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Validated UPLC Method for Organic Impurities of Phenazopyridine Hydrochloride Drug. A Green Analytical Technique

Omprakash Saini[a, b],Sanjay K. Sharma [a] and Yogesh Sharma [c]

[a] Green Chemistry and Sustainability Research Group, Department of Chemistry, JECRC University, Jaipur-303905, India

[b] Amol Pharmaceutical PVT.LTD, Jaipur, India

[c] Waters India PVT.LTD, Delhi, India

E-mail: drsannjaysharma@gmail.com, omprakash.20phsn001@jecrcu.edu.in

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KEYWORDS

Phenazopyridine Hydrochloride UPLC; Validation, Green Chemistry, High separation efficiency, Cost effective

ABSTRACT:

Introduction: Green Chemistry talks about any environmental technique or practice which may reduce waste, materials, hazards, risk, energy, environmental impact, and operational cost, also increase productivity. Ultra performance liquid chromatography (UPLC) is novel technique of High-performance Liquid chromatography (HPLC), which is a Green Technique in the true sense. To enhance those practices, a simple, sensitive, accurate, selective, and precise Ultra Performance Liquid Chromatography **(**UPLC) method was developed for the identification and quantification of the Phenazopyridine Hydrochloride Drug. The separation was performed on a BEH C18-column (1.7 µm, 2.1 mm X 100 mm) using a mobile phase20 mM ammonium acetate buffer as mobile phase-A and 100% Acetonitrile as a mobile phase-B ingredient elution. The detection was performed at fixed wavelength (λ=240nm) injection volume (1.5µl), with a flow rate of 0.40ml/min and Run Time 9.0 Minutes. The retention time of Phenazopyridine Hydrochloride was found at about 4.64 minutes. After developing the method, it was assured for future use by validation of the analytical parameters like System Suitability, Specificity, linearity, accuracy, precision, solution stability, The Limit of Detection (LOD) and Limit of Quantification (LOQ) and robustness. The results of all the parameters of the method were found within the acceptance criteria as per the International Council for Harmonization (ICH) guidelines and general practices of pharmaceutical industries.

Introduction: Phenazopyridine has antiseptic or local analgesic effects effect on urinary tract defense system to relieve pain, burning, urgency frequency and general discomfort due to infection, trauma, surgery, endoscopic procedures, or the way of equipment or catheters in lower part of urinary tract system (Bladder and Urethra) in adults.[1,2] In1932 Mr. Bernhard Joos discovered the analgesic properties of Phenazopyridine Hydrochloride.[3] as part of mechanism of action Phenazopyridine inhibited the voltage-gated sodium channel (Sodium channel protein type 1 subunit alpha-SCN1A) Inhibited by Phenazopyridine Hydrochloride.[2,4] These channels are enclosed with large pseudotetrameric pore-forming α-subunit which is associates with one or two β- subunits. α-subunits are large, single-chain polypeptides composed of approximately 2000 amino acid residues arranged in four homologous domains (DI to DIV). Each domain is composed of **six trans-membrane helical segments named S1 to S6**. The molecules of Phenazopyridine bind with voltage-gated sodium channels and temporarily disable the function of the ion channel means blocking the passing of sodium ions through voltage-gated sodium channels in cell membranes of nerve cells. Phenazopyridine Hydrochloride is affecting the lower part of the urinary tract system (Bladder and Urethra) to relieve pain or burning, increased urination, and increased the urge to urinate the bladder.[5] To analysis of organic impurities for Phenazopyridine hydrochloride at the stage of bulk/Tablets, HPLC methods are available in the pharmacopeia, the current HPLC method are used to analysis of Phenazopyridine hydrochloride bulk and tablets for organic impurity test in pharmaceuticals industries, testing laboratories, etc. The HPLC method of organic impurities test are required long run times & higher volume of the mobile phase resulting in more consumption of chemicals and solvents. To reduce the analysis time, consumption of

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solvents/chemicals and enhance productivity, a new analytical method has been developed and validated using, Ultra Performance Liquid Chromatography (UPLC) for the organic impurities test of Phenazopyridine Hydrochloride for bulk. Reviewed much literature/Articles and Pharmacopoeias there is no specific analytical method developed and validated to analyze the organic impurities test using the UPLC technique for Phenazopyridine Hydrochloride bulk. The structure, Molecular weight & IUPAC Name mention in

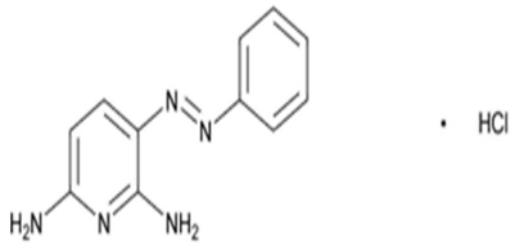

Figure-1 of Phenazopyridine Hydrochloride [6-7]

MATERIALS AND METHODS

Chemicals and drugs:

Known Impurities (2,6-Diaminopyridine) & Phenazopyridine Hydrochloride (Active Pharmaceutical Ingredient) and were get from Amol Pharmaceutical Pvt. Ltd. (Jaipur India). Ammonium Acetate and Acetonitrile were used by Merck.

Equipment: It consists of Ultra Performance Liquid Chromatography (UPLC) of Waters, a separation module, and a PDA detector. The output signal was monitored and processed by waters Empower 3 Software (Build 3471 SPs Installed: Feature Release 2 DB ID: 2373571950). The stationary phase employed was BEH C18 (1.7 µm, 2.1 mm X 100 mm) column.

Sensitivity: Take 2.5 mg of Phenazopyridine Hydrochloride standard and 2.5 mg 2, 6-di amino pyridine standard in 100 volumetric flasks, dissolved and dilute up to mark with diluents. Further transfer 1.0 of this solution to another 100 ml volumetric Flask & make up to mark with diluents (0.25µg/ml).

Standard Stock solution: Weight and transfer about 5 mg of Phenazopyridine HCL Working standard and 10 mg 2, 6-di amino pyridine standard in 100 ml volumetric flask and add about 50 ml diluents, swirl to dissolve, and make up the volume with diluents and mix well.

Standard Preparation: Transfer 1.0 ml of this solution to a 100 ml volumetric flask, makeup with diluent and mix well. The stock standard concentration is about (0.0005 mg/ml Phenazopyridine Hydrochloride and 0.001 mg/ml 2, 6-di amino pyridine).

Sample Preparation: Weight and transfer about 50mg of Phenazopyridine HCL sample to a 100 ml volumetric flask and add about 50 ml diluents, swirl to dissolve, and make up the volume with diluents and mix well.(0.5mg/ml).

UPLC method development: Acetonitrile and water (10:90%v/v) were chosen as Diluent. 20 mM ammonium acetate in water (1.54 gm Ammonium Acetate in 1000 ml of water), Multiple trials were done using 20 mM ammonium acetate as mobile phase-A and 100% Acetonitrile as mobile phase-B for the development of a UPLC method for Organic Impurities test. Various gradient programs were evaluated. Trials were continued till the method was optimized.

Quantitative Determination of known and unknown Impurities in Phenazopyridine HCL in sample solution:

Standard and sample solutions were injected and chromatograms were recorded at 240 nm with a PDA detector. The determination of the percentage of Phenazopyridine Hydrochloride is given by the following formula:

Calculate the percentage of 2, 6-diaminopyridine in the portion of Phenazopyridine Hydrochloride taken**:**

$$Result = (R_U/R_S) \times (C_S/C_U) \times 100$$

Where,

R_U = Peak response of 2, 6-diaminopyridine from the Sample solution

R_S = Peak response of 2, 6-diaminopyridine from the Standard solution

C_S = Concentration of 2, 6-diaminopyridine in the Standard solution (mg/ml)

C_U = Concentration of Phenazopyridine Hydrochloride In the Sample solution (mg/ml)

Calculate the percentage of any individual unspecified Impurities in the portion of Phenazopyridine Hydrochloride taken:

$$Result = (R_U/R_S) \times (C_S/C_U) \times 100$$

Where,

R_U = Peak response of each individual unspecified impurity from the Sample solution

R_S = Peak response of Phenazopyridine from the Standard solution

C_S = Concentration of USP Phenazopyridine Hydrochloride RS in the Standard solution (mg/ml)

C_U = Concentration of Phenazopyridine Hydrochloride in the Sample solution (mg/ml)

Method validation: Participants 'Analytical method validation was performed as per the ICH guidelines. [8] The developed method was validated for the **various** parameters like System Suitability, Specificity, linearity, accuracy, precision, solution stability, and robustness, LOD and LOQ.

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Specificity: Specificity is the ability to assess the analyte in the presence of components that may be expected to be present such as impurities, degradation products, and excipients. There must be inarguable data for a method to be specific. Specificity measures only the desired component without interference from other species which might be present; separation is not necessarily required. Selectivity is the ability of the analytical method to resolve each related compound in the mixture. Specificity is required for assay but selectivity is not. Both specificity and selectivity are required for impurities analysis. Specificity and selectivity are determined by analyzing blanks, sample matrix (placebo), and known related impurities to determine whether interferences occur. Specificity and selectivity are also demonstrated during the forced degradation Study.

Limit of Detection (LOD): Determination of the signal to noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. Acceptance limit for detection limit for Phenazopyridine hydrochloride was calculated using signal to noise ratio (S/N) must be Not Less Than (NLT) 3 for Detection Limit (DL).

Limit of Quantification (LOQ): Determination of the signal to noise ratio is performed by comparing measured signals from samples with known low concentrations of analytic with those of blank samples and by establishing the minimum concentration at which the analyst can be reliably quantified. Acceptance limit for quantification limit for Phenazopyridine hydrochloride peak was calculated using signal to noise ratio (S/N) must be Not Less Than (NLT) 10 for Detection Limit (DL).

Linearity: The linearity study covered the range of 80%-120% of the expected level of the analyte. Different concentration solutions (LOQ, 50%, 80%, 150%, & 200%) were prepared from the stock solution of working standard using diluent. The calibration curve was obtained by plotting peak area vs. concentration, using the least squares method. ICH recommends that, for the establishment of linearity, a minimum of five concentrations should normally be used. The acceptance criteria are correlation coefficient must be not less than 0.98.

Accuracy: The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. As per ICH guideline recommendation, the accuracy study was assessed by ***nine*** determinations i.e. **three** replicates at three concentrations across the specified range of the procedure. Acceptance criteria were kept as Individual % Recovery must be within 80.0%-120.0%.

Precision: The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions on different days. ICH guidelines recommend that repeatability should be assessed by using a minimum of six determinations at 100% of the specification level test concentration at days, different analysts, different equipment, etc. The Acceptance criteria are % RSD of six preparations must be not more than10.0% for 2, 6-Diaminopyridine and Not more than 3.0% for Phenazopyridine Hydrochloride.

Solution Stability: Solution stability is carried out to know the stability of the sample in analytical solution over the period during routine analysis, Solution stability is checked for 24 Hours. As per laboratory practices, the mobile phase should be clear without particles & Haziness, Retention time (RT) should be matched with the initial analysis and the %RSD of the Known impurity 2,6-Diaminopyridine and principal peak Phenazopyridine Hydrochloride should not be more than 3.0%.

Robustness: Robustness was evaluated by slightly changing the chromatographic conditions which include flow rate and column temperature.

Effect of a slight change in flow rate: The solution was analyzed at 0.36ml/minute and 0.44ml/minute rather than the optimized flow rate of 0.40ml/minute. Chromatograms were collected to compare with optimized chromatographic conditions. The %RSD of 6 preparations was compared with normal conditions.

Effect of slight changes in percent column temperature: The solution was analyzed by slightly varying the UPLC column temperature at 33°C & 27°C rather than the optimized UPLCcolumntemperatureof30°C.Chromatogramswerecollectedtocomparewithoptimizedchromatographicconditions.The % RSD of 6 preparations was compared with normal conditions.

RESULTS AND DISCUSSION

UPLC method development: The test method condition for Organic Impurities test of Phenazopyridine hydrochloride drug was developed using BEH C18-column (1.7 µm, 2.1 mm X 100 mm) using a mobile phase 20 mM ammonium acetate buffer as mobile phase -A and 100% Acetonitrile as a mobile phase-B at a different flow rate with various gradient programs, At last, optimized method conditions were developed, which employs 20 mM ammonium acetate buffer as mobile phase-A and 100% Acetonitrile as a

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mobile phase-B at a flow rate of 0.40 ml/min. The optimized chromatographic conditions are shown in Table-1 and the standard chromatogram is shown in Figure-2.

Table-1: Chromatographic condition

Mobile phase	20 mM ammonium acetate buffer as mobile phase -A	
	Mobile Phase-B: 100% Acetonitrile	
Column	BEH C18-column (1.7 µm, 2.1 mm X 100 mm)	
Elution	Gradient	
Flow Rate	0.40 ml/minute	
Injection Volume	1.5 µL	
Detection	240 nm	
Auto sampler Temperature	Ambient	
Column Temperature	30°C	
Retention Times	Phenazopyridine Hydrochloride: about 4.6min.	
Gradient Program		
Time (Minutes)	**Mobile Phase-A (%)**	**Mobile Phase B (%)**
0.00	95.0	5.0
0.40	95.0	5.0
3.20	50.0	50.0
4.20	50.0	50.0
5.90	30.0	70.0
6.90	30.0	70.0
7.30	95.0	5.0
9.00	95.0	5.0

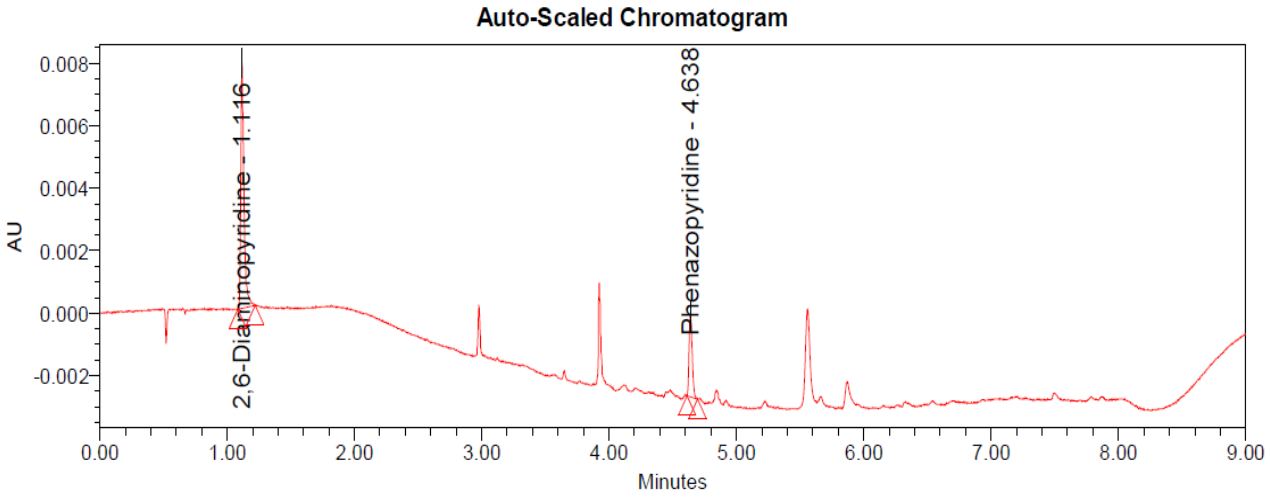

Figure 2: Chromatogram of Standard injection solution.

Method validation: Analytical method validation was performed as per the ICH guidelines.[8] The developed method was validated for various parameters like System Suitability, Specificity, LOD, LOQ, linearity, accuracy, precision, solution stability, and robustness.

Specificity: The specificity of the test method was evaluated by known Impurities (2, 6-Diaminopyridine) and active material Phenazopyridine Hydrochloride (Sample) was spiked. The specificity chromatogram is shown in Figure 3.

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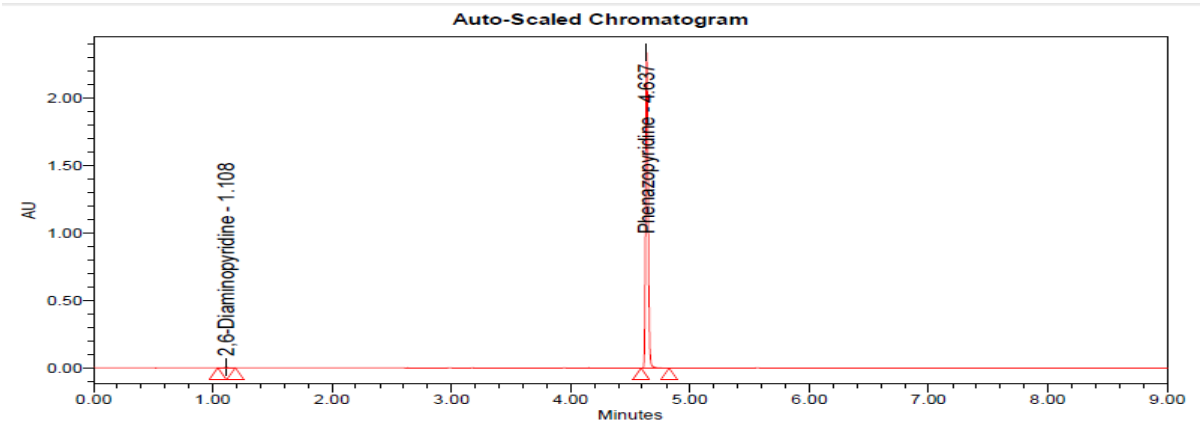

Peak Results

	Name	RT	Area	Height
1	2,6-Diaminopyridine	1.108	7602	4223
2	Phenazopyridine	4.637	3413304	2343100

Figure 3: Specificity solution Chromatogram

LOD and LOQ: The lowest possible concentrations of Phenazopyridine Hydrochloride that can be detected and quantified by the present method were found to be 0.0607µg/ml and 0.2503µg/ml. The low values of LOD and LOQ indicates that the method can be used for detection and quantification of Phenazopyridine hydrochloride drug over a very wide range of concentrations.

Accuracy: Accuracy study results are shown in Table2. An accuracy study was performed at different levels (i.e. 50%, 100% & 200%) of a concentrated solution of Phenazopyridine Hydrochloride. Accuracy Study sample solutions were prepared in triplicate. The mean percentage recovery was found to be within the acceptance limit of 98.0% to 102.0%. Hence, accuracy was established for the developed method and was found to be accurate.

Table 2: Accuracy study of 2, 6-Diaminopyridine

Level	**Amount of 2,6 DAP Added in (µg/ml)**	**Amount of 2,6 DAP Found in (µg/ml)**	**% Recovery**
50%	0.5233	0.5193	99.23
100%	1.0534	1.0333	98.12
200%	2.0811	2.0721	99.57
Overall Recovery =98.97%			

Linearity: Linearity study was performed at different concentration solutions (LOQ, 50%, 80%, 100%, 150% & 200%). Calibration curves were plotted using peak area vs. concentration for Impurities (2, 6-Di-aminopyridine) and active content (Phenazopyridine Hydrochloride) which is shown in Figure 4 & 5. The Correlation coefficient values for both were found 0.999 for 2, 6-Diaminopyridine known Impurities and 0.990 for Phenazopyridine Hydrochloride active content against the limit Not Less Than 0.98. As per ICH guidelines, these values were within an acceptable limit and hence the method was found to be linear.

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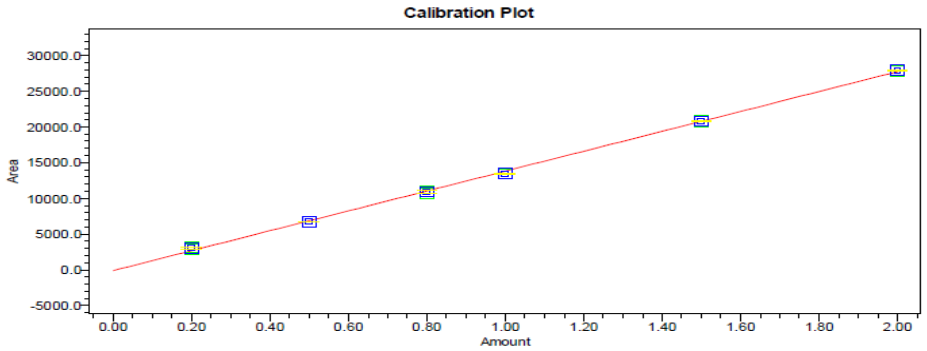

Figure 4: Calibration curve of 2, 6-Diaminopyridine known Impurities.

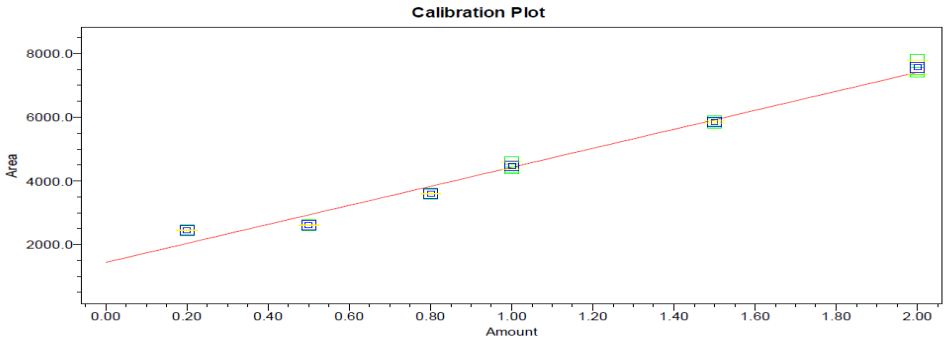

Figure 5: Calibration curve of Phenazopyridine Hydrochloride active content.

Precision: Repeatability and Intermediate precision (Ruggedness) performed and observed results from 6 preparations (0.5mg/ml) solutions were shown in Table 3. The percentage RSD values of 2, 6-di amino-pyridine obtained from both precision studies less than 10.0% which was well within the limits mentioned in the ICH guidelines. Hence, the method was found to be precise.

Table 3: A precision study of 2, 6-Diaminopyridine

Sr. No.	**Precision (Repeatability): Analyst-1**	**Intermediate precision: Analyst-2**
	Results in (%)	Results in (%)
1	0.013	0.010
2	0.012	0.013
3	0.012	0.011
4	0.012	0.013
5	0.012	0.013
6	0.010	0.011
Mean Results: 0.012% %RSD: 9.74		Mean Results: 0.012% %RSD: 8.82%
% RSD PPHCL: 1.06		% RSD of PPHCL:1.33

Solution Stability: Solution stability is carried out at the initial stage of the sample solution and after 24 Hours at room temperature, after 24 Hours the mobile phase was found clear without particles & Haziness, the Retention time (RT) and %RSD of the principal peak for 2,6-Diaminopyridine known Impurities and Phenazopyridine Hydrochloride active content obtained in line with initial analysis. Hence mobile phase, standard and sample solutions are stable for 24 Hrs. The observed results are shown in Table 4**.**

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Table 4: Solution stability study of Phenazopyridine Hydrochloride drug

Acceptance Criteria	**Observed results**		
% RSD: NMT 3.0% for 2-6 Di-amino pyridine and Phenazopyridine hydrochloride (PPHCL)	Initial (RT)	**2,6-DAP**	**PPHCL**
		1.12	1.18
	After 24Hours	1.19	1.76
The mobile phase should be clear without particles & Haziness	Clear no particle and haziness found after 24 hours		
Retention time (RT) should be matched with an initial analysis.		**2,6-DAP**	**PPHCL**
	Initial (RT)	1.121	4.645
	After 24Hours	1.116	4.632

Robustness: Mobile phase flow rate was slightly altered by (±10%) of an optimized flow rate of 0.40 ml/minute (0.36 ml/min and 0.44 ml/min) and the UPLC column temperature was slightly increased by 3 units (i.e. 33°C instead of the optimized temperature of 30°C) and decreased by 3 units (i.e. 27°C instead of the optimized temperature of 30°C). The % RSD of altered/changes condition for 2-6 Di-amino pyridine from 6 preparations was calculated and shown in Table 5. The % RSD were found to be not more than 3.0%for variation in flow rate and UPLC column temperature. Hence the method was found to be robust.

Table 5: Robustness of the method

Parameters	**Normal condition**	**Altered/Change Condition**	**% RSD of 2-6 DAP is NMT ±3.0%**
Flow rate	0.40 ml/minute	0.36 ml/minute	0.85%
		0.44 ml/minute	0.87%
Column temperature	30°C	27°C	0.80%
		33°C	1.01%

Conclusion: The present work was planned to development and validation an analytical method using UPLC-Instrument. The method is applicable only for "organic impurities test" for Phenazopyridine Hydrochloride bulk Product. It is simple, linear, accurate, precise, and robust method. The developed method improves productivity and reduced the analysis time, price, and consumption of chemical/solvent, comparison to currently used HPLC-Method which is already available in Pharmacopoeia. Discursively this method is part of the green practice. This method is reliable and convenient for routine quality control analysis of Phenazopyridine Hydrochloride bulk products for the test of Organic impurities.

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