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A VALIDATED STABILITY-INDICATING METHOD FOR DETERMINATION OF RELATED SUBSTANCES AND ASSAY OF PENCICLOVIR BY RP-UPLC IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT
A novel reverse-phase high performance liquid chromatography (HPLC) combined with photo-diode array detector method has been developed for determination of related substances (RS) in Penciclovir. The separation of penciclovir from its impurities has been successfully achieved using Kromasil C8 (150mm x 4.6 mm, 5µ) column maintained at 35°C±2°C. Due to longer runtime, development of an analytical method in ultra-performance liquid chromatography (UPLC) has been tried using Acquity UPLC column (100mm x 2.1mm, 1.7 µ). In optimized UPLC method, USP plate count is more and runtime is one fifth time lesser than HPLC method. The optimized UPLC method was validated with respect to specificity, accuracy, method precision, linearity, establishment of relative response factor (RRF) and limit of quantitation (LOQ), robustness, solution stability, forced degradation and filter validation studies. Same method was tested for determination of assay in UPLC using higher concentration of Penciclovir standard solution. Assay method was validated with respect to specificity, accuracy, method precision and linearity. It can be concluded that optimized UPLC method is suitable for determination of assay and related substances in Penciclovir with change in concentration of standard preparation.

KEYWORDS: Reverse-phase HPLC, Reverse-phase UPLC, related substances, assay, method development, validation.

1. INTRODUCTION
Cold sores are responsible for significant morbidity and mortality in the world caused due to Herpes simplex virus. Antivirals are the typical medication for this disease.[1] Penciclovir is one such medication for antiviral therapy. It is a guanosine, nucleoside analogue used for treatment of different types of herpes virus infections. It has low toxicity and good selectivity.

Research is going on for estimation of related substances in Penciclovir. Shiny Ganji et al developed a method for the analysis of Penciclovir and its related substances in bulk drug as well as pharmaceutical dosage forms.[2] This isocratic reverse-phase HPLC analysis was performed using Hypersil phenyl (250mm x 4.6mm, 5µ) with mobile phase consisting of 0.1% orthophosphoric acid in 1000 ml of water and acetonitrile in the ratio of 60:40. Column oven was maintained at 30°C with flow rate of 1.0 ml/min. The wavelength was fixed 254 nm with run time of 20 min. Asmaa A. Elzaher et al have developed a rapid and sensitive spectroscopic method for determination of Penciclovir and Entecavir.[3] The fluorescence intensity of Penciclovir was measured at 363 nm upon excitation at 260 nm. The calibration curve was linear over the concentration ranges of 0.1 – 0.8 µg/m.

The present study was carried out to develop a suitable reverse-phase HPLC method for estimation of related substances in Penciclovir [2-amino-9-[4-hydroxy-3-(hydroxymethyl) butyl]-6,9-dihydro-3H-purin-6-one] dosage form. Structures of Penciclovir and its related substances were represented in Fig. 1 to Fig. 5 with IUPAC names and molecular weight. The optimized method has longer runtime. To make it shorter, an analytical method was developed in UPLC. The developed method was validated with respect to specificity, accuracy, method precision, linearity, RRF and LOQ, robustness, solution stability, forced degradation and filter validation studies. Under forced degradation study, effect of acid & base treatment, oxidation, thermal degradation, photolysis was evaluated to know the degradation pathway of the product. Same method was tested for determination of assay in UPLC using higher concentration of Penciclovir standard solution. Assay method was validated with respect to specificity, accuracy, method precision and linearity.

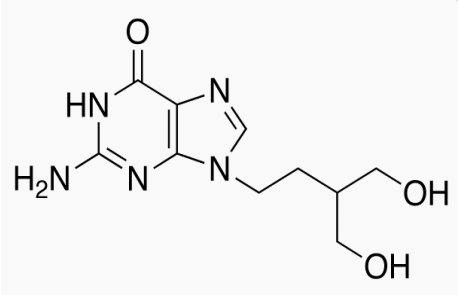

Fig. 1: Penciclovir (IUPAC Name: 2-amino-1,9-dihydro- 9-[4-hydroxy-3-(hydroxymethyl)butyl]-3H-purin-6-one, Mol Wt: 253.3).

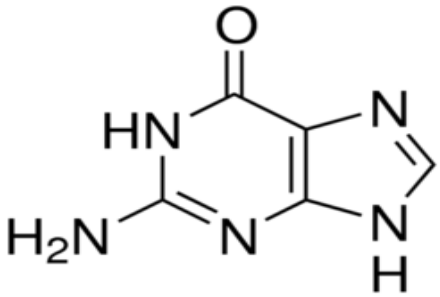

Fig. 2: Guanine (IUPAC Name: 2-amino-1,9-dihydro -6H-purin-6-one, Mol Wt: 151.1).

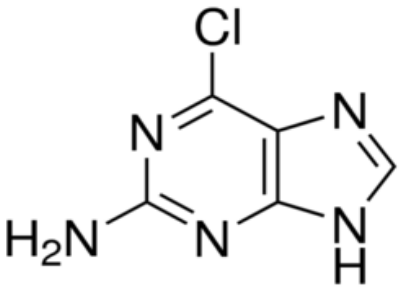

Fig. 3: 2-Amino-6-Chloropurine (IUPAC Name: 6-chloro-7H-purin-2-amine, Mol Wt: 169.6).

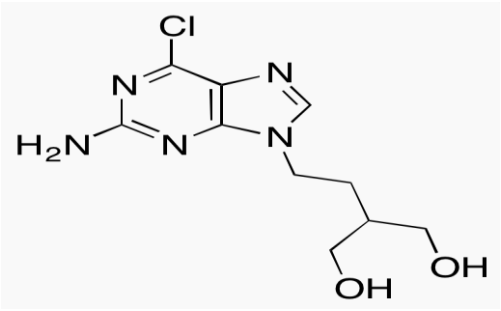

Fig. 4: 6-Chloropenciclovir (IUPAC Name: 2-[2-(2-amino-6-chloro-9H-purin-9yl)ethyl]-1,3-propanediol, Mol Wt: 271.7).

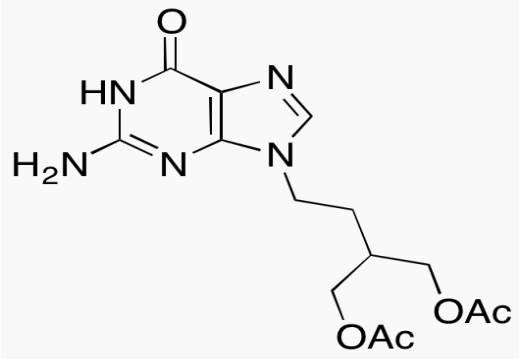

Fig. 5: Penciclovir Diacetate (or) Diacetyl penciclovir (IUPAC Name: 9-[4-(Acetyloxy)-3-[(acetyloxy) methyl]butyl]-2-amino-1,9-dihydro-6H-purin-6-one, Mol Wt: 337.3).

2. MATERIALS AND METHODS

2.1 Chemicals

Potassium dihydrogen phosphate, Ortho phosphoric acid, Acetonitrile, Methanol, Hydrochloric acid, Sodium hydroxide, Hydrogen peroxide were purchased from Rankem, India.

2.2 Instrument and chromatographic condition HPLC for RS

Waters (Model: e2695 with UV/Vis detector 2489) connected to Empower 3.0 data integrator module was used for method development. The separation was performed using an analytical column Kromasil C8 (150mm x 4.6mm, 5µ) maintained at 35°C±2°C whereas sampler temperature was kept at 5°C±2°C. The wavelength was optimized at 254 nm. Flow rate was set at 1.0 ml/min with injection volume of 50 µl. Two different mobile phases were mixed in a proportionate way to run this gradient programme. One mobile phase was mixture of buffer (phosphate buffer of pH 4) and acetonitrile (95:5) and another mobile phase was acetonitrile. At the beginning, two mobile phases were run with ratio of 95:5 up to 8 min and then reduced to 40:60 at 30 min. At 40 minutes, the ratio became 20:80. At 47 minutes, ratio was returned to its initial position i.e. 95:5 and hold up to 55 minutes.

2.3 Chromatographic condition in UPLC for RS

Waters Acuity H class was used for this purpose with Empower 3.0 data integrator module. Here, Acquity UPLC column (100mm x 2.1mm, 1.7 µ) was utilized for the separation of impurities from Penciclovir peak. Column oven temperature was kept at 40°C ± 2°C. But sampler temperature was reduced to 25°C ± 2°C. Flow rate was fixed at 0.3 ml/min with injection volume of 5 µl. Same combination of mobile phases were used for

this gradient programme. Here ratio was started with 100:0 and stayed up to 0.72 minute. At 2.72 minutes, it became 95:5 and was changed to 70:30 at 7.72 minutes with continuation up to 8.72 minutes. At 9.12 minutes, ratio was returned to initial condition and stayed up to 10.72 minutes.

2.4 Chromatographic condition in UPLC for Assay
Same chromatographic condition of UPLC in RS was used for determination of assay.

2.5 Preparation of Diluent
Degassed phosphate buffer of pH 4.0 was used for this purpose.

2.6 Preparation of Standard Solution for RS
2.5 ppm Penciclovir standard was prepared for this study.

2.7 Preparation of Sensitivity Solution for RS
5 ml of standard solution was diluted to 50 ml with diluent.

2.8 Preparation of Placebo Solution
1 ml of placebo was diluted to 20 ml. 5 ml of this solution was again diluted to 20 ml. Solution was filtered through 0.22 µ PVDF filter.

2.9 Preparation of Sample for RS
2.5 ml of Penciclovir injection (5 mg/ml) was transferred to 50 ml volumetric flask containing 35 ml of diluent. Volume was made up with diluent and mixed. Sample was filtered through 0.22 µ PVDF filter.

2.10 Preparation of Impurity Mixture of Penciclovir
2.10.1 Preparation of Stock solution of Guanine Impurity: 2 mg of Guanine Impurity of Penciclovir was transferred in to a 20 mL volumetric flask. 1 mL of 1 N Hydrochloric acid solution was added, dissolved and diluted to volume with methanol and mixed well.

2.10.2 Preparation of Stock solution of 2-Amino,6-Chloro purine Impurity: 2 mg of 2-Amino,6-Chloro purine Impurity of Penciclovir was transferred in to a 20 mL volumetric flask. 10 mL of methanol was added, dissolved and diluted to volume with methanol and mixed well.

2.10.3 Preparation of Stock solution of 6-Chloro Penciclovir Impurity: 2 mg of 6-Chloro penciclovir Impurity of Penciclovir was transferred in to a 20 mL volumetric flask. 10 mL of methanol was added, dissolved and diluted to volume with methanol and mixed well.

2.10.4 Preparation of Stock solution of Diacetyl Impurity: 2 mg of Diacetyl Impurity of Penciclovir was transferred in to a 20 mL volumetric flask. 10 mL of methanol was added, dissolved and diluted to volume with methanol and mixed well.
1 mL each of Impurities of Penciclovir was transferred in to a 100 mL volumetric flask, diluted to volume with diluent and mixed well.

2.11 Validation study for RS[5]
2.11.1 Specificity: Impurities were spiked in sample. Blank, placebo and spiked sample were injected to observe the interference of blank and placebo at the retention times of impurities.

2.11.2 Accuracy: Accuracy was executed at 50%, 100% and 150% level for all known impurities.

2.11.3 Method Precision: Method precision was performed through injection of sample in triplicate followed by determination of impurities present in those injections of sample.

2.11.4 Linearity: Linearity of the method was assessed through injections of linearity solutions in different concentration of Penciclovir and its impurities (LOQ level to 150% level). Preparation of solutions were summarized in Table 1.

Table 1: Preparation of Linearity solutions of Penciclovir and its related substances.

Name	Concentration of Linearity Solutions (in ppm)
Penciclovir	0.0124, 0.0254, 0.0635, 0.1269, 0.1904, 0.2539, 0.3174, 0.3808
Guanine Impurity	0.0059, 0.0259, 0.0647, 0.1293, 0.1940, 0.2587, 0.3233, 0.3880
2-Amino-6-chloro purine	0.0251, 0.0582, 0.0627, 0.1255, 0.1882, 0.2510, 0.3137, 0.3765
6-Chloro Penciclovir	0.0251, 0.0431, 0.0626, 0.1253, 0.1879, 0.2505, 0.3132, 0.3758
Diacetyl Impurity	0.0123, 0.0253, 0.0631, 0.1263, 0.1894, 0.2526, 0.3157, 0.3788

2.11.5 Establishment of RRF: From linearity curves of standard and impurities, slopes of standard as well impurities were obtained. RRF values were calculated through division of slope of impurity by slope of standard.

2.11.6 Establishment of LOQ: LOQ values of all impurities were established through signal to noise ratio method using baseline noise.

2.11.7 Robustness: Flow, column oven temperature and pH were selected for robustness study. During deliberate change in flow, temperature and pH (both increment and decrement), relative retention times of all impurities with respect to Penciclovir were measured.

2.11.8 Solution Stability: Solution stability of standard was performed for 76 hr in benchtop as well as refrigerated condition. Similarity factor was determined

between fresh standard and standard solution kept at refrigerated condition for 76 hrs.

For solution stability of sample, it was kept on bench top as well as refrigerated condition for 36 hr. After that time, percentage of impurities were calculated. From those values, percentage difference between initial and bench top, initial and refrigerated condition were calculated.

2.11.9 Forced Degradation: Forced degradation has been performed to know the degradation pathway of the sample. Under this study, acid & base degradation, oxidation, thermal degradation, photolysis (UV light as well as LUX light). For source of UV and LUX light, Newtronic photostability chamber (Mumbai, India) was utilised.

2.11.10 Filter Validation: Validation of 0.22µ PVDF filter and 0.45µ Nylon filter were performed for standard as well as sample. Unfiltered standard was used as reference to calculate the similarity factor of filtered standard solutions (both PVDF and Nylon). In case of sample, percentage impurities were calculated. Difference in content of impurities between unfiltered and filtered (both PVDF and Nylon) was measured.

2.12 Preparation of Standard Solution for Assay
50 ppm standard was prepared for Assay.

2.13 Preparation of Sample for Assay
50 ppm sample was prepared for Assay.

2.14 Validation study for Assay
2.14.1 Specificity: Specificity study was taken from RS.

2.14.2 Accuracy: It was performed in 50% level, 100% level and 150% level of Pencciclovir standard concentration in triplicate manner.

2.14.3 Method precision: Sample solution was injected in triplicate and assay was determined for each.

2.14.3 Linearity: For linearity study, 12.5 ppm, 25.0 ppm, 37.5 ppm, 50.0 ppm, 62.5 ppm, 75.0 ppm of Penciclovir standards were injected.

3. RESULTS AND DISCUSSION

3.1 Development of analytical method in HPLC and UPLC

Both optimized methods have fulfilled the criteria of system suitability. A comparative table of system suitability parameters along with elution times of Penciclovir and its related compounds was presented in Table 2. From that table, it was clear that in UPLC method, USP plate count was more and runtime was shorter than HPLC method. So, validation study of RS method was performed in UPLC method only.

Table 2: Comparative chart of system suitability parameters with elution time.

System	USP plate count of Penciclovir	USP tailing factor of Penciclovir	Elution time (min) of Penciclovir	Elution time (min) of Guanine	Elution time (min) of 2-Amino-6-Chloro-Purine	Elution time (min) of 6-Chloro Penciclovir	Elution time (min) of Diacetyl impurity
HPLC	19324	1.1	5.13	4.00	9.92	15.18	19.40
UPLC	113055	1.1	4.26	1.19	4.86	5.91	7.39

3.2 Validation Study for RS

3.2.1 Specificity: There was no interference either from blank or placebo at the retention times of related compounds of Penciclovir shown in Fig. 6.

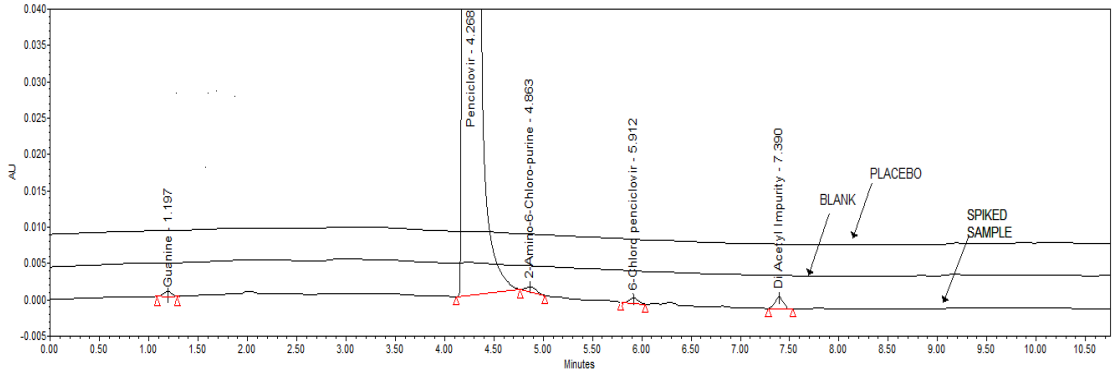

Fig. 6: Overlaid chromatograms of blank, placebo and spiked sample in UPLC.

3.2.2 Accuracy: This study was executed at 50%, 100% and 150% level for known impurities presented in Table 3. The results proved that all values were within limit.

Table 3: Accuracy study of different impurities.

% Level	% Recovery			
	Guanine	2-Amino-6-chloro purine	6-Chloro penciclovir	Diacetyl penciclovir
50	97.5	98.1	97.6	98.6
	97.1	98.5	97.9	98.4
	98.1	98.8	98.2	98.3
100	99.7	100.1	99.4	99.7
	99.4	99.8	99.7	99.3
	100.3	98.9	100.3	99.1
150	100.7	100.3	100.1	100.8
	100.5	100.9	100.6	100.7
	101.1	100.5	100.9	100.4

3.2.3 Method Precision: Method precision was performed through determination of impurities in sample in triplicate injections. Results were shown in Table 4.

Table 4: Results of impurities present in sample (triplicate preparation).

Name of Impurity	% Impurity			
	Sample-1	Sample-2	Sample-3	% RSD
Guanine	0.1	0.1	0.1	0.0
2-Amino-6-chloropurine	0.09	0.08	0.08	7.2
6-Chloro penciclovir	0.11	0.11	0.11	0.0
Diacetyl penciclovir	0.1	0.1	0.1	0.0
Total unknown impurities	0.08	0.08	0.07	7.8
Total impurities	0.57	0.56	0.55	1.8

3.2.4 Linearity: Linearity of the method was assessed through injections of linearity solutions with different concentration of Penciclovir and its impurities. Table 5 represented correlation coefficients of Penciclovir and its related compounds. The values proved that all were linear within these specified range of concentrations of Penciclovir and its related substances.

Table 5: Correlation coefficients in Linearity Study in RS.

Name of Compound	Correlation coefficient (R^2)
Penciclovir	0.999974
Guanine Impurity	0.999975
2-Amino-6-chloro purine Impurity	0.999039
6-Chloro penciclovir Impurity	0.999404
Diacetyl Impurity	0.999991

3.2.5 Establishment of RRF: RRF values of impurities were represented in Table 6.

Table 6: RRF values of impurities of Penciclovir.

Name of Impurity	RRF
Guanine	1.23
2-Amino-6-chloropurine	0.23
6-Chloro Penciclovir	0.18
Diacetyl Penciclovir	0.72

3.2.6 Establishment of LOQ: LOQ values of impurities were presented in Table 7.

Table 7: LOQ values of impurities of Penciclovir.

Name of Impurity	LOQ value (ppm/percentage with respect to test concentration)
Guanine	0.0059 ppm/0.002 %
2-Amino-6-chloropurine	0.0586 ppm/0.023 %
6-Chloro Penciclovir	0.0433 ppm/0.017 %
Diacetyl Penciclovir	0.0123 ppm/0.005 %

3.2.7 Robustness: Relative retention time of all impurities were shown in Table 8 after deliberate increment and decrement of flow, column oven temperature and pH. From that table, it was observed that there was no significant difference in relative retention time of all impurities proving the robustness of the method.

Table 8: Robustness study with respect to flow, column oven temperature and pH.

	Control		Decrement of Flow		Increment of Flow		Decrement of Temp		Increment of Temp		Decrement of pH		Increment of pH	
Name of Impurity	**RT**	**RRT**	**RT**	**RRT**	**RT**	**RRT**	**RT**	**RRT**	**RT**	**RRT**	**RT**	**RRT**	**RT**	**RRT**
Penciclovir	4.37	-	4.60	-	4.07	-	4.49	-	4.12	-	4.30	-	4.42	-
Guanine	1.82	0.42	1.95	0.42	1.67	0.41	1.94	0.43	1.67	0.41	1.75	0.41	1.76	0.40
2-Amino-6-chloropurine	5.01	1.15	5.29	1.15	4.67	1.15	5.15	1.15	4.76	1.15	4.94	1.15	5.07	1.15
6-Chloro penciclovir	6.08	1.39	6.36	1.38	5.81	1.43	6.14	1.37	5.96	1.44	6.03	1.44	6.18	1.40
Diacetyl Penciclovir	7.57	1.73	7.86	1.71	7.29	1.79	7.63	1.70	7.46	1.81	7.52	1.81	7.64	1.73

3.2.8 Solution Stability: Similarity factor of standard solution kept at benchtop as well as refrigerated condition for 76 hrs with respect to fresh standard was 0.99 represented in Table 9. It proves that standard solution is stable upto 76 hrs at both conditions.

For solution stability of sample, it was kept on bench top as well as refrigerated condition for 36 hr. It was observed that sample solution was stable for 36 hrs on bench top condition and 37 hrs on refrigerated condition through determination of impurities.

Table 9: Similarity factors of standard at different conditions.

	Benchtop condition	**Refrigerated condition**
Similarity factor with respect to fresh standard injected	0.99	0.99

3.2.9 Forced Degradation: In each stressed condition, percentage of impurity generated was calculated shown in Table 10. From this table, it was observed that maximum degradation occurred through oxidation whereas minimum degradation occurred under exposure to LUX light.

Table 10: Total percentage of degradation in each stressed condition.

Condition	**Total Impurity (%)**	**Purity Angle of Penciclovir**	**Purity threshold of Penciclovir**
Acid Degradation (5mL of 5N HCl at 80°C for 24 hrs)	0.8	0.826	1.463
Alkali Degradation (5mL of 5N NaOH at 85°C for 24 hrs)	0.8	0.079	0.268
Peroxide Degradation (5mL of 30% H_2O_2 on Bench top for 24 hrs)	1.33	0.962	1.002
Thermal Degradation (80°C for 48 hours)	0.38	0.046	0.261
UV Light Degradation (200 watt-hours/ Sq.meter)	0.45	0.045	0.260
LUX Light Degradation (1.2 Million Lux hours)	0.3	0.045	0.255

3.2.10 Filter Validation: For validation of filter, unfiltered condition, filtered through 0.22µ PVDF filter condition and standard filtered through 0.45µ Nylon filter condition were used. In case of Penciclovir standard, similarity factor was determined for PVDF filtered standard and Nylon filtered standard with respect to unfiltered standard shown in Table 11. In case of sample, percentage impurities were calculated. Percentage difference of impurities in unfiltered sample from impurities in two different filtered samples were determined.

Table 11: Similarity factor for standard.

Condition of Standard	**Similarity Factor**	
	Standard-1	**Standard-2**
Filtered through 0.22µ PVDF	1.00	1.00
Filtered through 0.45µ Nylon	1.00	1.00

3.3 Development of Assay in UPLC

In assay, USP tailing factor for Penciclovir was 1.0. USP plate count was 17288. %RSD of five replicates of Penciclovir was 0.0%.

3.4 Validation of Assay in UPLC

3.4.1 Specificity: As same method was used in both RS and assay. So, for specificity study, result of specificity in RS was considered for specificity in assay also.

3.4.2 Accuracy: Result of accuracy study was shown in Table 12. It showed that method is accurate.

Table 12: Accuracy study of Penciclovir for assay method.

% Level	**Accuracy results of Penciclovir**
50	99.4
	99.5
	99.5
100	98.7
	98.8
	98.5
150	98.1
	97.9
	97.9

3.4.3 Method Precision: Results of method precision was represented in Table 13. The result proved that the method is precise.

Table 13: Method precision study.

Name of Compound	% Assay			
	Sample-1	Sample-2	Sample-3	% RSD
Penciclovir	99.5	99.3	99.3	0.1

3.4.4 Linearity: Correlation co-efficient (R^2) of linearity curve of Penciclovir standard in assay was 0.9998 ascertaining that the method is linear in that specified range.

4. CONCLUSION

This reverse-phase UPLC method has been developed for quantification of related substances in Penciclovir injection. This method was stable, specific, accurate, precise and robust. The validation study showed satisfactory data for all the validation parameters tested. The UPLC method is better than HPLC method with respect to very shorter runtime and system suitability parameter. Same UPLC method is also suitable for assay analysis proved from the validation parameters tested. Hence, the optimized UPLC method is suitable for determination of assay and related substances in Penciclovir with change in concentration of standard preparation.

5. ACKNOWLEDGEMENTS

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6. REFRENCES

1. Richardson VN, Davis SA, Gustafson CJ, West CE, Feldman SR. Patterns of disease and treatment of cold sores. J Dermatolog Treat, 2013; 24(6): 439-443.
2. Ganji S, Satyavati D. Method development and validation for the analysis of Penciclovir and related impurity in bulk and pharmaceutical dosage forms by RP HPLC. Int J Pharm, 2016; 6(1): 137-148.
3. Elzaher AA, Fouad MA, Elhoussini OM, Behery YE. Validated spectrometric determination of penciclovir and entecavir in bulk and in pharmaceutical preparations. Bulletin of Faculty of Pharmacy, Cairo University, 2016; 54: 175-179.
4. https://www.trc-canada.com/product-detail/.
5. https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf.