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Liquid Chromatography/Positive-Ion Electrospray Tandem Mass Spectrometry Assay for Acyclovir in Human Plasma and Its Application to Pharmacokinetic Study

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ABSTRACT

A sensitive, accurate and rapid reverse phase Liquid chromatography/tandem mass spectrometry (LCMS/MS) method was described to estimate acyclovir in human plasma. Detection was made at m/z 226.1/152.2 for Acyclovir and 346.1/198.1 for internal standard using ESI Positive ion spray ionization mode. Analyst 1.5.1 software was used for the quantification. The stationary phase was Ascentis C18, 50 X 4.6 mm, 5µm column. The separation method developed produce recovery of 84.58%. Acceptable intra-day and inter-day precision (<15%) and accuracy (<10% diff.) were observed over the linear range of 82.967 to 4006.948 ng/mL. The absence of any matrix effects was displayed. The retention time of analyte and internal standard was 1.05 and 1.24 minutes. The developed and validated method was successfully applied for bioequivalence studies.

Keywords: Human plasma; Acyclovir; Liquid-Liquid Extraction; LCMS/MS.

INTRODUCTION

Acyclovir is a synthetic purine nucleoside analogue with in vitro and in vivo inhibitory activity against herpes simplex virus types 1 (HSV-1), 2 (HSV-2), and varicella-zoster virus (VZV). Acyclovir is a white, crystalline powder with the molecular formula $C_8H_{11}N_5O_3$ and a molecular weight of 225. The maximum solubility in water at 37°C is 2.5 mg/mL. The pka's of acyclovir are 2.27 and 9.25(1).

The chemical name of acyclovir is 2-amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6*H*-purin-6-one; it has the following structural formula:

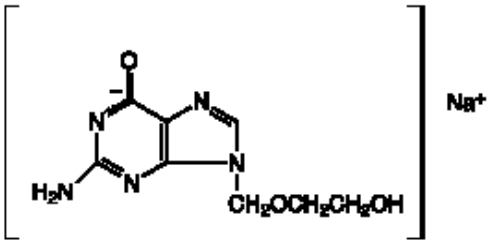


Figure No. 1: Molecular Structure

Several High performance liquid chromatography methods have been reported for determination of acyclovir in human serum using UV (2-27). Because acyclovir is soluble in aqueous medium and practically insoluble in most organic solvents, protein precipitation (PP) with perchloric acid or solid phase extraction (SPE) have been utilized to clean up the serum samples. However, these sample pre-treatment procedures suffer from some disadvantages. We have developed Liquid chromatography/tandem mass spectrometry method for acyclovir.

The goal of this study was to develop and validate a sensitive, specific and reproducible Liquid chromatography/tandem mass spectrometry (LC-MS/MS) method of Acyclovir estimation in human plasma by using Omeprazole as an internal standard.

UFLC offers advantage in shortening of analysis time through ultra fast analysis reduces delay risks. Omeprazole internal standard has been selected based on extraction efficiency which was equivalent to analyte and shorter retention time on column.

MATERIALS AND METHODS

Chemicals and Reagents

Acetonitrile and methanol of HPLC grade was procured from JT Becker. Water HPLC (High performance liquid chromatography) grade was obtained from a Milli-Q water purification system. Formic Acid was procured from CDH. Six lots of blank plasma were procured from Sai Labs Hyderabad, India. A reference standard of Acyclovir & Omeprazole internal standard was provided by SeQuent Research Limited Mangalore India.

Instrumentation and chromatographic conditions

The ultra flow liquid chromatography (UFLC) UFLC XR system (Shimadzu Corporation, Kyoto, Japan) is equipped with LC-20ADXR a binary pump, SIL-20ACXR auto sampler was used to keep temperature at 10°C, CTO-20AC column oven used to keep temperature at 35° C and CBM-20Alite system controller. Mass spectrometric detection was performed on an API 4000 triple quadrapole instrument using the multiple reaction monitoring (MRM) mode. Turbo electrospray ionization (ESI) interface in positive mode was used and detection was made at m/z (mass-to-charge ratios) 226.1/152.2 for Acyclovir and 346.1/198.1 for internal standard. Data processing was performed on Analyst 1.5.1 software package was used for the quantification (Applied Biosystems).

Optimization of LCMS method

The Liquid chromatography/tandem mass spectrometry procedures were optimized with a view to develop a method of estimation for Acyclovir. The mixed standard stock solution was diluted in methanol to a concentration containing 5 ng/mL (nanogram/milliliter) of Acyclovir. Then, the stock solution is injected into the Hypurity Advance C18 column. Different ratio of Acetonitrile

and water was tried (80:20, v/v) [volume/volume]. The optimum mobile phase was found to be Acetonitrile: Mill-Q water: formic acid (80:20:0.05, v/v/v) [volume/volume/volume]. The separation was carried out at ambient temperature with a flow rate of 0.4 mL (milliliter). The injection volume was 5 µL (microliter) and run time was 2 minutes. The retention time of analyte and internal standard was 1.05 and 1.24 minutes.

Preparation of standard and quality control samples:
Stock solutions of Acyclovir were made up in methanol at approximately 1 mg/ ml (milligram/milliliter) and these stock solutions were refrigerated. Working standard solutions of varying concentrations of Acyclovir were prepared on the day of analysis by diluting the stocks with dilution solution.

Calibration curve standards consisting of a set of eight non-zero concentrations ranging from 82.967 ng/mL to 4006.948 ng/mL for Acyclovir were prepared. Prepared quality control samples consisted of Acyclovir concentrations of 83.050 ng/mL Quality control - Lower limit of quantification (QCLLQ), 212.133 ng/mL Quality control - Low (QCL), 2121.325 ng/mL Quality control - Medium (QCM) and 3535.542 ng/mL Quality control - High (QCH). These samples were stored below -50 °C until used. Twelve sets of QCL and QCH were stored to below -20 °C freezer for generation of below -20 °C stability.

Sample Extraction (Liquid Liquid Extraction Method)
Before extraction, bulk spiked Calibration curve standards (CC) and Quality control (QC) samples were removed from the deep freezer and thawed at room temperature. Calibration standards and Quality control samples were then made ready for extraction in 4 mL polypropylene tubes. Exactly 50 µL of plasma was pipette out into prelabelled polypropylene tubes, to this 25 µL of internal standard dilution (2000 ng/mL) was added and vortex for 20 seconds. 2 mL of extraction solvent was added [Diethyl ether: Isopropyl alcohol] (1:1 v/v) and vortexed for 3 minute. Samples were centrifuged at 2000 RPM at 4°C for 2 minutes and the 1.9 mL supernatant layer was separated, evaporated to dryness under nitrogen gas stream, reconstituted 1 mL of dilution solvent and these samples were transferred to the auto sampler vial and 5 µL was injected into the chromatographic system.

Preparation of Mobile phase, Dilution and Washing Solution:
The mobile phase was prepared by mixing Acetonitrile: Mill-Q water: formic acid (80:20:0.05, v/v/v), was filtered using a 0.45 µm membrane filter (Millipore). The dilution solution was prepared by mixing milli-Q water and Acetonitrile in the ratio 20:80 v/v. The rising solution was prepared by mixing milli-Q water and Acetonitrile in the ratio of 20:80 v/v.

Method Validation Parameters:
The validation was performed as per the USFDA and ANVISA guidelines and in-house operating procedures. The optimized Liquid chromatography/tandem mass spectrometry method was validated as discussed below:

The system suitability was performed before starting each day's activity according to in-house. Specificity and Selectivity was performed in six different lots of plasma having K3EDTA as anticoagulant. Blank samples (plasma) from six independent sources of matrix were processed and then spiked with analyte at QCL level and internal standard at the concentration used in the method being validated just before injection into the Liquid chromatography/tandem mass spectrometry (LC-MS/MS). An aqueous solution of analyte was prepared at QCL level with internal standard in mobile phase. Processed and injected two blank, two LLOQ, two ULOQ

samples followed by re-injection of first two blank samples. A regression equation with a weighting factor of $1/x^2$ of drug to IS concentration was judged to produce the best fit for the concentration-detector response relationship for Acyclovir in human plasma. The representative calibration curves for regression analysis are illustrated in Figure 4. The precision of the assay was measured by the percent coefficient of variation over the concentration range of QCLLQ, QCL, QCM and QCH samples respectively during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the LLOQ, low, middle and high quality control samples to their respective nominal values, expressed in percentage. One complete precision and accuracy batch was processed and analyzed by different analyst using different column. Six sets of aqueous recovery comparison samples were prepared and injected. The recovery comparison samples of Acyclovir were compared against extracted samples of QCL, QCM and QCH samples. Six sets of dilution integrity samples (approximately 1.7 times of highest standard concentration) were processed by diluting them twice and another six sets by diluting them four times using pooled plasma. These quality control samples were analyzed along with a freshly spiked and processed calibration curve standards. The quality control sample concentrations were calculated using appropriate dilution factor.

Stabilities

Bench top stability was determined for 12 hours, using six sets each of QCL and QCH. The quality control samples were quantified against the freshly spiked calibration curve standards. Freeze-Thaw Stability Three Cycles The stability in human plasma was determined for three freeze-thaw cycles. Six replicates of QCL and QCH were analyzed after undergoing three freeze-thaw cycles. The freeze-thaw quality control samples were quantified against the freshly spiked calibration curve standards. Six replicates of QCL and QCH were stored below -20° C and below -50° C in the freezer and deep freezer respectively for 65 days. These samples were quantified against the freshly spiked calibration curve standards. The stability of the analytes was evaluated by comparing each of the back calculated concentrations of stability QCs with the mean concentrations of the respective QCs analyzed in the first accepted precision and accuracy batch (PA-1).Six replicates of QCL and QCH were analyzed and stored in autosampler to prove stability. These samples were injected after a period of 50 hours and were quantified against freshly spiked calibration curve standards. Six replicates of QCL and QCH of the precision and accuracy batch PA-3 were retained in the autosampler at 10°C for 50 hours to test the re-injection reproducibility of the method. Reinjection reproducibility concentrations were compared against the PA-3 batch concentrations. The stability of stock dilutions of analytes and the internal standard was evaluated at room temperature. Aqueous stock dilutions of the analytes and the internal standard were prepared. One portion of the stock dilution was placed in the refrigerator between 2-8°C, while the other portion was placed at room temperature for 21 hours. Stock solution stability was carried out for 65 days by injecting six replicates of stock dilution of stability standards (analyte and internal standard which prepared and stored in the refrigerator between 2 - 8° C) and freshly prepared stock dilutions of Comparison standard (analyte and internal standard). The response of stability sample was corrected by multiplying with correction factor.

Chromatography

Representative chromatograms of blank plasma, QCM & calibration curve of Acyclovir are given in Figure.2 to 4 as mentioned below:

Figure 2 - A Representative Chromatogram of Acyclovir for Blank

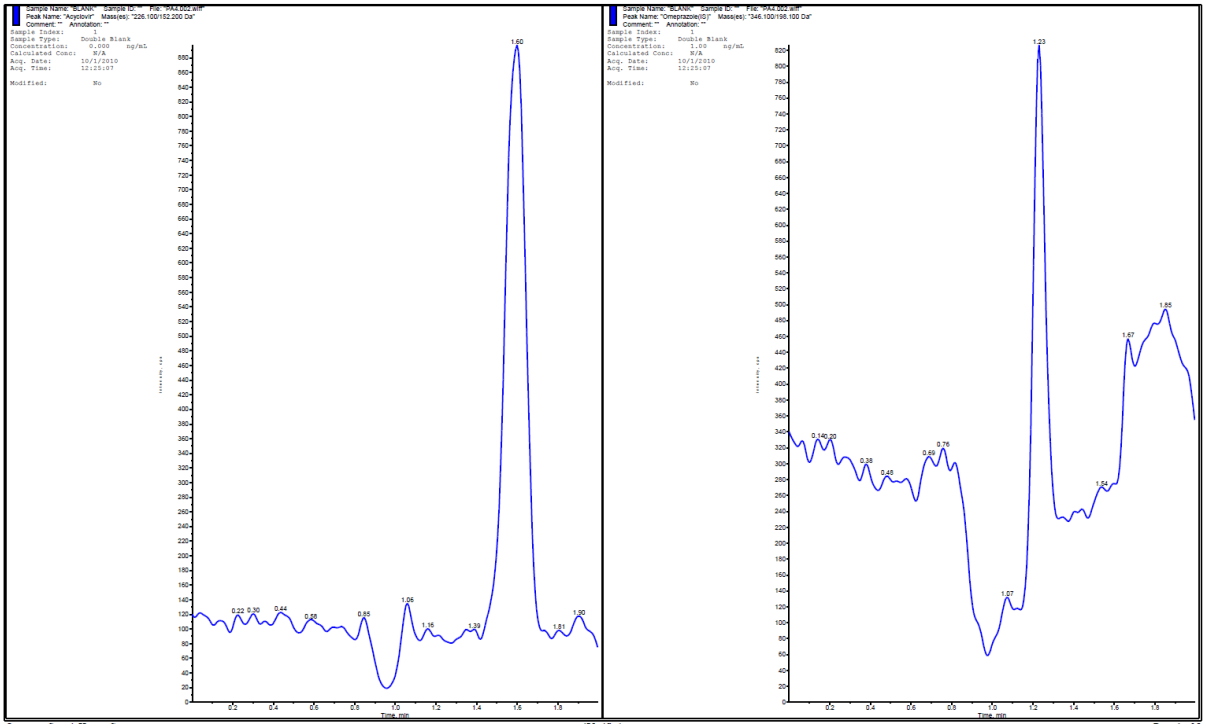
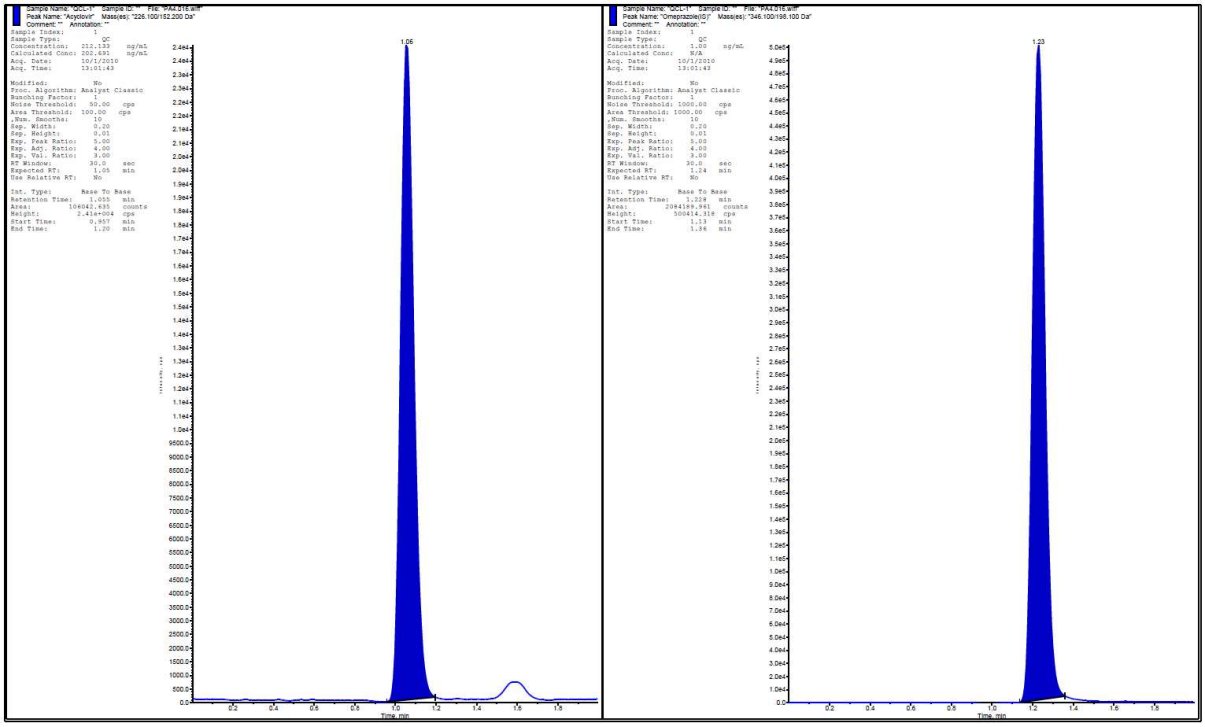


Figure 3 - A Representative Chromatogram of Acyclovir for QCL



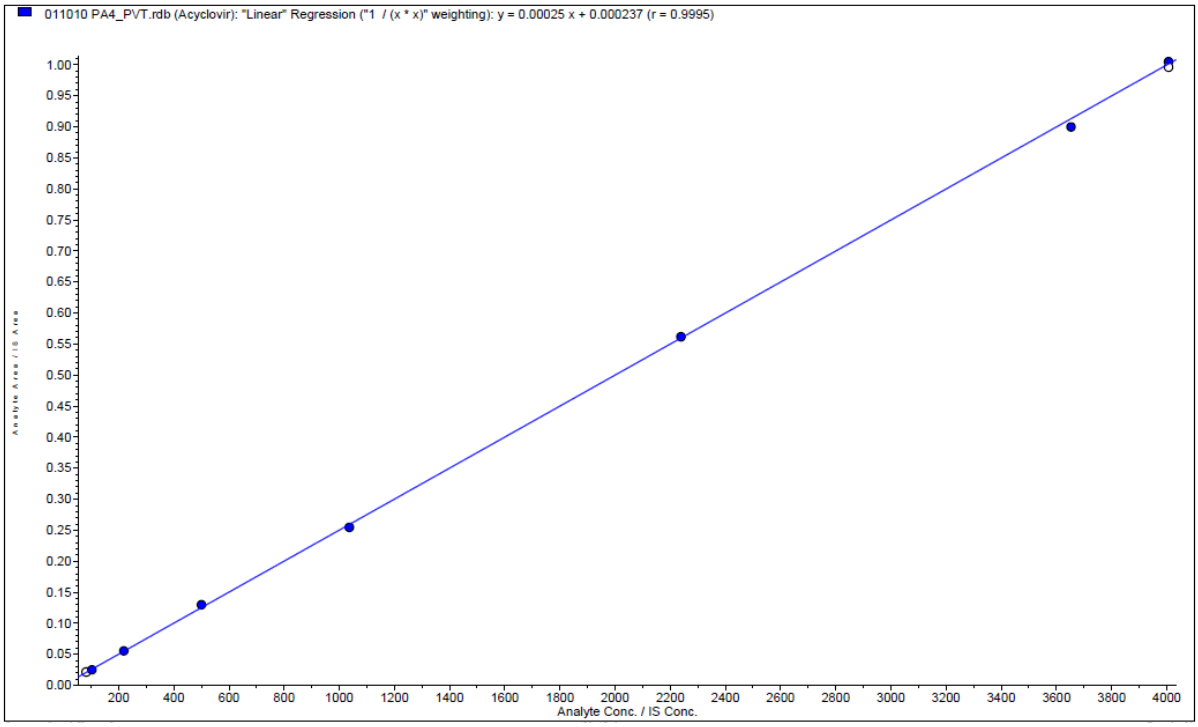
Data Processing

The chromatograms were acquired and were processed by peak area ratio method using the Analyst Version 1.5.1 Software. The concentration of the unknown was calculated from the following equation using regression analysis of spiked standard with the reciprocal of the ratio of the (drug concentration)^2 to internal standard concentration as a weighing factor ($1/x^2$):

$$y = mx + c$$

Where, y = peak area ratio of Acyclovir to internal standard
m = slope of calibration curve
x = concentration of Acyclovir
c = y-axis intercept of the calibration curve

Figure 4 - A Representative Calibration Curve for Acyclovir



RESULTS AND DISCUSSION

The method was validated in terms of limit of quantification, Recovery, Selectivity, Precision, accuracy and stability.

No significant interference was observed at the RT and m/z of Acyclovir and internal standard in all the batches screened. The IS-normalized matrix factor was found to be close to one for six different matrix lots for Acyclovir and % CV was 4.21%.The % carry over was found to be 0.00 for analyte and 0.00 for internal standard. The method was validated over the range of 82.967-4006.948 ng/ml. for Acyclovir. The correlation coefficient found to be greater than 0.9978.The recovery was determined by comparing the aqueous solution and the spiked drug. The percentage recovery was calculated Acyclovir 84.58% and 4.33% and for internal standard 101.71% and 2.99% respectively. The accuracy, precision and intraday precision were carried out by preparing six individual samples of QCH, QCM and QCL. The % CV and % nominal was calculated. Refer Table No: 1 for the results of Within-Batch Precision and Accuracy, Intraday Batch Precision and Accuracy and Between Batch Precision and Accuracy. The mean accuracy for Acyclovir ranged from 99.93% (QCL) to 105.71 % (QCH) and the precision ranged from 3.17 % (QCH) to 6.85 % (QCLLQ). The within batch precision and accuracy, for a dilution factor of two for Acyclovir was 93.33% and 3.85%. The within batch precision and accuracy, for a dilution factor of six for Acyclovir was 64.65% and 4.89%.

Table No. 1: Precision and Accuracy for Acyclovir

Within Batch Precision	PA1	2.53 % - 6.17 % (QCM-QCLLQ)
	PA2	3.68 % - 11.01 % (QCLLQ-QCL)
	PA3	1.65 % - 3.73 % (QCH-QCLLQ)
Within Batch Accuracy	PA1	85.47 % - 91.99 % (QCLLQ-QCM)
	PA2	87.86 % - 96.99 % (QCH-QCM)
	PA3	86.62 % - 99.79 % (QCLLQ -QCM)
Intraday Batch Precision	Day-1	5.99 % - 7.52 % (QCM-QCL)
	Day-2	3.61 % - 8.58 % (QCH-QCLLQ)
Intraday Batch Accuracy	Day-1	91.89 % - 98.39 % (QCL-QCM)
	Day-2	84.42 % - 98.43 % (QCLLQ -QCM)
Between Batch Precision		5.58 % - 7.33 % (QCLLQ -QCH)
Between Batch Accuracy		87.94 % - 96.80 % (QCLLQ - QCM)

Stability: Stability of the method was carried out by performing short term and long term stock stability. The percentage mean ratio of the drug and internal standard were calculated. Stability of the plasma samples was carried out bench top, freeze thaw, below -20°C and below -50°C, auto sampler, re-injection reproducibility, stock dilution and solution. The results for stability studies as mentioned below:

The percent nominal for bench top ranged from 90.41% (QCL) to 98.92% (QCH) and the precision ranged from 2.06% (QCH) to 3.88% (QCL).The percent nominal for Freeze Thaw ranged from 90.59% (QCL) to 99.75% (QCH) and precision ranged from 5.11% (QCH) to 6.62% (QCL) respectively. The percent nominal for long term below -20°C ranged from 94.47% (QCL) to 96.33% (QCH) and precision ranged from 2.15% (QCL) to 3.57% (QCH) respectively. The percent nominal for long term below -50°C ranged from 88.10% (QCL) to 93.98% (QCH) and precision ranged from 3.33% (QCH) to 3.55% (QCL) respectively. The percent nominal for auto sampler at around 50 hours ranged from 98.38% (QCL) to 114.14% (QCH) and precision ranged from 3.22% (QCH) to 6.97% (QCL) respectively. The percent change observed for the internal standard (Omeprazole) was 2.41%. : The % change of re-injection reproducibility is 0.49 % (QCL) and 10.28% (QCH). The percent change stock dilution for Acyclovir was 0.37 % and for Omeprazole is 0.81 %, respectively. The percent change stock solution for Acyclovir was 0.14 % and for Omeprazole is 0.19 % respectively.

Analyte and Internal elutes early in turn saves analysis time. Liquid Liquid Extraction Method is much cheaper compared to Solid Phase Extraction. Recovery of the method is very good which is more than 85% was for Analyte and Internal Standard) these are the advantages compare with Kanneti et al

CONCLUSION

The developed method was validated and found that the assay has good precision and accuracy over a wide concentration range, and no interference caused by endogenous compound was observed. The limit of quantification of acyclovir was 82.967 ng/mL. The separation method developed produce recovery of 84.58%. The developed and validated method was successfully applied for bioequivalence studies.

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