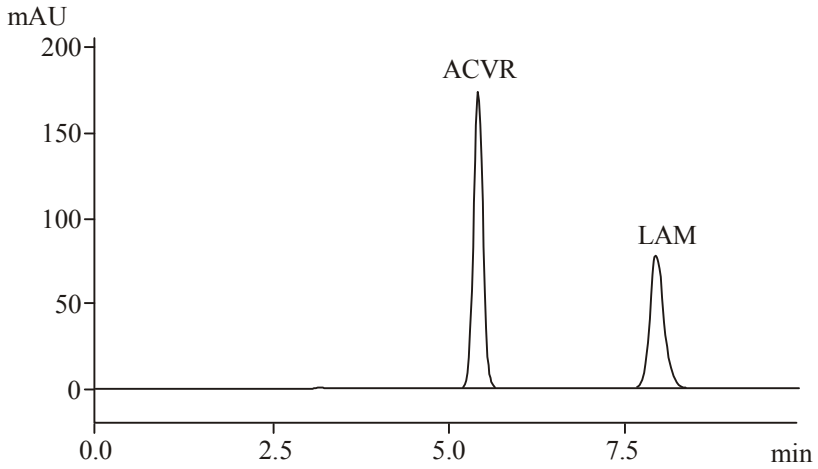


**Fig. 2: Representative chromatogram of acyclovir extracted standard**

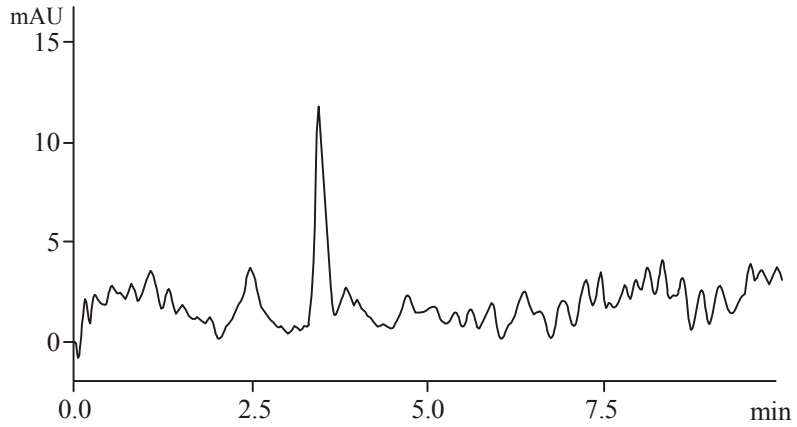


**Fig. 3: Representative chromatogram of acyclovir extracted blank plasma sample**

The representative chromatogram obtained for extracted blank plasma sample (Fig. 3) shows that the assay procedure is specific as there were no interfering peaks observed at the retention times corresponding to the drug and the internal standard.

## CONCLUSION

The proposed validated HPLC method is simple, selective, precise and accurate for quantification of acyclovir in human plasma. The method can also be applied to bio-availability and bio-equivalence studies.

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