

A NEW VALIDATED RP- HPLC METHOD FOR DETERMINATION OF PENCICLOVIR IN HUMAN PLASMA

CH. VENKATA KUMAR, D. ANANTAHAKUMAR, J. V. L. N. SESHAGIRI RAO

For author affiliations, see end of text

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ABSTRACT

A rapid, selective and sensitive high performance liquid chromatographic method for the estimation of *Penciclovir* in human plasma has been developed. Chromatography was carried out on a Hypersil BDS C₁₈ column using a mixture of 20mM Potassium dihydrogen phosphate buffer (pH 3.5 ± 0.05) and methanol (95:5 v/v) as the mobile phase. The eluents were monitored for the drug by UV detection at 254 nm. Acyclovir was used as an internal

standard for this study. The retention times for Penciclovir and Acyclovir were found to be 6.2 and 9.2 min respectively. The method was found to be linear in the concentration range of 50.1 ng/mL to 7000.9 ng/mL. The method was validated as per FDA guidelines and was found to be suitable for bioequivalence and pharmacokinetic studies.

KEY WORDS: *Penciclovir*, Determination, Plasma, HPLC

INTRODUCTION

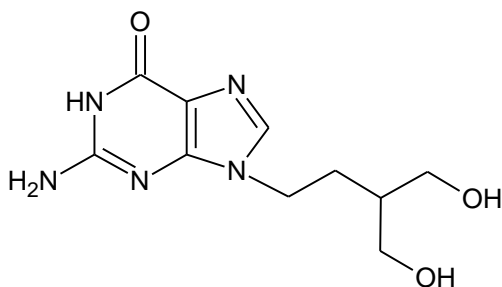


Fig. 1 Structure of Penciclovir

Penciclovir (2-Amino-1, 9-dihydro-9-[4-hydroxy-3-(hydroxymethyl) butyl]-6H-purin-6-one) is a guanine nucleoside analogue. It is active against herpes simplex virus type 1 and 2 and against Varicella zoster virus¹⁻². When famciclovir is taken orally it is converted to its active metabolite penciclovir. It inhibits enzyme thymidine kinase and interferes with DNA synthesis. It is official in USP and BP. Its empirical formula is C₁₀H₁₅N₅O₃. The structure of Penciclovir is shown in Fig. 1. A literature survey reveals the report of a few analytical methods for the determination of penciclovir in various biological fluids by HPLC³⁻⁹ and LC-MS/MS¹⁰. The authors now propose a simple, sensitive, accurate and precise RP-HPLC method for the determination of penciclovir by solid phase extraction technique.

EXPERIMENTAL PROCEDURE:

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 buffer was prepared by dissolving 2.72grams of Potassium dihydrogen phosphate in 1000 mL of milli-Q water. The pH of the buffer solution was adjusted to 3.5 ± 0.05 with ortho phosphoric acid. The working standard samples of Penciclovir and Acyclovir (internal standard) were obtained from Aurobindo Pharma (Hyderabad, India).

Chromatographic conditions

A Shimadzu HPLC equipment comprising of two LC-10AT VP pumps, VP CTO-10AS VP column oven, a Hypersil BDS C₁₈ (4.6 ID X 250 mm, 5 μ 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 ammonium acetate buffer (pH 3.5 ± 0.05) and methanol in a ratio of 95:5 v/v was used for elution at a flow rate of 1.4 mL/min. Data was acquired and processed with Class VP Software.

Working standard solutions and the calibration

The stock solution of the analyte was prepared in a mixture of water and methanol (80:20 v/v) at a free base concentration of 1 mg/mL. The working standard solutions were prepared from the stock solution by diluting with the above solvent mixture. These 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 penciclovir dilutions. The calibration curve was linear in the range of 50.1 to 7000.9 ng/mL ($r \geq 0.9900$). The calibration plot was drawn with a weighing factor of $1/X^2$. The quality control samples were prepared at four concentration levels of 50.3 ng/mL (LLOQ QC), 150.6 ng/mL (LQC), 3301.6 ng/mL (MQC) and 5240.7 ng/mL (HQC). The calibration curve results are presented in Table 1 & 2.

The stock solution of the internal standard was prepared in a mixture of water and methanol (80:20 v/v) at a free base concentration of 1 mg/mL. From this, the working internal standard solution was prepared by suitable dilution with the above solvent mixture to contain 40 ng/mL.

Sample extraction procedure:

Five hundred micro liters of spiked plasma calibration curve standards and quality control samples were transferred to pre-labeled polypropylene tubes containing 50 μ L of the internal standard. The tubes were vortexed for ten seconds.

curve:

Five hundred micro liters of 5% ortho phosphoric acid solution was added to each tube and vortexed for another ten seconds. Each of the MCX 30mg/1CC cartridges was conditioned with 1mL of methanol followed by equilibrating with 1mL of 0.5% ortho phosphoric acid in water on the solid phase extraction chamber. The above prepared samples were loaded on to the cartridges and the cartridges were washed with 1mL of ethyl acetate followed by 1mL of 10% methanol in water. The cartridges were dried for about one minute and eluted with 1mL of 1% ammonia in methanol. The eluents were evaporated in a stream of nitrogen for 20 minutes at 50°C and the residues in the dried tubes were reconstituted with 0.3mL of the mobile phase. The contents of the tubes were vortexed and transferred into auto-sampler vials and then analyzed by injecting 50 μ L of the sample into the column.

Validation:

The proposed method has been validated¹¹ for selectivity, sensitivity, linearity, precision, accuracy, recovery, and 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 are presented in Table 3.

Accuracy is defined as the percent relative (%RE) and was calculated using the formula $\%RE = (E-T) \times (100/T)$ where E is the experimentally determined concentration and T is the theoretical concentration of the drug. Assay precision was calculated using the formula $\%RSD = (SD/M) \times (100)$ 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 (comparison samples, t_0), with the

samples that were injected after keeping in auto-sampler at 10°C for 20.0 hrs (stability samples). The stability of spiked human plasma samples stored at room temperature (Bench top stability) was evaluated for a period of six 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°C (long – term stability) was evaluated by analyzing the quality control samples that were stored at -70°C for seven days together with the freshly spiked calibration standards and the quality control samples. All stability evaluations were based on back-calculated concentrations. The stability data is presented in Table 4. Analytes were considered stable if the deviations of the mean test responses were within 15% of the freshly prepared or comparison samples.

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

CONCLUSION

The proposed HPLC method is simple, selective, precise and accurate for quantification Penciclovir in human plasma. The method may be useful for bio-availability and bio-equivalence studies on the drug.

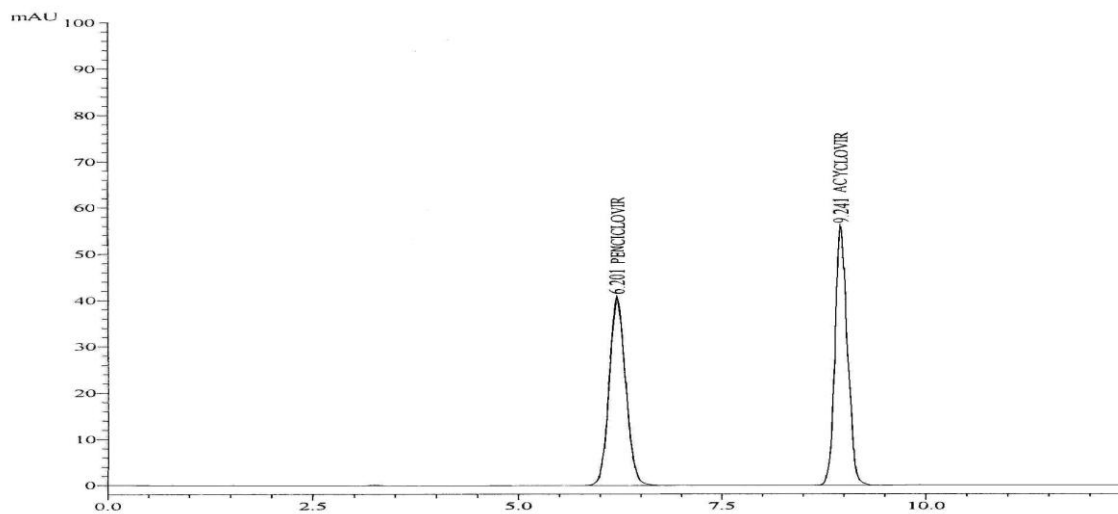


Fig. 2 Representative Chromatogram of Penciclovir extracted from plasma

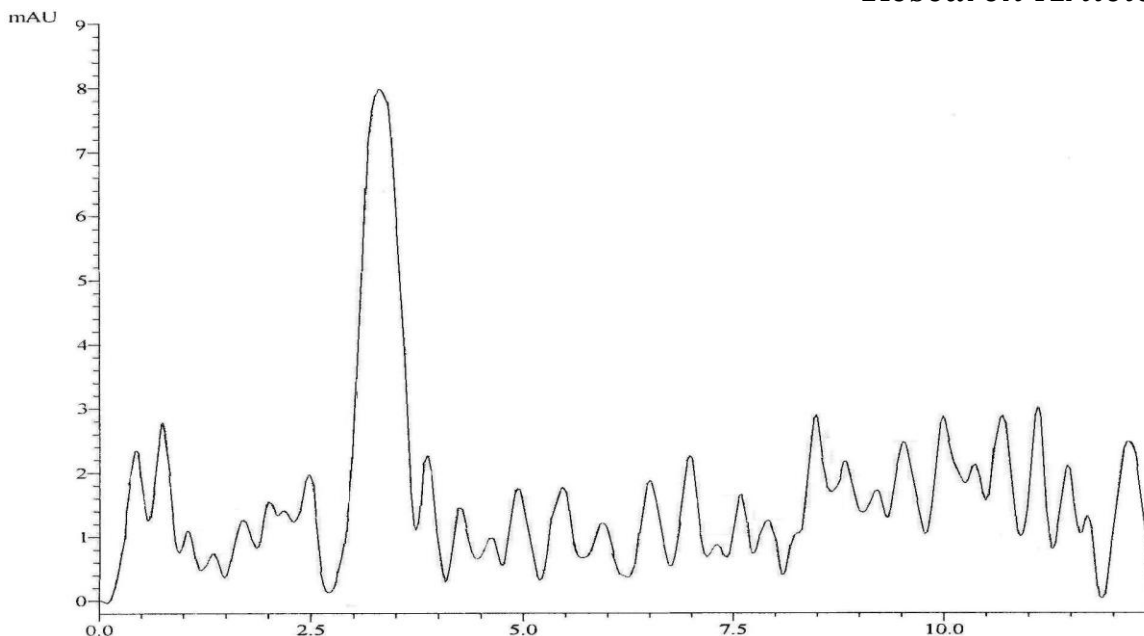


Fig. 3 Representative Chromatogram of extracted blank plasma sample

Table 1. Summary of calibration parameters

Calibration curve	Slope	Intercept	Correlation coefficient
1	0.000079	0.00012	0.9992
2	0.000077	0.00471	0.9995
3	0.000082	-0.00480	0.9992
4	0.000079	-0.00101	0.9989
5	0.000079	0.00553	0.9956
6	0.000077	0.00619	0.9967

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

Nominal concentration (ng/mL)	Mean calculated concentration	Percent Coefficient variation	Percent Relative Error
50.1	50.03	4.1	0.17
150.0	153.00	5.0	-1.97
750.2	738.77	7.7	1.52
1500.4	1554.20	7.3	-3.59
3000.8	2992.36	4.9	0.28
4001.0	4005.92	3.8	-0.12
5250.7	5291.35	5.0	-0.77
7000.9	6843.27	4.0	2.25

Table 3. Precision and accuracy

Details of Stability Experiment	n	Spiked concentration ng/mL	Mean calculated comparison sample concentration	Mean calculated stability sample concentration	Mean percent change
Bench top stability HQC	6	5240.7	4821.77	5002.75	-3.62
Bench top stability LQC	6	150.6	152.05	147.43	3.13
Auto-sampler stability HQC	6	5240.7	5043.37	5028.97	0.29
Auto-sampler stability LQC	6	150.6	152.82	147.00	3.96
Freeze-thaw stability HQC	6	5240.7	4821.77	5122.23	-5.87
Freeze-thaw stability LQC	6	150.6	152.05	149.65	1.60
Dry extract stability HQC	6	5240.7	5043.37	5215.13	-3.29
Dry extract stability LQC	6	150.6	152.82	146.65	4.21
Long term stability HQC	6	5240.7	5307.70	4881.95	8.72
Long term stability LQC	6	150.6	155.87	145.15	7.38

Table 4. Stability data

QC ID	Nominal concentration (ng/mL)	Intra batch				Inter batch			
		n	Mean concentration observed	% CV	% RE	n	Mean concentration observed	% CV	% RE
LLOQ QC	50.3	6	51.61	11.8	-2.63	36	48.48	9.1	3.59
LQC	150.6	6	150.96	6.7	-0.27	36	148.65	4.3	1.26
MQC	3301.6	6	3217.71	8.8	2.54	36	3321.02	8.0	-0.59
HQC	5240.7	6	5268.49	7.2	-0.53	36	5171.98	6.8	1.31

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AUTHOR AFFILIATIONS:

*Pharmaceutical Analysis and Quality Assurance
Division, A.U. College of Pharmaceutical Sciences,
Andhra University, Visakhapatnam – 530003.*

ADDRESS FOR CORRESPONDENCE:

Prof. J.V.L.N. SESHAGIRI RAO,
Pharmaceutical Analysis and Quality Assurance
Division
A.U. College of Pharmaceutical Sciences
Andhra University,
Visakhapatnam.
Off No: +91-891-2844933
Fax No: +91-891-2755324
Email: jvlns@yahoo.com