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Research Article

Validated UPLC Method for Identification and Quantification of Phenazopyridine Hydrochloride Drug: A Green Analytical Technique

Omprakash Saini[1,2*] , **Sanjay Kumar Sharma**[1] , **Yogesh Sharma**[3]

[1]Department of Chemistry, Green Chemistry and Sustainability Research Group, JECRC University, Jaipur, Rajasthan, India;[2]Amol Pharmaceuticals Private Limited, Jaipur, Rajasthan, India;[3]Waters India Private Limited, Jasola, Delhi, India

ABSTRACT

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 a modernized 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 × 50 mm) using a mobile phase 20 mM ammonium acetate buffer as mobile phase A and 100% Acetonitrile as a mobile phase-B in gradient elution. The detection was performed at a fixed wavelength (λ=280 nm), with a flow rate of 0.40 ml/min. The retention time of Phenazopyridine hydrochloride was found at about 4.67 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, 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.

Keywords: Phenazopyridine hydrochloride; UPLC; Validation; Green chemistry; High separation efficiency

INTRODUCTION

In 1932 Mr. Bernhard Joos discovered the analgesic properties of Phenazopyridine hydrochloride drug which use for local analgesic effects on the lower part (Bladder and Urethra) of the urinary tract system to relieve pain or burning [1,2]. Phenazopyridine hydrochloride has an antiseptic or analgesic effect on the Urinary system by the Inhibition of Voltage-gated sodium channel (Sodium channel protein type 1 subunit alpha) (Figure1). These channels are contained a large pseudotetrameric pore-forming α-subunit that 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 [3-6]. To Identification and Quantification for Phenazopyridine hydrochloride at the stage of bulk/Tablets, HPLC methods are available in the pharmacopeia, these methods are used to analysis of Phenazopyridine hydrochloride in pharmaceuticals industries, testing laboratories, etc. These methods are having long run times for analysis, higher volume of the mobile phase is required resulting in more consumption of chemicals and solvents.

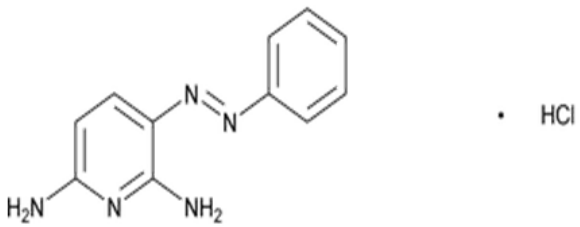

Figure 1: Structure of Phenazopyridine hydrochloride ($C_{11}H_{11}N_5 \cdot HCl$). **Note:** Molecular weight: 249.70; IUPAC Name: 2, 6-*Pyridinediamine*, 3-(*phenyl azo*)-*monohydrochloride*2,6-*Diamino*-3-(*phenyl azo*) *pyridine monohydrochloride*.

Correspondence to: Omprakash Saini, Department of Chemistry, Green Chemistry and Sustainability Research Group, JECRC University, Jaipur, Rajasthan, India, E-mail: omprakash.20phsnn001@jecrcu.edu.in

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To Enhance productivity and reduced the consumption of chemicals/solvents, A new analytical method has been developed and validated using, Ultra Performance Liquid Chromatography (UPLC) for the Assay test of Phenazopyridine Hydrochloride for bulk. Reviewed much literature, Journals and Pharmacopoeias there is no specific analytical method developed and validated to analyse the assay test using the UPLC technique for Phenazopyridine Hydrochloride bulk.

MATERIALS AND METHODS

Chemicals and drugs

Phenazopyridine hydrochloride (Active Pharmaceutical Ingredient) and Known impurity (2-6 Di-Aminopyridine) were procured from Amol Pharmaceutical Pvt. Ltd. (Jaipur,India). Ammonium Acetate and Acetonitrile are used by Merck.

Equipment

It consists of Ultra Performance Liquid Chromatography (UPLC) of Water, 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 × 50 mm) column.

Standard stock solution

Weight and transfer about 30 mg of Phenazopyridine HCL Working standard to a 50 ml volumetric flask and add about 20 ml diluents, swirl to dissolve, and make up the volume with diluents and mix well. The stock standard concentration is about 600 µg/mL of Phenazopyridine HCL.

Standard preparation

Transfer 5.0 ml of this solution to a 100 ml volumetric flask, dilute to volume and mix well. The stock standard concentration is about 30 µg/mL of Phenazopyridine HCL.

Sample stock solution

Weight and transfer about 30 mg of Phenazopyridine HCL sample to a 50 ml volumetric flask and add about 20 ml diluents, swirl to dissolve, and make up the volume with diluents and mix well. The stock standard concentration is about 600 µg/mL of Phenazopyridine HCL. Sample Preparation: Transfer 5.0 ml of this solution to a 100 ml volumetric flask, dilute to volume and mix well. The stock sample concentration is about 30 µg/mL of Phenazopyridine HCL.

UPLC method

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. Various gradient programs were evaluated. Trials were continued till the method was optimized [7].

Quantitative determination of phenazopyridine HCL in sample solution

Standard and sample solutions were injected and chromatograms were recorded at 280 nm with a PDA detector. The determination of the percentage of Phenazopyridine Hydrochloride is given by the following formula:

$$\%Assayasisthebasis\left(API\right)=\frac{At}{As}\times\frac{Ws}{50}\times\frac{5}{100}\times\frac{50}{Wt}\times\frac{100}{5}\times\frac{P}{100}\times100$$

Where:

At=Area of sample solution

As=Average area of standard solution

Ws=Standard weight in mg

Wt=weight of sample in mg

P=Purity of standard on is the basis.

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.

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.

Linearity: The linearity study covered the range of 80%-120% of the expected level of the analyte. Different concentration solutions (80%, 90%, 100%, 110% and 120%) 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 [9].

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 that are three replicates at three concentrations across the specified range of the procedure. Acceptance criteria kept as per USP monograph for Phenazopyridine hydrochloride must be within 98.0%-102.0%.

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 than 2.0% 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 and Haziness, Retention Time (RT) should be matched with the initial analysis. % Difference between the assay of initial analysis and after 24 Hrs. is not more than ±1.0% and the Cumulative % RSD of 10 standard injections should not be more than 1.0.

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

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

Effect of a slight change in wavelength: The solution was analyzed at 282 nm and 278 nm rather than the optimized wavelength of 280 nm. Chromatograms were collected to compare with optimized chromatographic conditions. The %RSD of 6 preparations was compared with normal condition.

Effect of slight changes in percent column temperature: The solution was analyzed by slightly varying the UPLC column temperature at 27°C rather than the optimized UPLC column temperature of 25°C. Chromatograms were collected to compare with optimized chromatographic conditions. The %RSD of 6 preparations was compared with normal conditions [10].

RESULTS AND DISCUSSION

UPLC method development

The test method condition for the identification and quantification of Phenazopyridine hydrochloride was developed using BEH C18-column (1.7 µm, 2.1 mm × 50 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 mobile phase-B at a flow rate of 0.40 mL/min. The optimized chromatographic conditions were 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 × 50 mm)
Elution	Gradient
Flow Rate	0.40 mL/minute
Injection Volume	10 µL
Detection	280 nm
Autosampler Temperature	Ambient
Column Temperature	25°C
Retention Times	Phenazopyridine hydrochloride: about 4.6min.

Gradient program		
Time (Minutes)	Mobile Phase-A (%)	Mobile Phase B (%)
0	95	5
0.4	95	5
3.2	50	50
4.2	50	50
5.9	30	70
6.9	30	70
7.3	95	5
9	95	5

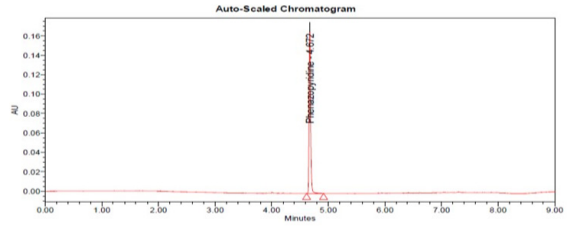

Figure 2: Chromatogram of Phenazopyridine hydrochloride standard solution.

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, linearity, accuracy, precision, solution stability, and robustness.

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

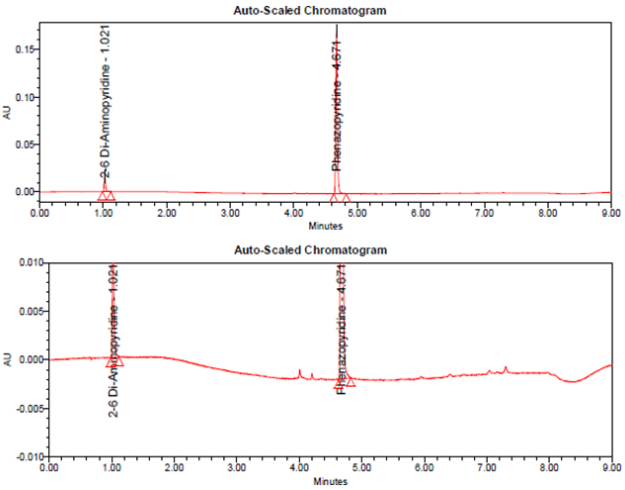

Figure 3: Full and expanded Chromatogram of specificity.

Accuracy: Accuracy study results are shown in Table 2. An accuracy study was performed at different levels (80%, 100% and 120%) 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 Phenazopyridine hydrochloride.

Level	Amount Added in (mg)	Amount found in (mg)	% Recovery
80%	24.018	24.13	100.48
100%	30.112	30.08	99.9
120%	36.201	36.03	99.54
Overall Recovery =99.97%			

Linearity: Linearity study was performed at different concentration solutions (80%, 90%, 100%, 110% and 120%). Calibration curves were plotted using peak area vs. concentration and which is shown in Figure 4. Correlation coefficient values were found to be 0.999. As per ICH guidelines, these values were within an acceptable limit and hence the method was found to be linear.

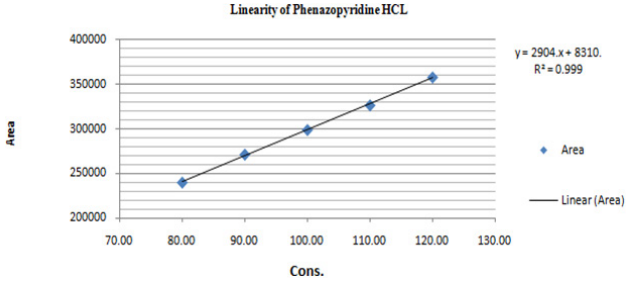

Figure 4: Calibration curve of Phenazopyridine hydrochloride. **Note:** (◆) Area, (—) Linear (Area).

Precision: Repeatability and Intermediate precision (Ruggedness) performed and observed results from 6 preparations at 100% concentration of test solutions were shown in Table 3. The percentage RSD values of Phenazopyridine Hydrochloride obtained from both precision studies were found to be less than 2.0% which was well within the limits mentioned in the ICH guidelines. Hence, the method was found to be precise [11].

Table 3: A precision study of Phenazopyridine hydrochloride.

Sr.No.	Precision: Analyst-1	Intermediate precision: Analyst-2
	% Assay	% Assay
1	99.59	100.53
2	100.11	99.76
3	100.39	100.98
4	98.95	99.95
5	99.13	100.93
6	99.06	100.88
	Mean assay: 99.54% SD: 0.60%RSD: 0.60%RSD: 0.60	Mean assay: 100.51%SD: 0.53 %RSD: 0.53%

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 and Haziness, and the Retention Time (RT) of the principal peak obtained in line with initial analysis, the %difference between the assay (initial analysis and after 24 Hrs.) found within a limit and Cumulative %RSD of 10 standard injections within a limit. Hence mobile phase, standard, and sample solutions are stable for 24 Hrs. The observed results are shown in Table 4.

Table 4: Solution stability study of Phenazopyridine hydrochloride.

Acceptance Criteria	Observed results
System Suitability: % RSD: NMT 0.73% Tailing Factor: NMT 1.5	% RSD: 0.1% ;Tailing Factor: 1.18
The mobile phase should be clear without particles and Haziness	Clear no particle and haziness found
Retention Time (RT) should be matched with an initial analysis.	Initial (RT): 4.671 RT; after 24 Hours: 4.657
%Difference between the assay of initial analysis and after 24 Hrs. is not more than ± 1.0%	Initial Assay: 100.08% ; After 24 Hours: 99.37% ; %Difference: 0.71
The cumulative % RSD of 10 standard injections should not be more than 1.0%	% RSD: 0.59%

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). The wavelength slightly Changed (± 2 nm) of the Optimized Nanometer of 280 nm (278 nm and 282 nm) and the UPLC column temperature was slightly increased by 2 units (27°C instead of the optimized temperature of 25°C). The % difference between the two analyses (Normal and altered/changes condition) of Phenazopyridine Hydrochloride from 6 preparations was calculated and shown in Table 5. The % difference values were found to be not more than ± 1.0% for variation in flow rate, wavelength, and UPLC column temperature. Hence the method was found to be robust [12].

Table 5: Robustness of the method.

Parameters	Normal condition	Condition opted	%Difference between the two analyses is NMT ± 1.0%
Flow rate	0.4 ml/minute	0.36 ml/minute	0.77%
		0.44 ml/minute	0.52%
Wavelength	280nm	278nm	0.57%
		282nm	0.53%
Column temperature	25°C	27°C	0.65%

CONCLUSION

The present work was planned for the identification and quantification of Phenazopyridine Hydrochloride for a Bulk product. Ultra Performance Liquid Chromatographic Method (UPLC), which was developed and validated in the present work was found to be simple, linear, accurate, precise, and robust, therefore, suitable for the intended use. The developed methods enhance productivity and reduced the time, cost, and consumption of solvent, and chemicals than the 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.

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CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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