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DEVELOPMENT OF ENANTIO-SELECTIVE REVERSE PHASE CAPILLARY ELECTROPHORESIS DIRECT SEPARATION METHOD FOR THE DETERMINATION OF VALACYCLOVIR D - ISOMER

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ABSTRACT

An accurate and reproducible capillary electrophoresis (CE) method has been developed and validated for the direct separation of individual enantiomers of valacyclovir, the active anti-viral drug. The enantiomers were resolved by reverse phase chromatography, on an extended light path bare fused silica capillary using 30 mM potassium hydrogen phosphate (KH_2PO_4), at pH = 2.5 ± 0.1 as background electrolyte. Baseline separation of the enantiomers of valacyclovir was obtained with a profound resolution of greater than 7.0. The developed method was extensively validated and proved its suitability and robustness. The standard curve for *(D)* - valacyclovir was linear (r > 0.999) in the concentration range from 0.25 µg mL^{-1} (LOQ) to 30 µg mL^{-1} for an analyte concentration of 0.5 mg mL^{-1}. The percentage recovery of *(D)* - valacyclovir was ranged from 89.753 to 102.273 in bulk drug samples of valacyclovir. The limit of detection and limit of quantitation of *(D)* – valacyclovir is found to be 0.084 µg mL^{-1} and 0.25 µg mL^{-1} respectively. The relative standard deviation of method precision and intermediate precision for the area % of *(D)* - valacyclovir is observed to be 2.4 and 2.6 respectively. valacyclovir sample solution stability and mobile-phase stability were studied for 48 h and found to be stable during the period. The validated method yielded good results

of selectivity, linearity, precision, accuracy and robustness. The method was also able to resolve the enantiomers from the degradation impurities of valacyclovir.

KEYWORDS: Valacyclovir, Capillary Electrophoresis, Extended light path bare fused-silica capillary, Method validation.

1. INTRODUCTION

Over one-third of the marketed drugs worldwide are chiral. Regulators are now approving only new chiral drugs in the single enantiomer form and even then insisting on full profiling of the role of the individual enantiomers in vivo.[1] Most of the molecules of importance to living systems are chiral e.g. amino acids, sugars, proteins and nucleic acids. An interesting feature of these biomolecules is that, in nature they usually exist in only one of the two possible enantiomeric forms.[2] Enantiomers of racemic drugs often differ in pharmacokinetic behavior and /or pharmacological action.[3] More recently, investigation of the molecular mechanism for stereo selective biological activities of the chiral molecules have received attracted attention. In order to assess the chiral purity, stability and pharmacokinetics of the isomers, it is necessary to achieve the enantiomeric separation of different enantiomeric forms. Development of analytical methods for the separation and determination of chiral compounds is extremely challenging due to the fact that enantiomers possess virtually identical properties.[4] Chirality is now a top-class subject for academic research as well as for pharmaceutical development. Both regulatory bodies recommend the use of validated methods to evaluate the enantiomeric purity of single-isomers.[5-6] Therefore, it is important to promote the chiral separation and analysis of racemic drugs in pharmaceutical industry as well as in clinic in order to eliminate the unwanted isomer from the preparation and to find an optimal treatment and a right therapeutic control for the patient.[7]

Valacyclovir, the active anti-viral drug is the L-valine ester of acyclovir pro-drug. Primary infection of Varicella causes chickenpox and reactivation is caused by herpes zoster virus leading to shingles, a painful acute inflammation of the nerve ganglia, with a skin eruption.
Valacyclovir is rapidly and completely transformed to acyclovir by esterase in the human body. Acyclovir is a specific inhibitor of the herpes viruses with invitro activity against herpes simplex viruses (HSV) type 1 and type 2, Varicella zoster virus (VZV), Cytomegalovirus (CMV), Epstein-Barr Virus (EBV), and Human herpes virus 6 (HHV-6). Primary infection of Varicella causes chickenpox and reactivation is caused by herpes zoster virus leading to shingles, a painful acute inflammation of the nerve ganglia, with a skin

eruption. Acyclovir in active triphosphate form competitively inhibits the virus DNA polymerase and incorporation of this nucleoside analogue results in obligate chain termination, halting virus DNA synthesis and thus blocking virus replication [8]. The chemical name of *(L)* - valacyclovir is L-Valine, 2-[(2-amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl)methoxy] ethyl ester, a white to almost white solid. The chemical structure of L-valacyclovir is represented in Fig-1.

Numerous HPLC methods been reported for the determination of valacyclovir and its pharmaceutical dosage.[9-21] A.S Jadhav et al. have developed and validated a chiral HPLC method for enantiomeric separation of valacyclovir.[22] With no end in sight to the worldwide shortage of solvents, power, time and many valuable resources, pharmaceutical laboratories are in search of cost-effective solutions to manage this impact on their research and business timeline. Green chemistry has emerged in the 1990's as a way that the skills, knowledge, and talents of chemists can be used to avoid threats to human health and the environment in all types of chemical processes and analytical aspects. One of the most active areas of Green Chemistry research and development is in analytical methodology development.[23]

It is clear from surveying developments that the need of the pharmaceutical industry, continues to be major driving force for development of new cost effective analytical methods for determination of optically active materials.[24-25] Recently, commercially available capillary electrophoresis technique (CE) has been proven to be one of the most promising developments in the area of chromatographic separations by reducing the cost, analysis time and maintaining high efficiency without compromising quality. Capillary electrophoresis has been emerged as an excellent alternate technique for chiral method development besides HPLC technique. CE has successfully gained a position in the pharmaceutical and biotech industry is demonstrated by the general chapters and monographs in pharmacopoeias e.g., the European Pharmacopoeia and the United States Pharmacopeial Convention.[26–27]

There is no chiral CE method reported in literature for the separation and quantitative determination of *(D)* - valacyclovir hydrochloride in bulk drug of *(L)* - valacyclovir hydrochloride. Therefore, with the advent of stereospecific analytical methods in recent years, more attention has been drawn to develop a simple and suitable method for the measurement of *(D)* - valacyclovir hydrochloride in bulk *(L)* - valacyclovir hydrochloride to be adapted for routine and in-process quality control analysis or similar studies.

In the current investigation; a simple, rapid, sensitive, selective and accurate method for the quantitation of individual enantiomers of valacyclovir hydrochloride is reported. A simple and accurate CE method for the separation and determination of (L) - valacyclovir hydrochloride and the undesired (D) - enantiomer using commercially available extended Light Path bare fused-silica capillaries, is developed. Superior resolution (> 7.0) between (D) and (L) valacyclovir was achieved by using the extended light Path bare fused-silica capillary, with in a short run time of 15 min, using 30 mM KH_2PO_4 buffer with pH 2.5 as back ground electrolyte. The method was also able to separate the known process impurities and degradation impurities from the enantiomers of valacyclovir.

2. EXPERIMENTAL

2.1. Compound

Valacyclovir, its pure desired and undesired enantiomer were received from process research department of custom pharmaceutical services, a business unit of DR. Reddy's laboratories ltd., Hyderabad, India. Qualified valacyclovir hydrochloride, its pure enantiomer, related impurities namely guanine, acyclovir, cbz-l-valine, alanine impurity, o-acetyl impurity of acyclovir, isoleucine impurity, n-formyl impurity were used for the preparation of solutions during the study. Deionized water was obtained from milli-Q water system (Millipore, Bedford, Massachusetts, USA). CE grade water was purchased from Agilent technologies (Mumbai, India). HPLC grade Methanol was procured from Merck (Mumbai, India), analytical grade orthophosphoric acid was procured from Spectrochem Pvt Ltd. (Mumbai, India). Potassium hydrogen phosphate, borate buffer with pH 9.3 and hydroxylmethyl alpha cyclodextrin from Sigma Aldrich (Bengaluru, India) were used for the study.

2.2. Instrumentation

CE separations were carried out on an Agilent ^{3D}CE ($HP^{3D,}$ HPCE, Model 7100) system equipped with a diode array ultra-violet (UV-Visible) detector. Instrument control and data evaluation were performed using chemstation software (version 5.0).

2.3. CE operating conditions

Capillary preparation: New extended light path bare fused-silica capillaries were flushed with 1M NaOH followed by CE grade water for about 30 min each and then flushed with back ground electrolyte (BGE) for 30 min before use. Analytical chromatographic separations were carried out on extended light path bare fused-silica capillaries (Agilent technologies, Phoenix, AZ). The mobile phase in CE technique is called as background electrolyte

consisted of 30 mM KH_2PO_4 buffer with pH 2.5. The background electrolyte was filtered through a 0.45 µm PTFE filter (Millipore, USA) and degassed by sonication just before use. Injection was done at the anodic side and detection was performed at the cathodic side of the capillary. A voltage of 30 kV was applied and the capillary temperature was controlled at 40 °C. Sample solutions were introduced by pressure of 50 mbar for 5 sec and all electrograms were recorded at 245 nm. The total analysis time for each run was 15 min. Capillary was washed between runs with deionized water for 3 min and conditioned with BGE (running buffer) for about 10 min.

2.4. Sample preparation

25 mg of *(L)* and *(D)* valacyclovir each were dissolved in 50 mL of acetonitrile: milli - Q water (1:1 v/v) for the method development study. For validation study, the stock solutions of *(L)* - valacyclovir (0.5 mg mL^{-1}) and *(D)* - valacyclovir hydrochloride (0.5 mg mL^{-1}) were prepared separately by dissolving the appropriate amounts of the substances in acetonitrile: milli - Q water (1:1 v/v). The analyte concentration of *(L)* - valacyclovir hydrochloride was fixed as 0.5 mg mL^{-1}. Working solutions of *(L)* and *(D)* - valacyclovir hydrochloride were prepared in acetonitrile: milli - Q water (1:1 v/v).

2.5. Method Development

The solution containing pure *(L)* - valacyclovir hydrochloride and *(D)* - valacyclovir hydrochloride mixture was used for the method development. A systematic study had been conducted to achieve the proper separation of the enantiomers using different conditions, experimented to achieve optimum resolution and selectivity for the two enantiomers and all achiral impurities (guanine, acyclovir, cbz-l-valine, alanine impurity, o-acetyl impurity of acyclovir, isoleucine impurity, n-formyl impurity) within a shorter run time. During the method development, the injection volume (50 mbr for 5 sec) and UV detection wavelength (245 nm) were maintained constant in all the experimental trials. The method development trials and results are presented in **Table-1**. Typical chromatogram indicating the separation of valacyclovir from its enantiomer is shown in **Fig-2**.

2.6. Validation of the method

2.6.1. System suitability test

System suitability testing is an integral part of liquid chromatographic analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such [28].

System suitability test is used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. The system suitability test results of the chiral CE method on extended light path bare fused-silica capillary column are presented in **Table-2**.

2.6.2 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present, such as impurities, degradants, matrix etc (ICH Q2R1 2005),[29] The ability to separate all the compounds was assessed by the resolution between the peaks corresponding to the various substances. The specificity of the developed chiral analytical method was evaluated by the analysis of a solution containing valacyclovir enantiomers and its related substances (Fig-3 (a)). This solution was injected into a CE system equipped with PDA detector. The resolution between the enantiomers and other known impurities was recorded. valacyclovir peak was checked for peak purity spectra, to ensure that there is no interference of known impurities in the analyte peak (Fig-3 (b)).

Method selectivity was also challenged by performing the forced degradation of *(L)* -valacyclovir. Sample was exposed to 200 watt hours/ square meter of UV light, 1.2 million lux hours visible light for photolytic degradation, and subjected to 105 ℃ in oven for 7 days for thermal degradation. Acid, base and oxidative degradations of valacyclovir sample were performed to demonstrate the specificity of the method (Fig-4). The drug was found to degrade extensively in alkaline, acidic, oxidative condition, mild degradation in light, but is stable to thermal and humidity stress. But no change in the concentration of *(D)* -valacyclovir during the study period was observed. The peak purity of exposed and degraded *(L)* -valacyclovir was confirmed by diode array detector.

2.6.3. Limit of Detection and Limit of Quantitation

The limit of detection (LOD) represents the concentration of analyte that would yield a signal to noise ratio of 3. The limit of quantitation (LOQ) represents the concentration of analyte that would yield a signal to noise ratio of 10. (ICH Q2R1 2005) [28, 30]. The LOD and LOQ were determined by injecting a series of dilute solutions of desired isomer and undesired isomer of valacyclovir. The precision of this sensitive and enantio-selective method at LOQ concentration was checked by analyzing six LOQ solutions of desired and undesired isomers, and the percentage relative standard deviation of area was calculated.

2.6.4. Precision

The precision of an analytical procedure expresses the closeness of an agreement among series of sample measurements obtained from multiple samplings of the same homogeneous sample under prescribed conditions. Method precision was determined by measuring the repeatability (intra-day precision) and the intermediate precision was determined by measuring the reproducibility (inter-day precision) of retention times of both the isomers and area percent for *(D)*-valacyclovir. The intra-day variability was performed by injecting six separate preparations of valacyclovir containing about 1.9 % of its *(D)* - enantiomer by same analyst over a day, while inter-day precision was carried out similarly, by another independent analyst on a capillary from different lot over 7 days. The relative standard deviation of intra-day and inter-day repeatability experiments in the test sample is evaluated to ascertain the ruggedness of the method.

2.6.5. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of the analyte in the sample [28]. The linearity evaluation was performed with the standard solutions of *(D)*-valacyclovir at the concentrations ranging from 251 ng mL^{-1} (LOQ) to 30132 ng $mL^{-1.}$ The peak areas response of undesired isomer was plotted against the corresponding concentration and the linear regression equations were computed.

2.6.6. Accuracy

The valacyclovir bulk drug sample contained about 1.9 % of *(D)* - enantiomer. Standard addition and recovery experiments were conducted to determine the accuracy of the present method for the quantitation of *(D)* - enantiomer in bulk drug samples. The quantitation study for *(D)* - enantiomer in the developed LC analytical method for valacyclovir drug substance was evaluated in triplicate by recovery study of impurity spiked at LOQ level and at 50 %, 100 % and 150 % of target analyte concentration. The results of accuracy are tabulated in Table-3.

2.6.7. Robustness

The capability of the method to remain unaffected by small deliberate variations in the method parameters was studied in order to anticipate the problems that may arise during the regular application of the developed method. To determine the robustness of the method, experimental conditions were purposely altered and chromatographic resolution between

valacyclovir and *(D)* - enantiomer was evaluated. The capillary voltage in the method is 30 kV. To study the effect of capillary voltage on the resolution of enantiomers, the capillary voltage was varied to 28 and 32 kV from 30 kV. To study the effect of variation in the pH of the background electrolyte on the resolution of the enantiomers, it was carried out by changing the pH to 2.4 and 2.6 from 2.5. The effect of capillary temperature on resolution was studied at 35 ºC and 45 ºC instead of 40 ºC, while the other mobile phase components were held constant. The effect due to the variation in the wavelength on resolution was studied at 243 nm and 247 nm instead of 245 nm. The resolution between critical pair, valacyclovir and *(D)* - enantiomer in all the above varied conditions was greater than 7.0, indicating the method robustness. The results of robustness are tabulated in Table-4.

2.6.8. Solution stability and mobile phase stability

Solution Stability of *(L)* -valacyclovir sample solution containing *(D)* - enantiomer was studied by keeping the solution in tightly capped volumetric flask at room temperature on a laboratory bench for 48 h. Content of *(D)* – enantiomer was checked for every 6 h interval up to the study period and compared with 0'h solution.

Mobile phase/BGE stability was carried out by evaluating the content of *(D)*–enantiomer in valacyclovir sample solutions containing *(D)* – enantiomer, which were prepared freshly at 6 h interval up to 48 h, while the same BGE was used during the study period (Table-5).

Table-1: Method development summary

Trial	Chromatographic conditions	Remarks
1	Capillary : Extended light path bare fused silica capillary of length 56 cm, internal diameter of 50 µm BGE : 20 mM borate buffer with pH 9.3 Voltage : +30 KV Wavelength : 245 nm Injection volume : 50 mbr for 5 sec Capillary temperature : 30 °C	The peaks were not eluted. It may be due to its low interaction with the background electrolyte.
2	Capillary : Extended light path bare fused silica capillary of length 56 cm, internal diameter of 50 µm BGE : 100 mM phosphoric acid Capillary temperature : 30 °C	Though the peaks were eluted, the resolution is observed to be less. Buffer concentration and Capillary length could be increased.
3	Capillary : Extended light path bare fused silica capillary of length 72 cm, internal diameter of 50 µm. BGE : 100 mM KH_2PO_4 Capillary temperature : 30 °C	The peaks were resolved. But, tailing factor was greater than 1.5. Buffer pH could be adjusted to more acidic for better peak shapes.

4	Capillary : Extended light path bare fused silica capillary of length 72 cm, internal diameter of 50 µm BGE : 50 mM KH_2PO_4 pH 2.5 ± 0.1 Capillary temperature : 30 °C	The resolution was improved. But, tailing is more than 1.5. Cyclodextrins can be used, to check the impact on improving the peak shape.
5	Capillary : Extended light path bare fused silica capillary of length 72 cm, internal diameter of 50 µm. BGE : 50 mM KH_2PO_4 + 10 mM Hydroxy methyl alpha cylodextrine. Capillary temperature : 30 °C	No improvement in peak shape observed. The capillary length could be reduced to decrease the migration time (run time).
6	Capillary : Extended light path bare fused silica capillary of length 40 cm, internal diameter of 50 µm, BGE : 50 mM KH_2PO_4 pH 2.5 ± 0.1 Capillary temperature : 40 °C	The run time is reduced to 20 min from 60 min. But, no improvement in peak shape observed.
7	Capillary : Extended light path bare fused silica capillary of length 40 cm, internal diameter of 50 µm BGE : 50 mM KH_2PO_4 pH 2.5 ± 0.1: MeOH (80:20 v/v) Capillary temperature : 40 °C	Tailing is still observed to be more than 1.5. The buffer concentration shall be reduced to 30 Mm KH_2PO_4 pH 2.5 ± 0.1
8	Capillary : Extended light path bare fused silica capillary of length 40 cm, internal diameter of 50 µm BGE : 30 mM KH_2PO_4 pH 2.5 ± 0.1 Voltage : +30 KV ; Wavelength : 245 nm Injection volume : 50 mbr for 5 sec Capillary temperature : 40 °C.	Tailing of both the peaks is obtained as 1.0 with resolution of greater than 7.0 between the peaks, with optimum run time (~15 min).

Table-2: System suitability results

Capillary	Compound	K	Rs	N	T	α
Extended light path bare fused silica capillary	*(L)* -valacyclovir	6.5	---	15574	1.0	--
	***(D)* -valacyclovir**	9.5	7.2	9182	1.0	1.4

k=capacity factor; Rs=USP resolution; N= number of theoretical plates (USP tangent method); T=USP tailing factor; α=enantio-selectivity

Table 3: Recovery results of *(D)* - enantiomer in bulk drug samples

Added (ng)	Concentration level to the nominal concentration	Recovered (ng)	% Recovery
250.8	LOQ	225.1	89.753
7524	50 %	7433	98.791
15048	100 %	14962	99.428
30096	200 %	30780	102.273

Table 4: Robustness results

Method parameter variation	USP Resolution (*Rs*) between *(L)* and *(D)* - valacyclovir	
Capillary Voltage (kV)	28	7.0
	30	7.2
	32	7.2
Capillary temperature (ºC)	35	7.0

	40	7.2
	45	7.3
UV Wavelength (nm)	243	7.2
	245	7.2
	247	7.2
Background electrolyte (BGE) pH	2.4	7.0
	2.5	7.2
	2.6	7.2

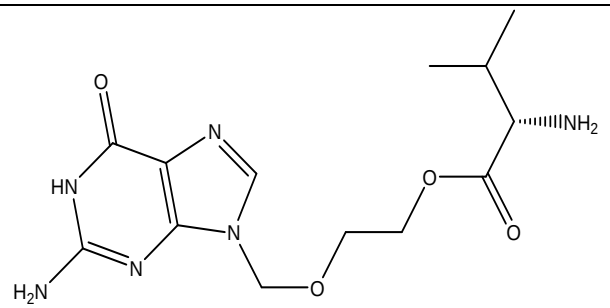
Table 5: Solution stability and mobile phase stability results

Time interval (h)	Solution Stability (D) - valacyclovir (% area)	BGE Stability (D) - valacyclovir (% area)
Initial	1.92	1.92
6	1.92	1.92
12	1.93	1.93
18	1.92	1.93
24	1.93	1.92
48	1.93	1.93

Table 6: Validation summary

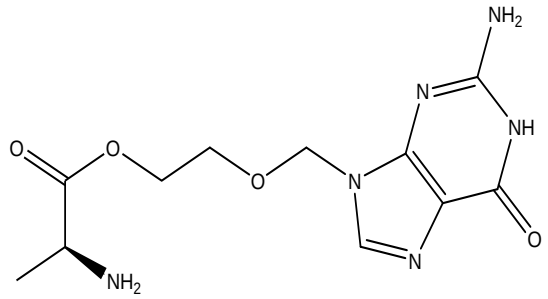
Validation parameter	Results
Repeatability (n=6, %RSD)	
Retention time (L - valacyclovir)	0.03
Retention time (D - isomer)	0.02
Percent area (D - isomer)	2.4
Intermediate Precision(n=6, %RSD)	
Retention time (L -valacyclovir)	0.8
Retention time (D - isomer)	0.8
Percent area (D - isomer)	2.6
LOD-LOQ (D- isomer)	
Limit of detection (ng/mL)	83.6
Limit of quantitation (ng/mL)	250.8
Precision at LOQ (%RSD)	7.1
Linearity (D - isomer)	
Range (ng)	251 – 30132
No. of points	6
Correlation coefficient	0.999
Accuracy (D -isomer) % Recovery	
LOQ	89.753
50% level	98.791
100 % level	99.428
200 % level	102.273

Figures



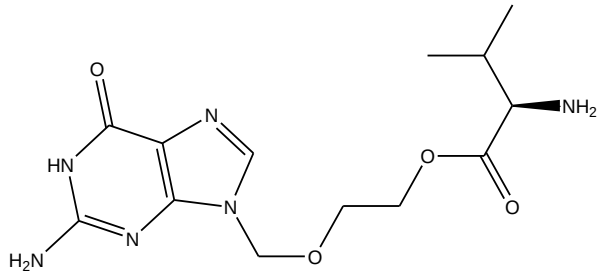
L-Valine, 2-[(2-amino-1, 6-dihydro-6-oxo-9*H*-purin-9-yl)methoxy] ethyl ester

Chemical structure of L-valacyclovir (main drug).



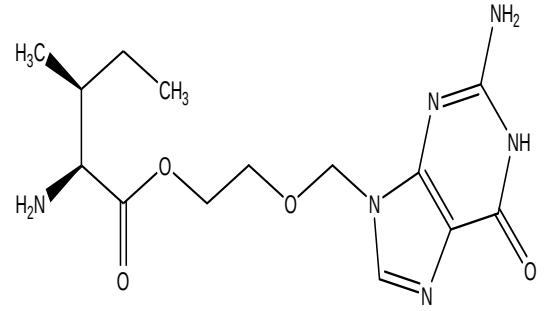
9-[(2-Hydroxyethoxy)methyl]guanine L-alaninate

(d) Chemical structure of acyclovir alaninate.



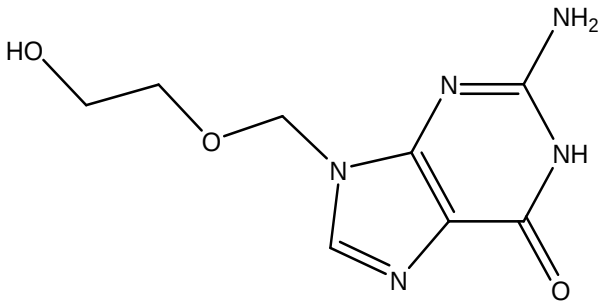
D-Valine, 2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methoxy] ethyl ester

(b) Chemical structure of D-valacyclovir hydrochloride.



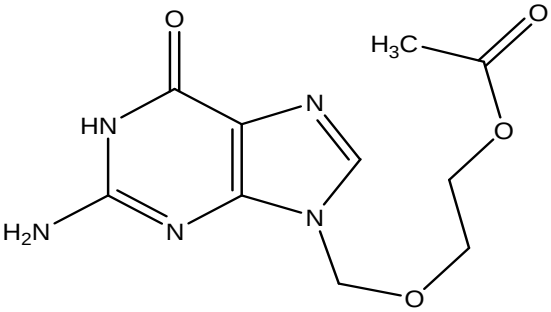
9-[(2-Hydroxyethoxy)methyl]guanine L-isoleucinate

(e) Chemical structure of acyclovir isoleucinate.



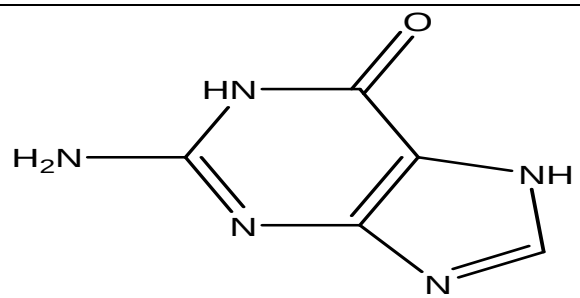
9-[(2-Hydroxyethoxy)methyl]guanine

(c) Chemical structure of acyclovir



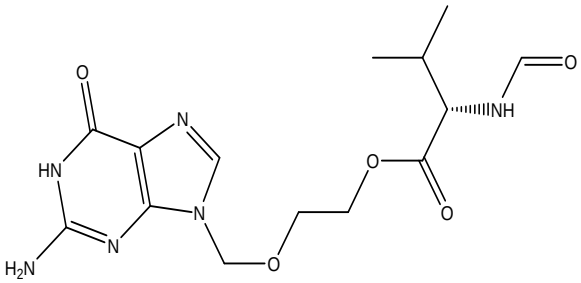
2-((2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl)methoxy)ethyl acetate

(f) Chemical structure O-acetyl impurity of acyclovir.



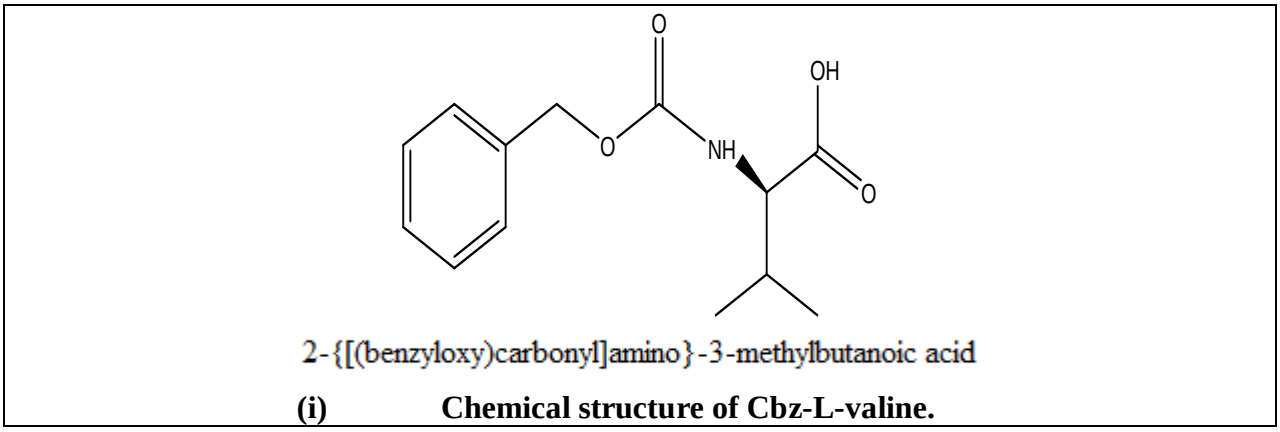
2-Amino-1H-purin-6(9H)-one

(g) Chemical structure of guanine.



9-[(2-Hydroxyethoxy)methyl]guanine N-formyl-L-valinate

(h) Chemical structure of N-formyl impurity.



(i) Chemical structure of Cbz-L-valine.

Fig-1 (a) to (i): Chemical structures of valacyclovir and related impurities

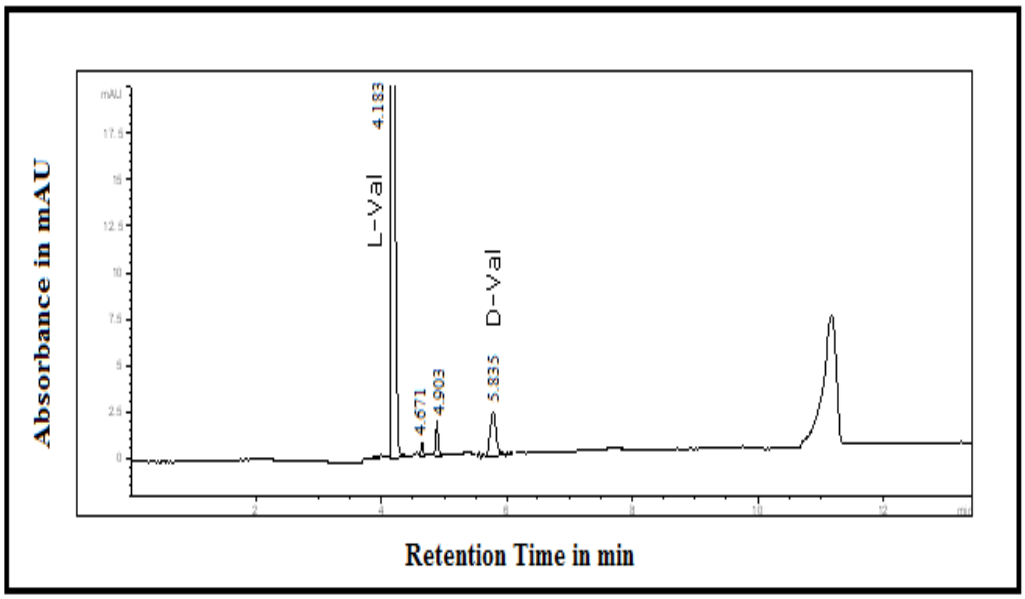


Fig 2: SST and Specificity chromatogram of valacyclovir spiked with 0.5 % of *(D)* - enantiomer and their purity plots.

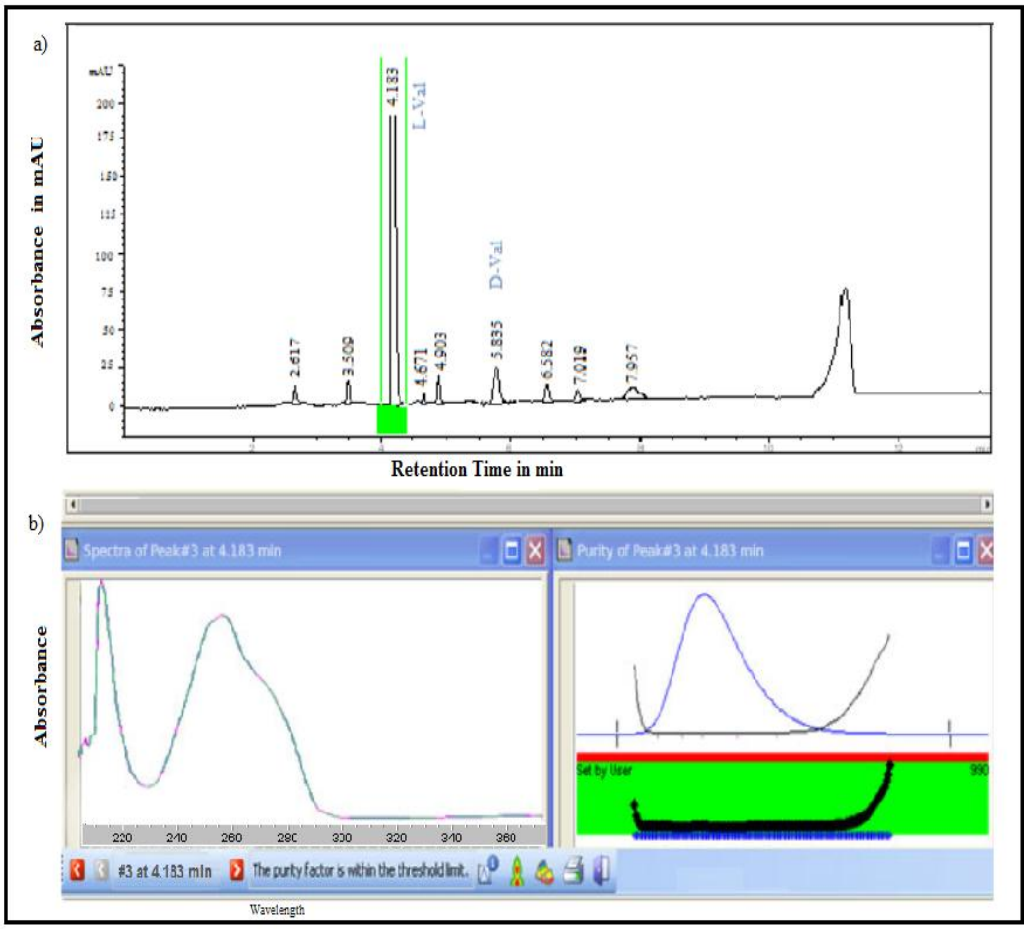


Fig: 3 (a) SST and Specificity chromatogram of valacyclovir spiked with related impurities (b) Purity plot for valacyclovir peak.

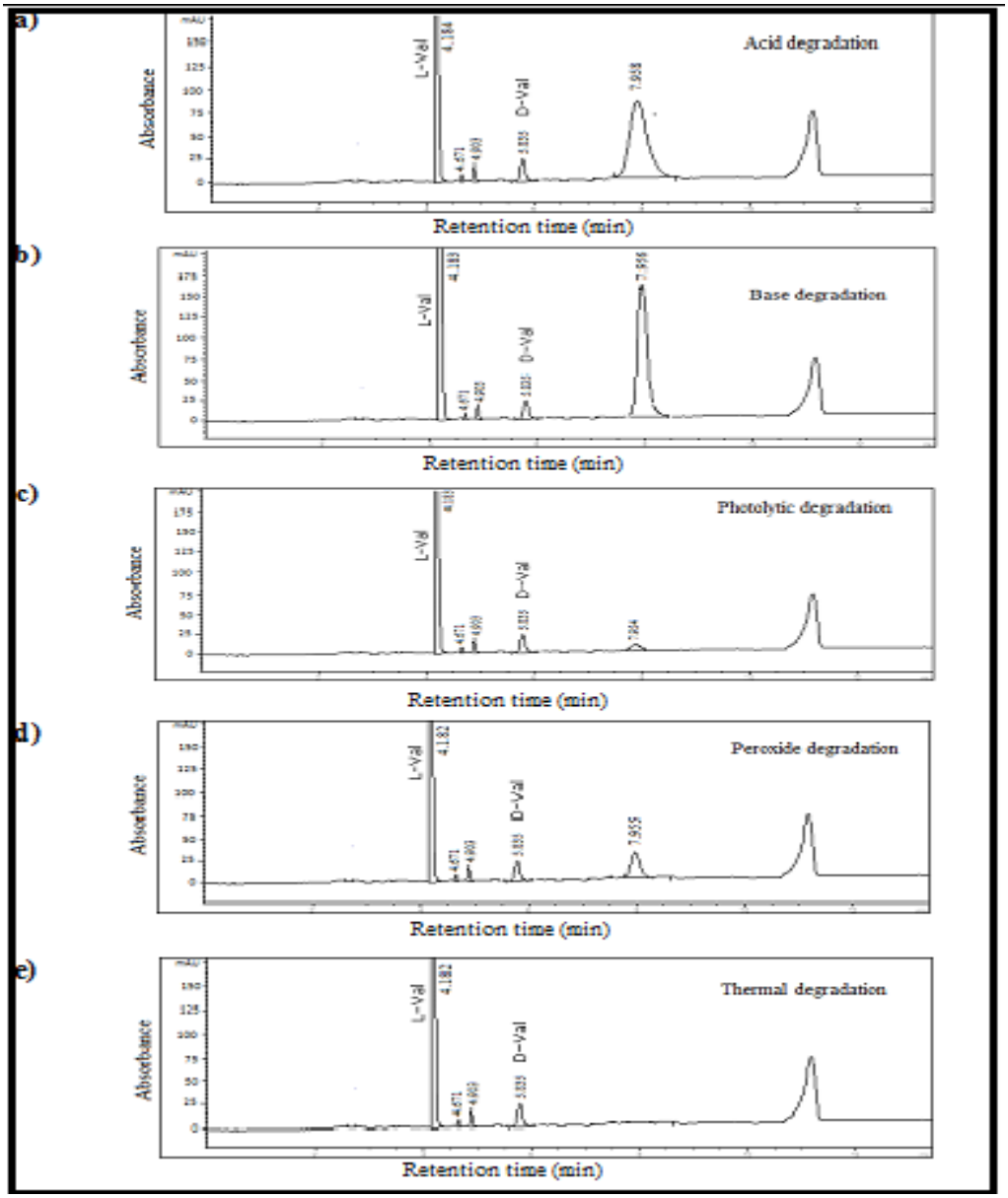


Fig 4: Specificity of valacyclovir in presence of degradation products

3. CONCLUSION

The chiral CE has become a powerful separation technique complementary to HPLC, owing to minute sample and buffer requirements. New methods and techniques that reduce and eliminate the use and generation of hazardous substances through all aspects of the chemical analysis lifecycle are the manifestations of the recent interest in Green Analytical Chemistry. In view of which, a simple isocratic and selective chiral CE method was developed for the enantiomeric separation and determination of *(D)* - valacyclovir in *(L)* - valacyclovir. Good enantioselectivity is obtained on extended light path bare fused-silica CE capillary. The developed method is selective for *(D)* -valacyclovir and *(L)* - valacyclovir, in presence of achiral impurities. The method is validated for all the method validation parameters as per

ICH (Table-6). The developed method is stability indicating and can be used for the quantitative determination of chiral impurity *(D)*–enantiomer in valacyclovir bulk drug material.

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