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Method Validation and Quantitative Determination of Antiviral Drug Acyclovir in Human Plasma by a LCMS/MS

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ABSTRACT : A simple, sensitive and selective LCMS/MS method has been developed for the determination of acyclovir in human plasma. Since acyclovir is a polar compound and soluble in aqueous medium and practically insoluble in most of organic solvents its analysis in biological fluids in currently published HPLC methods, involve pre-treatment of acyclovir plasma sample including deproteinization or solid phase extraction, Acyclovir in plasma were concentrated by solid phase extraction and chromatographed on a C_{18} column using a mobile phase of 0.1% formic acid : methanol (30 : 70% *v/v*). The method was validated over a linear range of 20 - 1000 ng mL^{-1} for acyclovir. The LOQs were 60.0 and 900 ng mL^{-1}. The validated method was applied for the quantitation of acyclovir from plasma samples in a pharmacokinetic study.

Keywords: Acyclovir; LCMS/MS; Plasma; solid phase extraction

INTRODUCTION

Acyclovir {9-[(2-hydroxyethoxy) methyl] guanine, zovirax} is a guanosine analogue with an acyclic side chain at the cella zoster viruses. As acyclovir is structurally similar to endogenous substances, its analysis in human serum is complicated and requires high selective analytical methods. Immunological techniques and HPLC are the most common used methods for determination of acyclovir in biological samples. Acyclovir is a nucleoside analog with antiviral activity against herpes viruses. This drug is an effective agent in the treatment of herpes virus infections and may also used in the prophylaxis of cytomegalovirus infections in immune compromised patients. Some HPLC methods for the analysis of acyclovir in plasma, serum or urine has been published [2-5]. However, these analytical methods require the use of an ion-pairing agent [6], column thermostating and fluorimetric detection [7] or are limited by the lack of sensitivity.

Several HPLC methods [8-9] have been published for determination of acyclovir in human serum using UV or fluorescence detection. Since acyclovir is a polar compound and soluble in aqueous medium and practically insoluble in most of organic solvents, protein precipitation with perchloric acid [10-11] or solid phase extraction [12] are applied for pre-treatment of the drug in serum samples. While the sensitivity of analysis is significantly reduced due to dilution of the samples after deproteinization, injection of the acid supernatant after precipitation of proteins by perchloric acid leads to numerous late eluting peaks and significant reduction of the lifetime of analytical column. Time consuming gradient elution is needed for removing of the late-eluting peaks, and deterioration of column performance significantly reduces the number of samples which can be analyzed. Solid phase extraction is expensive and moreover about 1 ml of solvent is required for elution of the drug from the cartridges. How ever, in solid phase extraction methods, the drug is eluted by application of aqueous solvents. As these solvents can-not easily be evaporated, dilution of the samples reduces the method sensitivity. Different limits of quantification (LOQ) ranging from 10 to 200 ng/mL of serum have been reported in published methods, however; LOQ of less than 50 ng/mL has been achieved in these methods by either increasing the injection volume [13-14] and/or application of highly acidic mobile phase [15] and at the expense of rapid deterioration of the analytical column.

EXPERIMENTAL

A. Chemicals and reagents

Acyclovir (99.8%) was provided by Cipla Inc. and dexchlorpheniramine (99.9%) was obtained from Sigma Aldrich Co., HPLC grade methanol was purchased from Mallinckrodt Baker, S.A.de C.V. (Estado de Mexico, Mexico). GR grade formic acid, GR grade Hydrochloric acid was obtained from Merck Specialties Pvt. Ltd. (Mumbai, India). Ultra-pure water was obtained from an TKA Water Purification System. Blank human blood was collected from healthy drug-free volunteers by Bhosale Pathology. Plasma was obtained by centrifugation of blood treated with K2 EDTA plasma as anticoagulant. Pooled plasma was prepared and then stored at approximately “20°C until needed.

B. Calibration Standards and Quality Control

Stock solutions of acyclovir and internal standard (dexchlorpheniramine) were prepared in Methanol at concentrations of 100ppm. Calibration curves of acyclovir were prepared by spiking blank plasma at concentrations of 20.0, 60.0, 120.0, 200.0, 350.0, 600.0, 800.0 and 1000.0 ng/ml.

The analysis was carried out in duplicate for each concentration. The quality control samples were prepared in blank plasma at concentrations of 60.0, 300.0 and 900.0 ng/ml (LQC, MQC and HQC, respectively). The spiked plasma samples (standards and quality controls) were extracted from each analytical batch along with the unknown samples.

C. Sample preparation

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 0.500 mL of spiked plasma samples, 50 mL internal standard was added and vortexed for 10 s. Further, 0.500 mL of 0.1 N hydrochloric acid was added and vortex mixed for another 10 s. Centrifugation of the samples was done at 3200 × g for 2 min at 10°C. The samples were loaded on Oasis HLB (1 cc, 30 mg) extraction cartridges which were preconditioned with 1 mL of methanol followed by 1 mL of water. Drying of cartridges was done for 1 min by applying nitrogen (1.72 × 105 Pa) at 2.4 L/min flow rate. Elution of analytes and IS from the cartridges was carried out with 0.200 mL of water, followed by 0.300 mL of methanol into pre-labeled tubes. The contents were vortexed to mix and 5 µL was used for injection in the chromatographic system.

D. Chromatographic conditions

A Shimadzu LC–VP HPLC system (Kyoto, Japan) consisting of LC-10AD prominence pump, SIL-HTc auto sampler, CTO 10 ASvp column oven and a DGU-14A degasser was used for setting the reverse phase liquid chromatographic conditions. The separation of ACV and IS was performed on a Phenomenex analytical column type Gemini, C18, 150 mm × 4.6 mm (length × inner diameter) with 5 µm particle size and was maintained at 40°C in column oven. The mobile phase consisted of 0.1% formic acid and methanol (30:70, v/v), For isocratic elution, the flow rate of the mobile phase was kept at 0.8 mL/min. The total eluate from the column was split in 70:30 (v/v) ratio; flow directed to the ISP interface was equivalent to 240 L/min. The chromatographic run time was 2.0 min. The sample manager temperature was maintained at 5°C and the pressure of the system was 4.13 × 106 Pa. Under these conditions, typical standard retention times were 0.86 ± 0.1min for acyclovir and 0.71 ± 0.2 min for dexchlorpheniramine, and back-pressure values of approximately 60 bar were observed. Temperature of the auto-sampler was kept at 6°C and run-time was set to 2.0 min.

E. Mass-Spectrometric conditions

Ionization and detection of analyte and IS was carried out on a triple quadrupole mass spectrometer, MDS SCIEX API-4000 (Toronto, Canada), equipped with electrospray ionization (TIS interface of the API 4000) and operating in positive ion mode. Quantitation was performed using multiple reaction monitoring mode to monitor precursor → product ion transitions at m/z 226.2 → 152.2 for ACV and 307.1 → 220.3 for IS respectively . The source dependent parameters maintained for ACV and IS were Gas 1 (Nebuliser gas): 40.0 psig; Gas 2 (heater gas flow):60.0 psig; ion spray voltage (ISV): 5500.0 V, turbo heater temperature (TEM): 400.0°C; interface heater (Ihe): ON; entrance potential (EP): 10.0 V; collision activation dissociation (CAD): 5 psig and curtain gas (CUR), nitrogen: 20 psig. The optimum values for compound dependent parameters like declustering potential (DP), collision energy (CE) and cell exit potential (CXP) set were 50 and 39 V; 25 and 28 eV; 10 and 13 V for ACV and IS respectively. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 200 ms. Analyst software version 1.4.2 was used to control all parameters of the LC and MS.

F. Linearity

Linearity was determined to assess the performance of the method. A linear least-squares regression with a weighting index of $1/x^2$ order was performed on the peak-area ratios of acyclovir and I.S. versus Acyclovir concentrations of the eight plasma standards (20.0, 60.0, 120.0, 200.0, 350.0, 600.0, 800.0 and 1000.0 ng/ml) to generate a calibration curve. Unknown sample peak-area ratios were then interpolated from the calibration curve to provide concentrations of acyclovir.

G. Specificity and selectivity

Blank samples from six different pools of plasma, including one lipemic and one haemolyzed, were tested for interference using the proposed extraction procedure and analytical conditions.

H. Recovery

The recovery was evaluated by calculating the mean (and RSD) of the response of each concentration and dividing the extracted sample response by the unextracted sample mean of the corresponding concentration. Comparison with the unextracted samples, spiked on plasma residues, was performed in order to eliminate the matrix effect, providing a true recovery. Matrix effect experiments were carried out using the ratio between spiked mobile phase solutions and unextracted samples, spiked on plasma residues.

I. Stability

Stability quality control plasma samples (20.0 and 1000.0 ng/ml) were subjected to short-term (8 hrs) room temperature, three freeze/thaw (“15 to 25°C) cycles, 24 hrs autosampler stability (6°C) and long-term stability at “15 to 25°C (24 days) tests. Subsequently, the acyclovir concentrations were measured compared to freshly prepared samples.

J. Precision and Accuracy

To assess precision and accuracy of the developed analytical method, four distinct concentrations in the range of expected concentrations were evaluated using eight determinations per concentration.

Precision and accuracy was assessed at within-day basis (intra-batch) during a single analytical run and at a between-day basis (inter-batch), which measures the between day variability, possibly involving different analysts and regents.

K. Ionic Suppression

A procedure to assess the effect of ion suppression on the MS/MS was performed. The experimental set-up consisted of an infusion pump connected to the system by a “zero volume tee” before the split and the HPLC system pumping the mobile phase, which was the same as that used in the routine analysis of acyclovir, i.e. 0.1% formic acid and methanol (30:70, v/v), at a flow-rate of 0.800 ml min”1. The infusion pump (Harvard Apparatus, Holliston, MA, USA) was set to transfer (10 ìl/min) a mixture of analyte and internal standard in mobile phase (both 50 ng/ml). A sample of human pooled blank plasma was extracted and the reconstituted extract was injected into the HPLC system while the standard mixture was being infused. In this system any ion suppression would be observed as a depression of the MS signal.

SAMPLE COLLECTED

Blood samples (4 ml) from a suitable antecubital vein were collected by an indwelling catheter into heparin containing tubes prior to administration and at 20 min, 40 min, 1 h, 1 h 20 min, 1 h 40 min, 2 h, 2 h 20 min, 2 h 40 min, 3 h, 3 h 30 min, 4 h, 4 h 30 min, 5 h, 5 h 30 min, 6 h, 8 h, 10 h, 12 h, 18 h, 24 h, 48 h, 96 h and 120 h after administration the of 1 mg acyclovir tablet formulation. Blood samples were centrifuged at approximately 2000 × g for 10 min at room temperature and the plasma was stored at “20 °C until assayed for acyclovir content.

RESULTS

Plasma calibration curves were prepared and assayed in triplicate on three different days to evaluate linearity, precision, accuracy, recovery, limit of quantitation (LOQ), selectivity and stability.

A. Linearity and LOQ

The method was considered linear at the concentration range between 20.0 ng/mL and 1000 ng/mL. Linear regression analysis was determined with correlation coefficients (r^2) greater than 0.99 for the calibration curves. The representative linearity curve is presented in Fig. 1

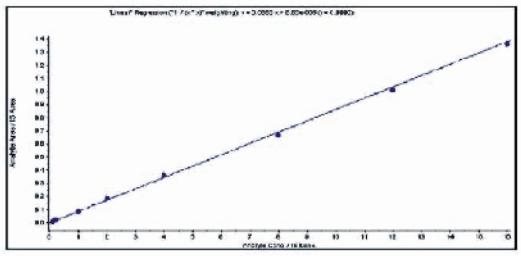

Fig. 1. Linearity Curve for Acyclovir.

The LOQ were estimated and were 60.0 ng/mL. The representative chromatogram for LOQ is presented in Fig. 2

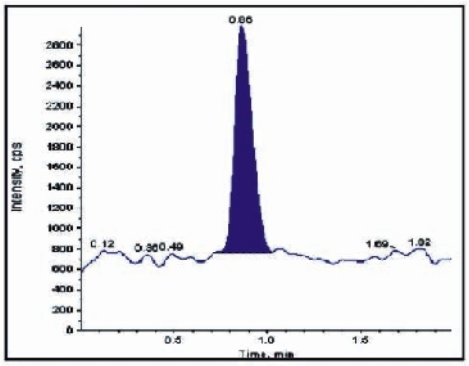

Fig. 2. chromatogram for LOQ of Acyclovir.

B. Precision, Accuracy, and Recovery

Results of intra- and inter-day precision showed CV% values not exceeding 15%, which mean that the method is precise.

As the calculated values of accuracy were always within 15% of the nominal value, the method could be considered accurate. Results are summarized in Table 1 and 2.

Table 1: Intra-day Precision And Accuracy For Acyclovir.

	LQC 60.0 ng/mL		MQC 300.0 ng/mL		HQC 900.0 ng/mL	
File Name	Calculated Conc. (ng/mL)	% Nominal Conc.	Calculated Conc. (ng/mL)	% Nominal Conc.	Calculated Conc. (ng/mL)	% Nominal Conc.
P&A_2	30.012	99.96	299.814	100.06	897.556	100.27
P&A_2	29.988	100.04	299.884	100.04	885.012	101.69

P&A_2	29.778	100.75	299.589	100.14	884.869	101.71
P&A_2	30.003	99.99	298.754	100.42	899.987	100.00
P&A_2	30.091	99.70	289.741	103.54	900.566	99.94
P&A_2	30.003	99.99	300.521	99.83	898.902	100.12
N	6	6	6	6	6	6
Mean	29.98	100.07	298.05	100.67	894.48	100.62
SD (±)	0.11	0.35	4.11	1.42	7.46	0.84
CV (%)	0.35	0.35	1.38	1.41	0.83	0.84

Table 2: Inter-day Precision And Accuracy For Acyclovir.

File Name	LQC 60.0 ng/mL		MQC 300.0 ng/mL		HQC 900.0 ng/mL	
	Calculated Conc. (ng/mL)	% Nominal Conc.	Calculated Conc. (ng/mL)	% Nominal Conc.	Calculated Conc. (ng/mL)	% Nominal Conc.
P&A_1	30.066	99.78	298.003	100.67	901.003	99.89
P&A_2	29.979	100.07	298.051	100.67	894.482	100.62
P&A_3	29.899	100.34	300.002	100.00	894.911	100.57
N	3	3	3	3	3	3
Mean	29.98	100.06	298.69	100.45	896.80	100.36
SD (±)	0.08	0.28	1.14	0.39	3.65	0.41
CV (%)	0.28	0.28	0.38	0.39	0.41	0.41

Recoveries varied between 94.85% and 98.59% for Acyclovir. Recovery for Dexchlorpheniramine was 78.93%. Results are summarized in Table 3 and 4.

Table 3: Recovery of Analyte From Biological Matrix.

	LQC 60.0 ng/mL		MQC 300.0 ng/mL		HQC 900.0 ng/mL	
File Name	Test Area	Comparison	Test Area	Comparison	Test Area	Comparison
P&A_2	4532	4553	21570	22721	67659	67664
P&A_2	4432	4566	20394	23844	64215	64545
P&A_2	4472	4573	22413	22730	62887	66887
P&A_2	4483	4463	22376	22632	62589	64395
P&A_2	4477	4590	21245	21946	60024	69842
P&A_2	4399	4433	20860	21985	64483	65810
N	6	6	6	6	6	6
Mean	4466	4530	21476	22643	63643	66524
SD (±)	45.71	65.07	812.45	689.19	2527.48	2068.79
% CV	1.02	1.44	3.78	3.04	3.97	3.11
% Recovery	98.59			94.85	95.67	

Table 4: Recovery of Internal Standard From Biological Matrix.

File Name	Internal Standard (330 ng/ml)	
	Test Area	Comparison
	28724	39022
	28520	33390
	25366	30489
	26344	32462
	26493	38441
	28084	37372
	27597	35747
	27048	31933
P&A_2	28617	31661
	26786	34693
	25302	31411
	27961	33844
	26472	35730
	27785	31632
	26364	37946
	26310	35985
	27188	32735
	26736	33380
N	18	18
Mean	27094	34326
SD (±)	1039.27	2651.98
% CV	3.84	7.73
% Recovery	78.93	

C. Specificity

Blank samples from six different pools of plasma, including one lipemic and one haemolyzed, were tested for interference using the proposed extraction procedure the results are summarized in Table 5.

Table 5: Blank Matrix Specificity.

Matrix Identification (Number)	Anticoagulant	Interference with Analyte (% of LLOQ)	Interference with Analyte Standard (% of IS)
BLK-KKP/A/756-1	K2 EDTA	*	*
BLK-KKP/A/757-1	K2 EDTA	*	*
BLK-KKP/A/758-1	K2 EDTA	*	*
BLK-KKP/A/759-1	K2 EDTA	*	*
BLK-KKP/A/771-1 (H)	K2 EDTA	*	*
BLK-KKP/A/772-1 (L)	K2 EDTA	*	*

D. Stability

Plasma stability was tested at different conditions such as Bench top, Auto sampler stability and Dry Extract. Results are summarized in Table 6, 7 and 8. Freeze – Thaw stability was evaluated after three cycle and found within acceptance limit. Results are summarized in Table 9.

Table 6: Bench Top Stability.

	LQC 60.0 ng/mL Calculated Conc.		HQC 900.0 ng/mL Calculated Conc.	
File Name	Test	Comparison	Test	Comparison
P&A_2	59.477	59.499	895.006	884.978
P&A_2	59.500	59.509	895.098	885.12
P&A_2	58.509	59.501	894.882	884.888

P&A_2	58.508	59.498	894.578	894.991
P&A_2	58.488	59.478	894.756	895.003
P&A_2	58.469	59.499	895.091	894.833
N	6	6	6	6
Mean	58.825	59.497	894.902	889.969
SD (±)	0.51	0.01	0.21	5.45
CV (%)	0.87	0.02	0.02	0.61
% Changes	-1.13	—	0.55	—

Table 7: Auto Sampler Stability.

	LQC 60.0 ng/mL Calculated Conc.		HQC 900.0 ng/mL Calculated Conc.	
File Name	**Test**	**Comparison**	**Test**	**Comparison**
P&A_3	58.477	59.242	888.006	894.978
P&A_3	58.500	59.902	899.098	897.120
P&A_3	57.509	59.581	891.882	877.888
P&A_3	57.508	59.786	892.578	899.991
P&A_3	57.488	59.657	884.756	891.003
P&A_3	59.469	59.902	885.091	892.833
N	6	6	6	6
Mean	58.159	59.678	890.235	892.302
SD (±)	0.80	0.25	5.44	7.74
CV (%)	1.38	0.42	0.61	0.87
% Changes	-2.55	-0.23		

Table 8: Dry Extract Stability.

	LQC 60.0 ng/mL Calculated Conc.		HQC 900.0 ng/mL Calculated Conc.	
File Name	**Test**	**Comparison**	**Test**	**Comparison**
P&A_3	58.344	59.242	898.006	894.978
P&A_3	58.211	59.902	890.038	897.12
P&A_3	58.219	59.581	881.222	877.888
P&A_3	56.476	59.786	879.499	899.991
P&A_3	58.908	59.657	894.721	891.003
P&A_3	58.744	59.902	895.922	892.833
N	6	6	6	6
Mean	58.150	59.678	889.901	892.302
SD (±)	0.87		0.25	7.86
CV (%)	1.49	0.42	0.88	0.87
% Changes		-2.56	-0.27	

Table 9: Freeze Thaw Stability.

	LQC 60.0 ng/mL Calculated Conc.		HQC 900.0 ng/mL Calculated Conc.	
File Name	**Test**	**Comparison**	**Test**	**Comparison**
P&A_3	57.598	59.242	898.006	894.978
P&A_3	59.908	59.902	897.022	897.12
P&A_3	58.309	59.581	895.462	877.888
P&A_3	56.998	59.786	898.533	899.991
P&A_3	57.779	59.657	899.956	891.003
P&A_3	58.903	59.902	884.091	892.833
N	6	6	6	6
Mean	58.249	59.678	895.512	892.302
SD (±)	1.04	0.25	5.79	7.74
CV(%)	1.78	0.42	0.65	0.87
% Changes	-2.39	0.36		

V. CONCLUSION

The proposed HPLC-MS/MS method can be regarded as selective, accurate, precise, and valid for determination of acyclovir with a total running time of 2.0 min. Through this method it was possible to evaluate, acyclovir quantification in human plasma and offers advantages over methods previously reported. The present method is more sensitive, while the analytical run is shorter, permitting a high throughput.

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