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Role of RBC Partitioning and Whole Blood to Plasma Ratio in Bioanalysis: A Case Study With Valacyclovir and Acyclovir

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Abstract

A LC-MS/MS method was developed for simultaneous estimation of valacyclovir and acyclovir in human plasma. Plasma sample was extracted with solid phase extraction technique and chromatographic condition was set with Inertsil CN-3 (5 µm) column and mobile phase (1 mM ammonium acetate buffer - methanol, 50:50 v/v). Valacyclovir, acyclovir, Valacyclovir D4 and acyclovir D4 were detected in positive polarity in multiple reactions monitoring mode at mass transitions (m/z) 325.2→152.1, 226.2→152.1, 329.3→152.1 and 230.2→152.1, respectively. The validated calibration curve range for valacyclovir is 4.09 to 725.63 ng/mL and for acyclovir is 50.35 to 10017.29 ng/mL. During method development, stability of acyclovir in whole blood could not be established over the period for 2 hr as the $K_{WB}/_{P}$ ratio for acyclovir is greater than 1 and although for valacyclovir it is less than 1. Therefore, the drug distribution of acyclovir was investigated in whole blood and plasma. Experimental data showed that an initial drop of acyclovir level in plasma due to the cellular uptake of acyclovir by erythrocytes. Hence, the spiked comparison samples were allowed to reach equilibrium (between RBC and plasma). After reaching the equilibration time (30.0 min), plasma was harvested from the spiked whole blood and processed as per the proposed protocol. From the blood stability data, we concluded that valacyclovir and acyclovir both are stable in blood for 2 hrs. The developed method was validated as per current regulatory guidelines and applied for valacyclovir and acyclovir bio-equivalence study.

Keywords: Acyclovir; LC-MS/MS; RBC partitioning; Stability; Valacyclovir; Whole blood to plasma ratio

Introduction

Valacyclovir the L-valyl ester of acyclovir is an oral prodrug that undergoes rapid and extensive first-pass metabolism to yield acyclovir and the essential amino acid L-valine [1]. Acyclovir, the active antiviral component of valacyclovir, shows good *in vitro* activity against the HSV-1, HSV-2 and varicella zoster virus. The bioavailability of acyclovir after oral administration of valacyclovir is considerably greater than that achieved after oral administration acyclovir. Thus, valacyclovir delivers therapeutic acyclovir concentrations when administered in a less frequent oral dosage regimen than is required for acyclovir. The estimated plasma concentrations of valacyclovir is very low after 3 hours and practically non-quantifiable. The maximum plasma concentrations (C_{max}) of valacyclovir are usually less than 0.5 µg/mL at all doses [2]. The plasma half-life of acyclovir is approximately 2.5 to 3.3 hrs for all oral doses of valacyclovir [3].

Perrottet et al. [4] investigated the distribution of ganciclovir and acyclovir in red blood cell (RBC) and plasma and concluded that an initial drop of ganciclovir and acyclovir level in plasma (~25%) due to the cellular uptake of acyclovir and ganciclovir by RBC and probably due to high value of blood to plasma ratio of these antiviral drugs. Henceforth, the blood to plasma ratio is crucial parameter for these antiviral drugs.

Usually blood to plasma ratio defines as the concentration of drug in whole blood (that is, target drug containing both erythrocytes and plasma) to the concentration of target drug in plasma. The red blood cell partition coefficient is the ratio of the concentration of drug in the red blood cells (that is, not including plasma) to concentration of drug in plasma. The PK parameters are generally determined by using plasma concentrations of drug rather than whole blood. So, PK analysis would be difficult if the blood to plasma ratio value is less than 1 for target drug.

Literature survey revealed that there are many methods for the qualitative and quantitaion estimation of acyclovir in biological fluids alone, with valacyclovir or with other antiviral drugs, such as high performance fluorescence [4-6], amperometric detection [7], high performance capillary electrophoresis [8], UV detection [9-15] and LC-MS/MS technique [16-19]. However, all these methods have their own disadvantages and which includes complicated sample pretreatment, time consuming chromatographic separation, low sensitivity and large volume of plasma (>500 µL). No research data was published for RBC partitioning and whole blood to plasma ratio of acyclovir and no study was conducted for determination of equilibration time for acyclovir till date. Thus a new LC-MS/MS method was developed with several advantages over other existing methods. The advantages of the developed method includes: less aliquot volume (100 µL), higher sensitivity, short chromatographic run time and null matrix effect observed in all type of matrices (like normal plasma, hemolyzed plasma, lipemic plasma or whole blood). After determination of equilibration time, precise estimation of valacyclovir and acuclovir concentration in incurred samples was possible and this was not reported by any of the published assay methods.

Experimental

Chemicals and reagents

Working standard of valacyclovir (VAL) and acyclovir (ACL) were obtained from USP. Working standard of valacyclovir D4 (VAL D4) and acyclovir D4 (ACL D4) were obtained from Clearsynth labs Ltd, Mumbai, India. were procured from Fluka (Sigma-aldrich, Steinheim, USA). All the reagents (like formic acid, ortho phosphoric

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acid, hydrochloric acid and liquor ammonia solution) with analytical grade was used for sample preparation and LC-MS grade solvent (like methanol) was used analysis. Oasis MCX cartridge (30 mg/1 cc) were used for extraction purpose. Human K_3EDTA plasma was procured from Yash Path Lab, Mumbai, India.

Chromatographic conditions

Suitable chromatographic conditions was achieved with Inertsil CN-3 (75 mm × 4.6 mm, 5 µm) column and mobile phase composed with 1 mM ammonium acetate buffer and methanol (50:50, v/v). Mobile phase was delivered with 0.8 mL/min flow, where 50% of the flow was splitted. For autosampler (in injector) 10°C and for column oven 35°C temperature was maintained.

Mass spectrometric conditions

Mass spectrometer parameters were optimized, by infusing individual neat solution of each compound (100.0 ng/mL) into the LC-MS/MS. The mass spectrometer (API-3000) equipped with electrospray ionization operated in positive polarity using multiple reaction monitoring (MRM). The mass transitions (m/z) were selected as 325.2→152.1, 226.2→152.1, 329.3→152.1 and 230.2→152.1 for VAL, ACL, VAL D4 and ACL D4, respectively. The optimized compound parameters for monitoring VAL and VAL D4 were set as follows: declustering potential (DP), 35 V; entrance potential (EP), 10 V; focusing potential (FP), 140 V; collision energy (CE), 23 V; and collision cell exit potential (CXP), 8 V. The optimized compound parameters for monitoring ACL and ACL D4 were set as follows: declustering potential (DP), 20 V; entrance potential (EP), 10 V; focusing potential (FP), 100 V; collision energy (CE), 15 V; and collision cell exit potential (CXP), 8 V. The source parameters of the mass spectrometer were optimized and maintained as follows: collision activated dissociation gas (CAD), 10 psi; curtain gas (CUR), 8 psi; nebulizer gas, 8 psi; turbo ion spray voltage, -5500 V; and source temperature, 475°C.

Bulk spiking

All the stock solutions of working standard was prepared in 0.5(N) hydrochloric acid solution at a concentration of 1 mg/mL except for the ACL, for which the strength is 3 mg/mL and were stored in refrigerator (1-10°C). The stock solutions of VAL and ACL were further diluted with methanol-water (50:50, v/v) to yield working solution at different concentration levels. Non zero-calibration standards (CC) and quality control (QC) samples were prepared by spiking (1%, v/v) of secondary solutions in human K_3EDTA plasma. The final concentration for the CC in human K_3EDTA plasma were 4.09, 11.61, 23.22, 58.05, 145.13, 290.25, 580.50, 725.63 ng/mL for VAL and 50.35, 141.04, 320.55, 801.38, 2003.46, 4006.91, 8013.83, 10017.29 ng/mL for ACL. Separate stock solutions were prepared for spiking of QC samples. Working solutions prepared from the stock solutions were used to spike QC samples in human K_3EDTA plasma at 4.10 ng/mL (LOQQC); limit of quantification, 11.72 ng/mL (LQC); lower QC, 285.92 ng/mL (MQC); middle QC and 571.84 ng/mL (HQC); higher QC for VAL and 50.46 ng/mL (LOQQC); limit of quantification, 145.41 ng/mL (LQC); lower QC, 4039.06 ng/mL (MQC); middle QC and 8078.12 ng/mL (HQC); higher QC for ACL. During bulk spiking, ice cold water bath and under low light conditioned was maintained. Bulk spiked CC and QC samples were stored below -50°C and protected from light till use. The secondary solution of ISTD (1000.0 ng/mL of VAL D4 and 2000.0 ng/mL of ACL D4) for regular use was prepared in methanol-water (50:50, v/v) from standard stock solution.

Plasma sample extraction

The SPE method was used to extract VAL, ACL and their respective internal standard from human plasma. For this purpose, 100 µL of plasma and 50 µL of internal standard was added in labeled polypropylene tubes. Thereafter, 750 µL of solution-1 (5% ortho phosphoric acid in water, v/v) was added to each sample and vortexed for 10 sec. The pretreated samples was loaded on the equilibrated Oasis MCX cartridge (30 mg/1 cc) and centrifuged at 4000 rpm for 1 min. After that 1 mL of methanol and 1 mL of water were used to washed the SPE cartridges and 1 mL of elution solution (5% ammonia in methanol, v/v) was used to elute the analyte and internal standard from the cartridges. The eluted samples were evaporated to dryness at 50°C under nitrogen gas and then reconstituted with 500 µL of reconstitution solution (1 mM ammonium acetate buffer-methanol, 50:50 v/v). Reconstituted sample was transferred into autosampler vials and 10 µL of sample was injected into the LC-MS/MS for analysis.

Impact of RBC partitioning and whole blood to plasma ratio in bioanalysis

After spiking of ACL working solution into human whole blood, a substantial decrease in ACL concentration was observed *in vitro* plasma sample. It may be due to the reason of drug uptake by erythrocyte before reaching the equilibrium between erythrocyte and plasma, that is, the value of blood to plasma ratio ($K_{WB}/_{P}$) for ACL is high. This it could lead to pseudo estimation of ACL and VAL in human plasma after oral dose of VAL. To avoid such occurence, it is essential to identified the time to reach equilibrium between RBC and plasma before separation of plasma from the incurred blood samples. Therefore, it was essential to determine the RBC partitioning, whole blood to plasma ratio and equilibration time for VAL and ACL.

Phase I: Determination of RBC partitioning and K_{WB}/p ratio: Aliquots of fresh whole blood and control plasma (separated from fresh whole blood in parallel) were spiked with working solutions of ACL and VAL (at HQC level) and then incubated at 37°C. After completion of the incubation period, plasma was separated from the incubated whole blood. Four aliquots of each sample (that is, isolated plasma from whole blood and control plasma) were processed as per proposed sample processing technique and the concentrations of target analytes in plasma samples was analyzed by LC-MS/MS. The $K_{WB/P}$ ratio and RBC partitioning were calculated using following equations:

Whole blood to plasma ratio: $$KWB/\mathrm{P} = \frac{Ccp}{cp}$$

Where, C_{cp} is the concentration of the drug in control plasma and C_p is the concentration of the drug in separated plasma.

RBC partitioning: $$KRBC/\mathrm{P} = 1 + \left(\frac{1}{H}\right)\left[\left(\frac{Ccp}{Cp}\right) - 1\right]$$

Where, H is the hematocrit value.

Phase II: Determination of equilibration time: Working solutions of VAL and ACL were spiked into human K_3EDTA whole blood (at LQC and HQC level). After spiking, samples were shaken gently for drug distribution and incubated at 37°C. Blood samples were then centrifuged at 4 ± 2°C and 4000 rpm for 15 minutes to separate plasma from the blood. At 0.0 min and 15.0 min, 30.0 min, 45.0 min and 60.0 min after spiking, the blood samples were centrifuged for plasma isolation and samples were kept in ice cold water bath till processing. Plasma samples separated at each time points (including both QC levels) were processed as per proposed processing protocol and analyzed in LC-MS/MS system. Peak area ratio (peak area response of analyte/ peak area response of internal standard) was obtained at each time interval was compared with the adjacent time point that is, 0.0 min

vs 15 min, 15 min vs 30 min and so on. The % difference was calculated using following equation:

$$\%\ difference = \frac{Absolute(mean\ area\ ratio\ of\ \ preceding\ time\ point - mean\ peak\ area\ ratio\ of\ \ subsequent\ time\ point)}{Average\ of\ \ mean\ area\ ratio\ of\ \ preceding\ and\ subsequent\ time\ points} \times 100$$

Phase III: Assessment of whole blood stability: Secondary solutions of both analytes (at LQC and HQC level) were added in human whole blood and incubated at 37°C to maintained equilibrium between RBC and plasma. After incubation, samples were stored for a period of approximately 2 hrs in ice cold water bath and these samples were served as stability samples. After completion of the storage period, secondary solutions of analytes were again spiked in blood to prepared comparison samples and incubated at 37°C. Comparison samples were allowed to reach equilibrium between RBC and plasma and after that all samples were centrifuged together at 4 ± 2°C and 4000 rpm for 15 mins to separate plasma from blood. Four aliquots of all samples were processed and analyzed in LC-MS/MS system. The stability duration was calculated as the difference between the times of spiking of comparison samples less the time of spiking of stability samples. % Stability was calculated by using the following equation:

$$\%\ Stability = \frac{Stability\ samples\left(Analyte\ peak\ area\ /\ ISTD\ peak\ area\right)}{Comparison\ samples\ \left(Analyte\ peak\ area\ /\ \ ISTD\ peak\ area\right)} \times 100$$

Validation parameters

For procedure and acceptance criteria of method validation exercises were performed as per USFDA and EMEA guidelines [20,21].

Selectivity

To demonstrate the method selectivity, ten different lots of human plasma (including two hemolyzed plasma lots and two lipemic plasma lots) and six aliquots of LOQ samples were processed and analyzed. The processed plasma lots were compared with LOQ sample for any interference at the retention time (RT) of analyte and internal standard. The degree of interference was calculated by comparing the peak area response observed at RT of target analyte against the mean peak area response of extracted LOQ sample.

Linearity

Calibartion curve (CC) set prepared in three validation batches were used determine for the linearity and after applying the least square regression analysis best fit curve was determined. During least square regression analysis, it was noted that (1/concentration^2) was to be more suitable for obtaining the best fit line for CC, where eight non-zero concentration was used. The concentration of VAL and ACL in human plasma (both in QC samples as well as incurred samples) were back-calculated from the corresponding CC.

Precision and accuracy

For determining the method intra-day precision and accuracy, two validation batches were processed and analyzed on the same day. The validation batch consisted of a CC set (eight non-zero calibration standards, double blank sample, single-blank sample) and six replicates of each QC samples (LOQQC, LQC, MQC and HQC). The inter-day precision and accuracy, were determined by analyzing the three validation batches on two consecutive days. The precision (%CV) of the method was determined at each QC level from the nominal concentration and it should be less than 15%, but for LOQQC it should be less than 20%. Similarly for all QC level, mean accuracy should be within 85-115%, except for LOQQC samples, for which it can be within 80-120%, by calculating the %CV at each QC level. The deviation at each concentration level from the nominal concentration should be within ± 15%, excluding at LOQQC level (± 20%). Similarly, the mean accuracy should not deviate ± 15%, excluding at LOQQC level (± 20%).

Process efficiency and matrix effect

The process efficiency (PE) or recovery of VAL and ACL was estimated in three QC levels (LQC, MQC and HQC level). To perform recovery exercise, six aliquots of LQC, MQC and HQC (extracted samples) were processed and analyzed in LC-MS/MS with neat samples (unextracted samples) and peak area response of extracted samples were compared against the unextracted sample. The PE of internal standards (VAL D4 and ACL D4) was determined at the working concentration (1000.0 ng/mL for VAL D4 and 2000.0 ng/mL for ACL D4) in a similar way. Process efficiency (PE) of analytes and internal standard were determined by using the following equation:

$$\%\ AME = \frac{Mean\ Peak\ Area\ of\ \ analyte\ in\ post\ extracted\ samples}{Mean\ Peak\ Area\ of\ \ analyte\ in\ neat\ solution} \times 100$$

'T' joint experiment is a post column infusion technique [22], where the effect of co-elute matrix on ionization of target analytes is determined qualitatively. An individual working solution of VAL, ACL, VAL D4 and ACL D4 was prepared at a concentration of 500 ng/mL and was infused through infusion pump at a flow of 10 µL/min into the mobile phase (post column via a 'T' connector). Extracted 10 µL double blank sample was then injected from the autosampler through LC column. In Analyst software, version 1.4.2, chromatograms were acquired and monitored for both analytes and internal standards. The 'T-Joint' experiment data showed that there was no effect of matrix at retention times of both analytes and internal standards.

The absolute matrix effect (AME) was determined by the following equation:

$$\%\ AME = \frac{Mean\ Peak\ Area\ of\ \ analyte\ in\ post\ extracted\ samples}{Mean\ Peak\ Area\ of\ \ analyte\ in\ neat\ solution} \times 100$$

Case I: When AME=1 it indicates there is no matrix effect on ionization of target analyte(s).

Case II: When AME value is <1, ionization of the analyte in mass spetrometer ion source is suppressed by the co-elute matrix components (that is, ion-suppression).

Case III: When AME value is >1, ionization of the analyte in mass spetrometer is enhanced by the co-elute matrix components (that is, ion-enhancement).

Required number of aliquots of human plasma with different lots were processed as per our developed sample processing technique till drying step and after drying the analyte and internal standard dilution were added into the dried plasma samples to prepared the post-extracted samples and analyzed in LC-MS/MS system along with the neat samples. The neat samples prepared by assuming the 100% extracted concentrations for analyte as well as internal standard in LQC, MQC and HQC levels.

Evaluation of relative Matrix effect (RME) was carried out in six lots of normal plasma, hemolyzed plasma and lipemic plasma. Secondary solution of VAL and ACL were spiked at two level of QC (that is, LOQQC and HQC) in each plasma lot (including hemolyzed and lipemic plasma). Two aliquots of matrix effect QC samples were processed along with freshly spiked CC set and control QC samples and analyzed in LC-MS/MS system.

Stability

Stability of analytes was investigated in stock solutions as well as in human plasma under different storage conditions and for internal standard only stock solution and working solution stability were performed. In stock solution stability, secondary working solution of analyte as well as internal standard were prepared from the old and new stock solution and the peak area response observed in stability sample was compared with the comparison sample. Stability QC samples were retrived from the deep freezer (below -50°C) and stored in ice cold water bath to performed the bench top stability and after completion of the required time (for ~7.1 hr), stability samples were processed with the freshly spiked QC samples. Stability QC samples were processed and stored in auto sampler (at 10°C) and after complete the required storage time (~98 hr), freshly spiked QC samples (comparision sample) and CC set were processed and analyzed with the stability samples. The freeze-thaw stability was performed by comparing the concentrations of the stability samples that were frozen (at -50°C) and thaw (in ice cold water bath) for three times with freshly prepared QC samples. To demonstrate the long term stability experiment, QC samples were stored in deep freezer (below -50°C) for longer duration. After completion of the storage period, freshly spiked CC set and comparision QC samples were processed with the stability QC samples. For evaluation purpose, the observed concentrations of stability QC samples were compared with the original spiked concentrations. Stability experiment in human plasma were performed at both level of QC (that is. LQC and HQC). % Change was determined by following formula:

$$\%\,change = \left[\frac{S}{F} - 1\right] \times 100$$

If % change is within 15, then analyte is considered as stable in that storage condition. Where, S=Mean observed concentration of stability samples and F=Mean observed concentration of comparison samples.

Results and Discussion

Optimization of mass parameters

Chemical structures of VAL, VAL D4, ACL and ACL D4 are shown in Figure 1. Due to presence of an amino moiety in the chemical structure of the analyte, scanning also performed in positive polarity. During daughter ion scan, the major ions was observed at m/z 152.1, 146.2 and 174.1 for VAL and ACL. The predominant and most intense ion of m/z 152.1 was selected as a daughter ion for both analytes. During product ion scan of both the internal standards, simillar fragmentation pattern was observed and thus 152.1 was also selected as daughter ion for both the internal standard. During mass parameter optimization it was noted that compound parameters like CE and CAD are the most crucial compound parameters to achieve peak sensitivity and stable response for all analytes. The parent ion (Q1 mass) /daughter ion (Q3 mass) 325.2→152.1, 226.2→152.1, 329.3→152.1 and 230.2→152.1 were selected for VAL, ACL, VAL D4 and ACL D4, respectively. The daughter ion mass spectra of VAL, ACL, VAL D4 and ACL D4 are shown in Figure 2.

Optimization of sample extraction

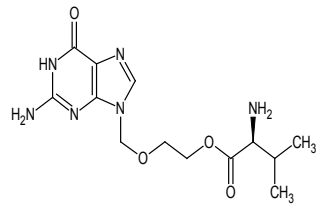
Initially, the extraction of VAL and ACL was carried out via protein precipitation with common solvents like acetonitrile, methanol and acetone, but the recovery was poor (<15%) in all solvents with frequent clogging of the column. LLE technique was also evaluated for extraction purpose using organic solvents like isopropyl alcohol, diethyl ether, dichloromethane, tert methyl butyl ether. However, due to ion enhancement the recovery was inconsistent at all three level of QC (%CV >40) for ACL. Finally, optimization of the SPE process was done on Waters Oasis® HLB, Waters Oasis® MCX, Waters Oasis® MAX and Phenomenex Strata cartridges. Addition of strong acid like ortho phosphoric acid (5%, v/v) during sample preparation helped in breaking the drug-protein binding and maintaining the analyte in the ionized form. Thus, better retention was provided on the Waters Oasis® MCX as compared to other cartridges. Elution step was optimized with different solvent/solutions and it was observed that 2% ammoniated methanol is optimum to get higher and consistent recovery for both analytes.

Selection of internal standard

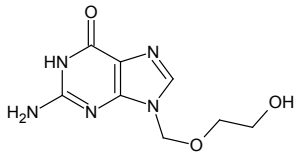
According to the US FDA guideline, the internal standard should ideally mirror the analyte in as many ways as possible. Henceforth, the isotopic labeled compound VAL D4 and ACL D4 were selected as internal satdards for simultaneous estimation of VAL and ACL in human plasma.

Estimation of RBC partitioning and $K_{WB/P}$ ratio

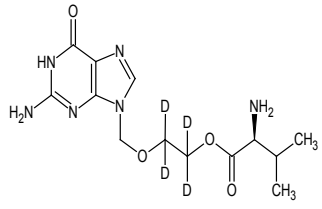
The estimated value of $K_{WB}/_{P}$ is less than 1 for VAL, but for ACL it is greater than 1. Due to high value of $K_{WB}/_{P}$, ACL is available in low concntration in separated plasma samples which was harvested



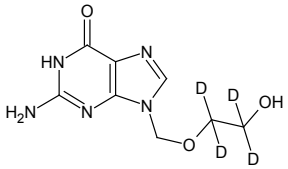
Molecular Formula=$C_{13}H_{20}N_6O_4$ Formula Weight=324.34
Valacyclovir



Molecular Formula=$C_8H_{11}N_5O_3$ Formula Weight=225.20
Acyclovir



Molecular Formula=$C_{13}H_{16}D_4N_6O_4$ Formula Weight=328.36
Valaciclovor D4



Molecular Formula=$C_8H_7D_4N_5O_3$ Formula Weight=229.23
Acyclovir D4

Figure 1: Chemical structure of valacyclovir, Acyclovir, Valacyclovir D4 and Acyclovir D4.

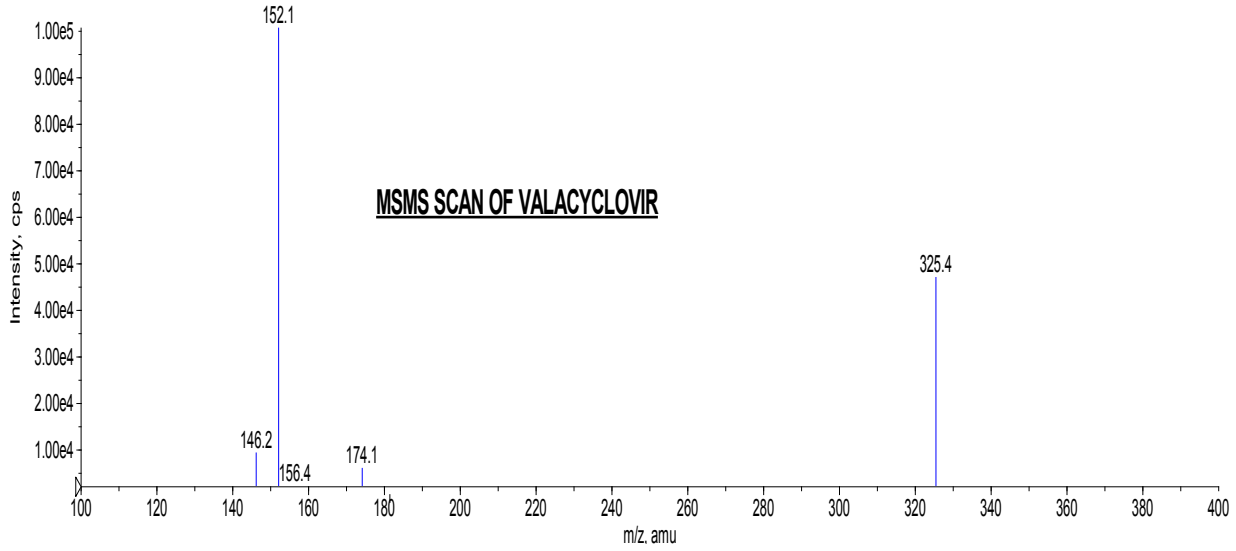


Figure 2a: MSMS scan of Valacyclovir.

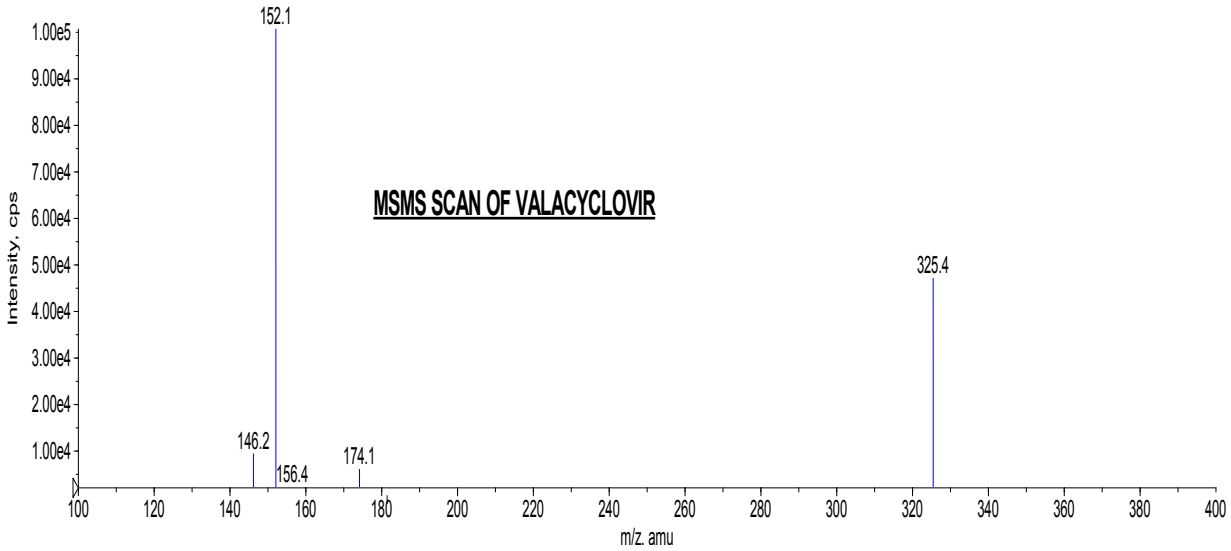


Figure 2b: MSMS scans of Acyclovir.

from the whole blood before reaching the equilibrium time. VAL is rapidly converted to its active metabolite ACL, therefore it is required to estimate the ACL concentration in incurred sample in plasma. Henceforth, it is essential to determine the equilibration time for ACL to avoid any pseudo concentration in incurred plasma samples.

Determination of equilibration time

No significant difference was observed within mean area ratio in all the processed samples (including all time points) for VAL. But, concentration dependent partitioning was observed for ACL, that is. at high QC level RBC partitioning value is more as compare to low QC level. This is probably due to the high protein binding characteristic of ACL or within the blood active transport of drug is take place. At high drug concentration, this process is become saturate. For evaluation purpose, an through invstigation was carried out with complete concentration range.

Mean peak area ratio was compared between two neighboring time points and similar peak area ratio was observed between time point 30.0 min and 45.0 min for ACL and represented graphically in Figure 3. So, it was concluded that equilibration was achieved after 30 min between RBC and plasma (Tables 1a and 1b). For this reason, during whole blood stability the comparison samples (freshly spiked) were kept in ice cold water bath for 30 min to achieve equilibrium between erythrocyte/plasma before plasma seperation.

Whole blood stability: In human K_3EDTA whole blood, the target analytes (that is. VAL and ACL) were found to be stable for ~2.45 h and the calculated % stability is within ± 15% for both the analytes. Whole blood stability data are presented in Table 2.

Time (min)	Mean peak area ratio (At LQC level)	
	Acyclovir	Valacyclovir
0.00	0.2420	0.0422
15.00	0.1924	0.0402
30.00	0.1579	0.0366
45.00	0.1572	0.0368
60.00	0.1571	0.0378
Time (min)	**Mean peak area ratio (At HQC level)**	
	Acyclovir	**Valacyclovir**
0.00	13.8010	2.0682
15.00	11.6395	2.0906
30.00	9.2473	1.9445
45.00	9.5016	1.9921
60.00	9.2038	1.9403

Table 1a: Time v/s area ratio (n=4).

Time intervals	% Difference			
	Acyclovir		Valacyclovir	
	LQC level	HQC level	LQC level	HQC level
0.0 min vs 15.0 min	5.71	4.25	1.21	-0.27
15.0 min vs 30.0 min	4.93	5.73	2.36	1.81
30.0 min vs 45.0 min	0.11	-0.68	-0.14	-0.61
45.0 min vs 60.0 min	0.01	0.80	-0.65	0.66

Table 1b: %Difference of area ratio with time (n=4).

Analyte	QC level	Mean area ratio		% Stability
		Stability sample	Comparison sample	
VAL	LQC	0.0364	0.0371	98.11
	HQC	1.9628	2.0238	96.99
ACL	LQC	0.1408	0.1322	106.51
	HQC	7.9912	8.2579	96.77

Table 2: Whole blood stability (n=4).

Analyte name	QC level	Nominal conc. (ng/mL)	Intra-run (n=6)			Inter-run (n=18)		
			Mean observed conc. (ng/mL)	% CV	% Accuracy	Mean observed conc. (ng/mL)	% CV	% Accuracy
VAL	LOQQC	4.10	4.26	6.1	104.0	4.29	5.8	104.6
	LQC	11.72	11.81	2.7	100.8	11.71	2.9	99.9
	MQC	285.92	289.16	0.6	101.1	289.06	1.4	101.1
	HQC	571.84	585.49	2.6	102.4	582.49	1.6	101.9
ACL	LOQQC	50.46	47.43	1.9	94.0	49.57	4.0	98.2
	LQC	145.41	141.08	1.3	97.0	140.60	1.5	96.7
	MQC	4039.06	3991.93	1.1	98.8	3999.87	1.8	99.0
	HQC	8078.12	8175.51	3.1	101.2	8058.67	2.3	99.8

Table 3: Intra-and inter-day precision and accuracy data.

Results of method validation

The calculated % peak area response at the RT of VAL and ACL in the processed human plasma lots are less than 20% when compared with mean peak area of analyte that was observed at LOQ levels. The chromatograms of double blank samples, single blank samples (where double blank samples are processed with internal standard) and LOQ are shown in Figure 4. The evaluation of a representative calibration curve for weighing factor was statistically determined using the formula $|\Sigma\%\ \text{dev}| + \sqrt{\Sigma\ (\%\ \text{dev})^2}$. The least value was choosen for best fit by statically evaluation. The r-square was greater than 0.99.

In Table 3, precision and accuracy data for intra-day and inter-day run VAL and ACL are tabulated. The process efficiency at LQC, MQC and HQC levels were 81.5%, 77.9%, 76.7% for VAL and 70.2%, 70.1%, 73.2% for ACL, whereas it was 92.0% and 83.0% for VAL D4 and ACL D4, respectively. The process efficiencies were consistent and reproducible with this extraction method (Table 4).

In LC-MS/MS method, ionization of analyte is effected by the co-elute matrix ions specially when, ESI is applied as an ionization mode.

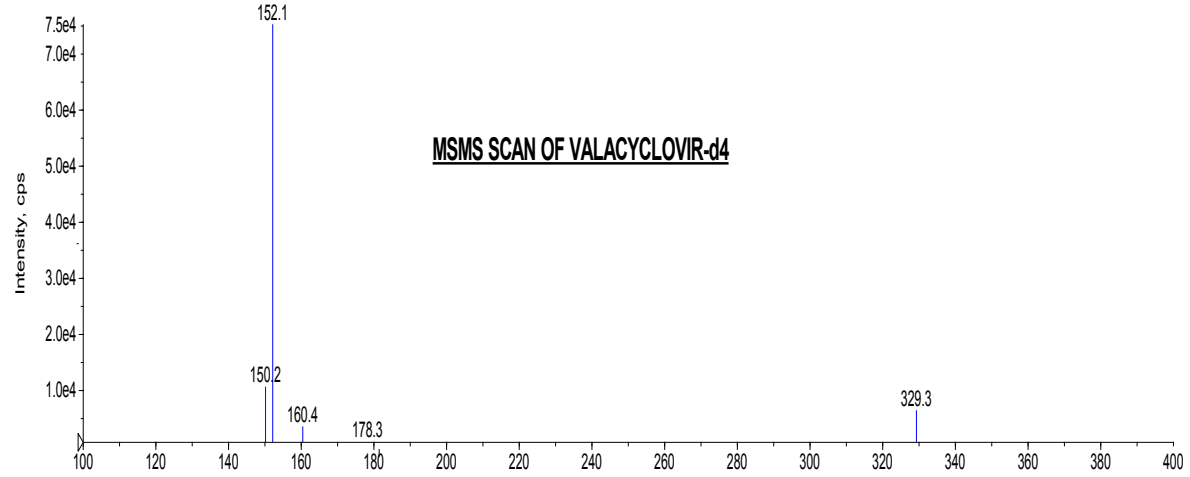


Figure 2c: MSMS scan of Valacyclovir D4.

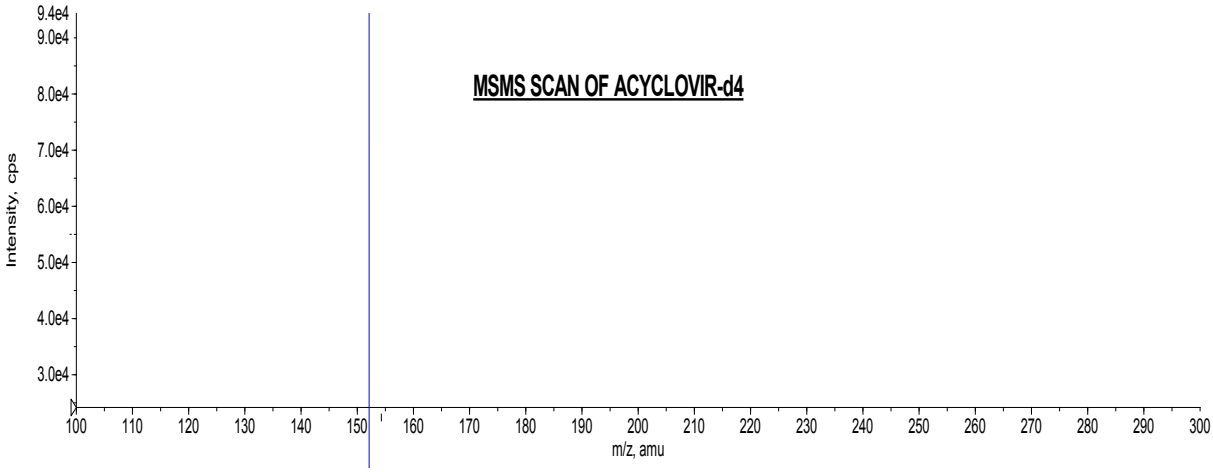


Figure 2d: MSMS scan of Acyclovir D4.

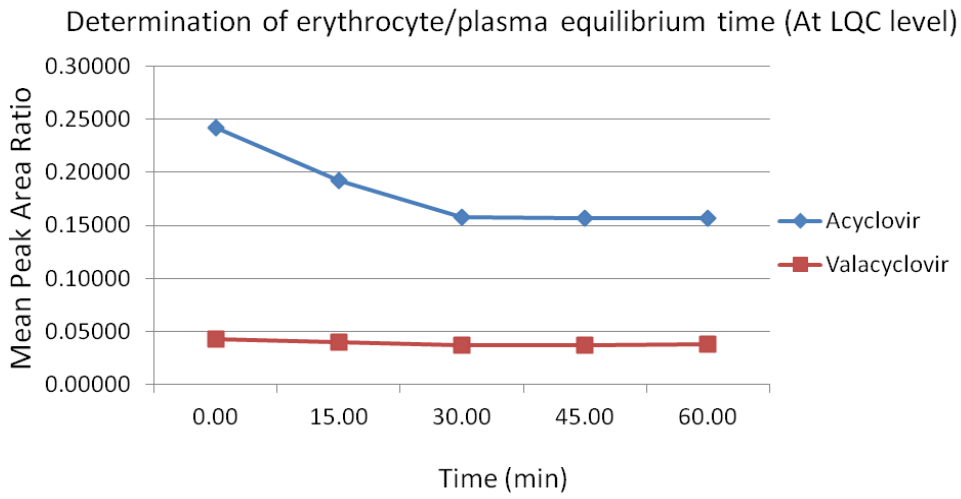


Figure 3a: Erythrocyte/plasma equilibration curve (at LQC level).

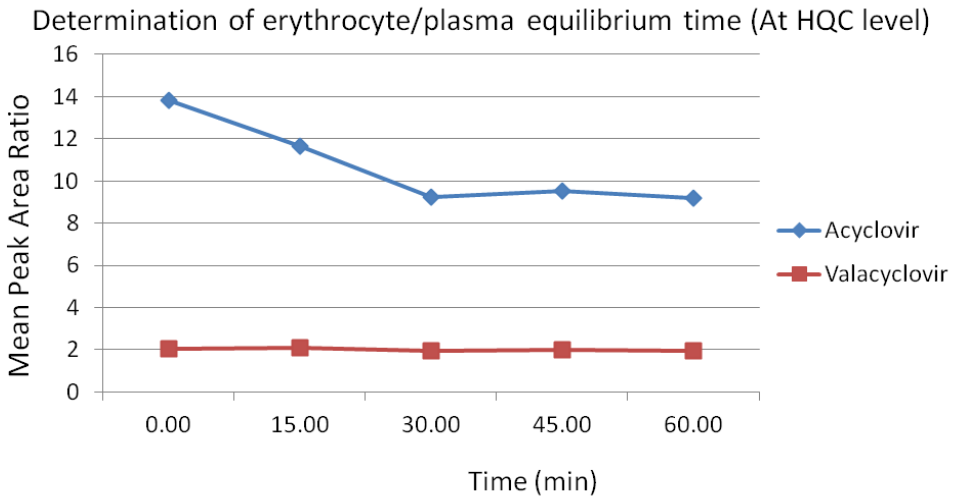


Figure 3b: Erythrocyte/plasma equilibration curve (at HQC level).

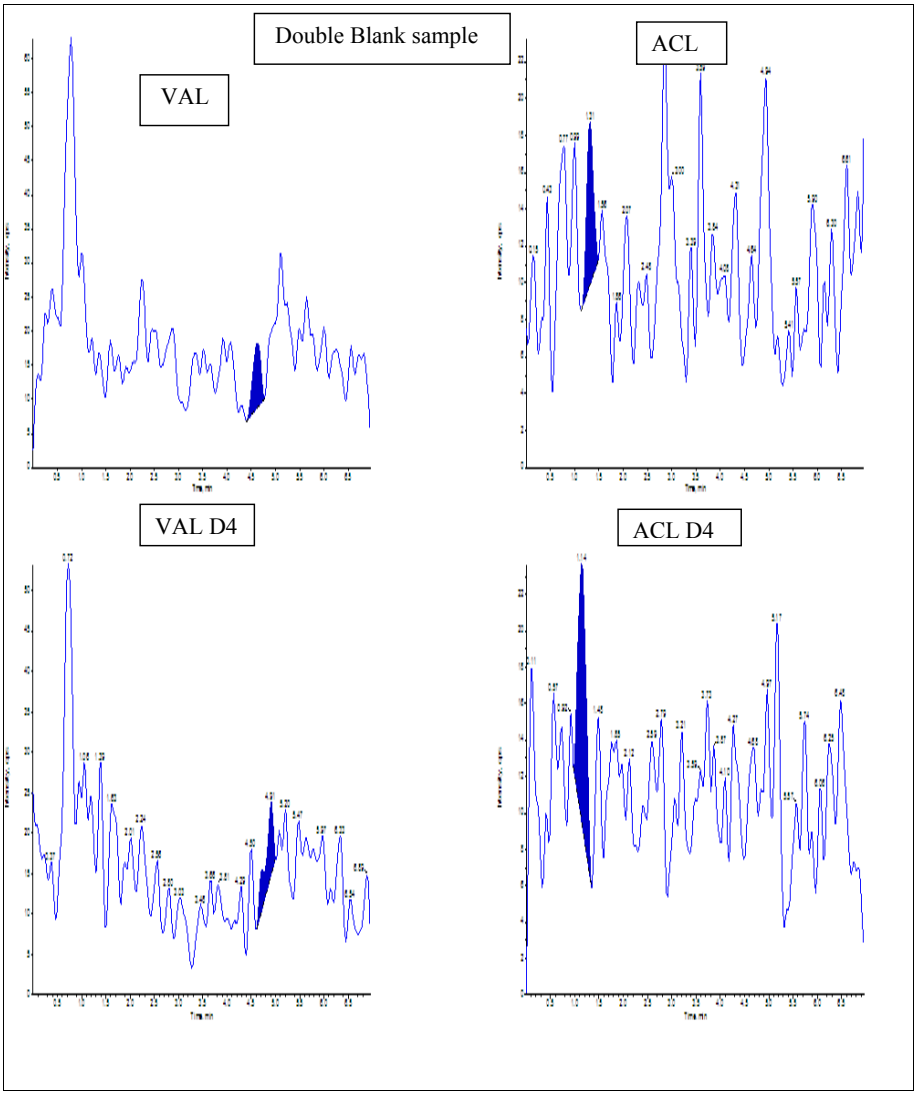


Figure 4a: Representative chromatograms of extracted double blank sample.

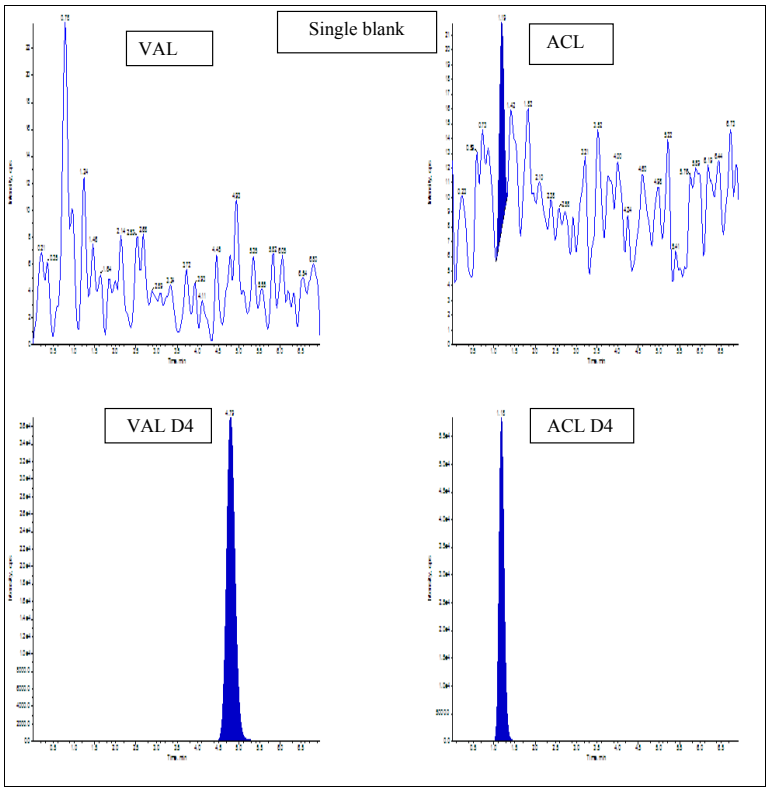


Figure 4b: Representative chromatograms of extracted single blank sample (processed with internal standard).

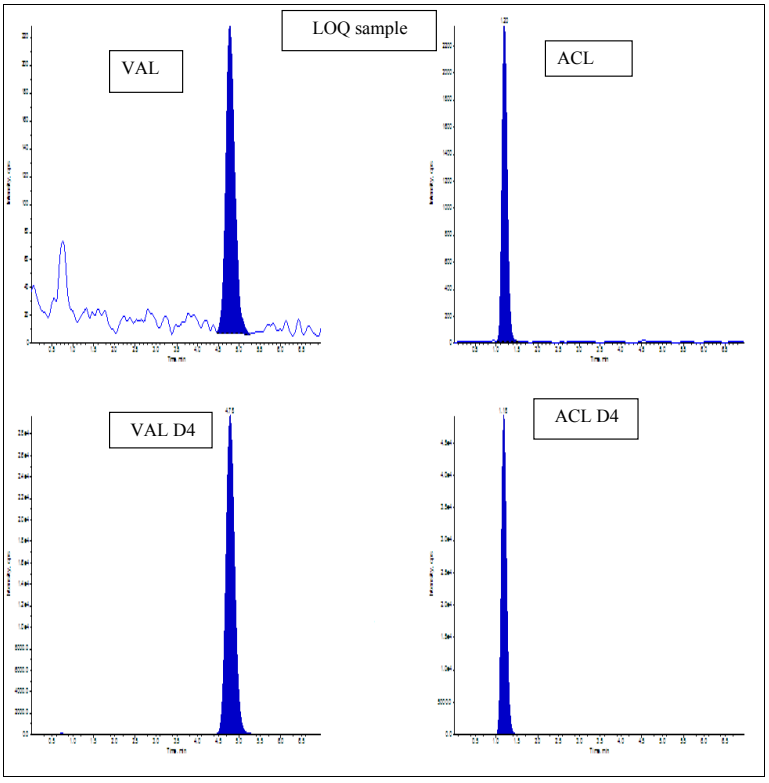


Figure 4c: Representative chromatograms of extracted LOQ sample.

Analyte Name	QC level	A[a] (%CV)[b]	B[c] (%CV)[b]	Process efficiency (%PE)[d]
VAL	LQC	11046(1.3)	13561(3.3)	81.5
	MQC	275189(0.4)	353211(4.2)	77.9
	HQC	557174(0.6)	726391(1.3)	76.7
VAL D4	MQC	479920 (1.2)	521639 (1.6)	92.0
ACL	LQC	53017(4.9)	75500(3.0)	70.2
	MQC	1451893(0.9)	2070523(7.7)	70.1
	HQC	2804447(8.8)	3830040(1.3)	73.2
ACL D4	MQC	340501 (0.4)	410164 (1.4)	83.0
[a]Mean area response of six replicate samples prepared by spiking before extraction (n=6).				
[b]Coefficient of variation.				
[c]Mean area response of six replicate samples prepared in reconstitution solution (n=6).				
[d]A/B × 100				

Table 4: Process efficiency (n=6).

Plasma lot	Absolute matrix effect					
	VAL			ACL		
	LQC	MQC	HQC	LQC	MQC	HQC
Lot- 1	1.01	0.97	0.99	0.97	0.96	0.98
Lot-2	1.00	0.98	1.02	0.97	0.97	1.03
Lot-3	0.98	0.96	1.03	0.96	0.96	1.02
Lot-4	1.00	0.96	1.02	0.98	0.96	1.03
Lot-5[a]	1.00	0.98	1.02	0.97	0.96	1.01
Lot-6[b]	1.02	0.97	1.04	0.97	0.95	1.00
Mean	1.00	0.97	1.02	0.97	0.96	1.01
%CV	1.3	0.9	1.6	0.7	0.7	1.9
[a]Hemolyzed plasma lot; [b]Lipemic plasma lot						

Table 5: Absolute matrix effect.

Plasma lot	Calculated conc. (ng/mL)			
	VAL		ACL	
	LOQQC	HQC	LOQQC	HQC
Lot- 1	4.31	565.76	50.98	8216.28
Lot- 1[a]	4.37	570.94	48.12	7888.28
Lot- 2	4.35	561.38	50.68	7916.33
Lot- 2[a]	4.34	565.75	46.43	7877.58
Lot- 3	4.85	555.03	49.52	7709.73
Lot- 3[a]	4.11	567.00	48.36	7994.62
Lot- 4	4.27	565.56	46.36	7766.52
Lot- 4[a]	4.08	561.43	48.77	7917.68
Lot- 5	4.23	557.66	49.98	7913.19
Lot- 5[a]	4.33	573.01	47.52	7986.57
Lot- 6	4.32	573.26	48.85	7954.17
Lot- 6[a]	4.32	573.15	47.88	7876.83
Mean	4.32	565.83	48.62	7918.15
%CV	4.4	1.1	3.1	1.6
Nominal conc.(ng/mL)	4.1	571.84	50.53	8078.12
%Nominal	105.4	98.9	96.2	98.0
[a]Duplicate; Lot 5 is haemolyzed plasma; Lot 6 is lipemic plasma				

Table 6: Relative matrix effect.

So, it is essential to eliminate the effect of co-elute matrix in ionization, to get the exact concentration of analyte in the incurred samples. Hence, the two most important validation parameters like, AME and RME were evaluated during validataion. The % CV of AME at each level QC were in the range of 0.9-1.6 and 0.7-1.9 for VAL and ACL, respectively and between three QC levels it was 2.5 and 2.8 for VAL and ACL, respectively. This data suggested that, when the target analytes are ionized in ion source no interference (that is. ion-suppression or ion-enhancement) was observed from the co-elute matrix ions. The AME data is tabutaed in Table 5. The relative matrix effect (RME) data was also acceptable as per regulatory guidelines and tabulated in Table 6.

Stock solution stability of VAL, VAL D4, ACL and ACL D4 were stable for 22 days at refrigerated temperature (1-10°C) and the calculated % stability were 96.1%, 100.7% and 105.8%, respectively. After retrival of the spiked plasma samples from the deep freezer were found stable for ~7.17 h in ice cold water bath and under low light conditions and for atleast

Stability	Analyte	Level	A	%CV	B	%CV	% Change
Bench top stability (~7.17 hr, in ice-cold water bath)	VAL	LQC	11.720	7.00	12.075	1.57	-2.94
		HQC	587.865	1.27	584.835	1.38	0.52
	ACL	LQC	142.183	1.36	140.94	1.65	0.88
		HQC	8094.908	0.84	8090.82	1.19	0.05
Auto sampler stability (~98.98 hr, 10°C)	VAL	LQC	10.873	1.46	10.938	2.60	-0.59
		HQC	577.873	1.82	580.095	1.85	-0.38
	ACL	LQC	138.763	1.55	135.893	0.64	2.11
		HQC	8043.100	1.27	7824.948	0.76	2.79
Freeze-thaw stability (Thee freeze-thaw cycle)	VAL	LQC	11.550	1.46	12.075	2.75	-4.35
		HQC	579.098	1.82	584.835	1.85	-0.98
	ACL	LQC	138.513	1.65	140.94	1.46	-1.72
		HQC	7913.433	2.59	8090.82	2.37	-2.19
Long term stability (107 days, below -50°C)	VAL	LQC	11.555	2.35	11.378	2.43	1.56
		HQC	578.805	0.63	584.283	0.96	-0.94
	ACL	LQC	137.813	0.91	139.21	1.65	-1.00
		HQC	7930.883	0.96	8090.820	1.19	-1.98

A=Stability sample concentration (ng/mL); B=Comparison sample concentration (ng/mL); CV=Coefficient of variation;

Table 7: Stability exercises

three freeze (below -50°C) and thaw (in ice cold water bath) cycles. The final extracted samples was stable for ~98 h in auto sampler temperature (10°C) without any drug loss. To performed the long term stability, spiked plasma samples were stored below -50°C and were found stable for 107 days. All the stability experiment in human plasma were performed at two levels of QC (LQC and HQC) and the data are shown in Table 7.

Conclusion

The work described in this article for simultaneous estimation of valacyclovir and acyclovir in human plasma that reports a significant advancement over existing LC-MS/MS methods for simulataneous analysis of VAL and ACL in human plasma. From the experimental data it reflects that $K_{WB}/_{P}$ ratio of ACL is high, hence it is recommended that plasma to be separated from the spiked and/or clinical blood sample after equilibration attained between RBC and plasma.

Overall the developed method is highly selective and sensitive with no matrix interference and successfully applied for estimation of VAL and ACL in human plasma to conduct a bioequivalence study.

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