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SYNTHESIS OF 2, 6-DIHYDROXY-3-PHENYLDIAZENYLPYRIDINE AND 6-AMINO-2-HYDROXY-3-PHENYLDIAZENYLPYRIDINE: AN IMPURITY IN THE PROCESS FOR PHENAZOPYRIDINE HYDROCHLORIDE A GENITO-URINARY ANTISEPTIC DRUG

Dr. Ajit Anerao*, Dr. Vihar Telange, Nitin Bondre, Vikram Dighe, K. Swamy Reddy, A. V. V. Somasekhar, Dr. Nitin Pradhan

R&D Centre (API), Wanbury Ltd., EL-16, TTC Industrial Estate, Mahape, Navi Mumbai 400710, India.

***Corresponding Author: Dr. Ajit Anerao**
R&D Centre (API), Wanbury Ltd., EL-16, TTC Industrial Estate, Mahape, Navi Mumbai 400710, India.

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ABSTRACT
2,6-Dihydroxy-3-phenyldiazenylpyridine and 6-Amino-2-hydroxy-3-phenyldiazenylpyridine an impurity formed in the process for the genitourinary antiseptic drug Phenazopyridine hydrochloride. Impurities were synthesized; characterized and reverse phase high-pressure liquid chromatography (HPLC) method has been developed and validated for estimation of related substances of Phenazopyridine hydrochloride active pharmaceutical ingredient (API). Efficient chromatographic separation was achieved on a Cosmosil 5µm particles size, 250mm × 4.6mm column. The method was validated for accuracy, precision, specificity, robustness, and detection and quantification limits, in accordance with ICH guidelines. Statistical analysis proved the method was precise, reproducible, selective, specific, and accurate for analysis of Phenazopyridine hydrochloride and its related impurities.

HIGHLIGHTS
- Related substances method is developed for quantification of specified and unspecified impurities of Phenazopyridine hydrochloride.
- Reliability and reproducibility of results is confirmed by performing analytical method validation as per ICH guideline.
- Force degradation study is carried out by exposing the API to stress conditions and found all degradants are separating from the main component and can be quantified accurately.
- In acidic degradation two degrading impurities generated are synthesized and identified.

KEYWORDS: Phenzopyridine; Synthesis; characterization; impurity; NMR; validation.

INTRODUCTION
The impurity profile of a drug substance is critical to its safety assessment and its manufacturing process. As per guidelines of Center for drug evaluation and research (CDER), the impurities that exceed 0.10% in a drug must be identified prior to clinical trials.[1] Because the impurities are usually process related, they are most probably structurally similar to the synthesized target drugs. High performance liquid chromatography in combination with multistage mass spectrometry (HPLC/MS) is extremely useful for its capability to afford both molecular masses and structural information. Phenazopyridine hydrochloride (PPH) has been used for long time in conjunction with antibacterial agents for the treatment of urinary-tract infections.[2–4] It exerts an analgesic effect on the mucosa of the urinary tract and is used to provide symptomatic relief of pain in conditions such as cystitis and urethritis.[5–7] It is absorbed from the gastrointestinal tract and is excreted mainly from the urine.[8] During its production in large scale, an unknown impurity was detected by HPLC in all batches of the drug substance. In literature 2, 6-diamino-3-phenyl-5-Phenazopyridine impurity synthesis and characterization is studied.[9]

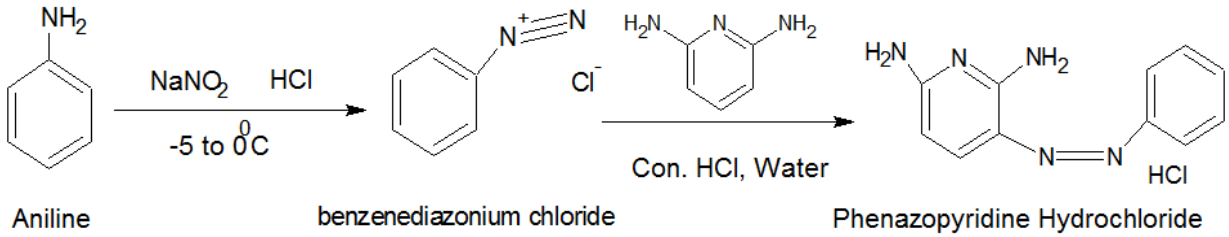
The present manuscript describes a comprehensive investigation on isolation and characterization of a major process related degradant impurities of Phenazopyridine hydrochloride6-Amino-2-hydroxy-3-phenyldiazenylpyridine and 2,6-Dihydroxy-3-phenyl diazenylpyridine. The related substances method is developed using HPLC followed by characterization using LC-MS, 1H NMR and IR spectroscopy techniques. The possible mechanism of its formation was proposed. After structure elucidation the qualified impurity standards were used for analytical method development. The developed analytical method is validated as per International conference on harmonization guidelines

(ICH Q2-R1).[10]

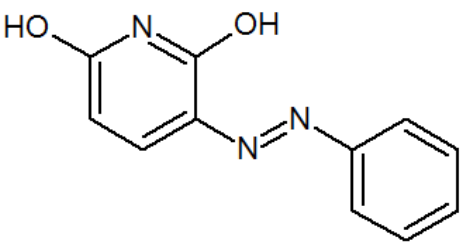
MATERIALS AND METHODS

1.0 Manufacturing process

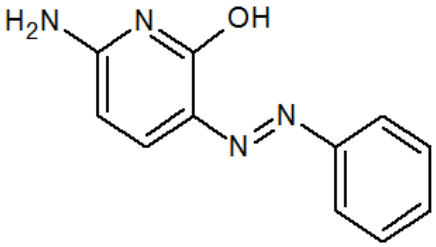
The chronology of development of the commercial process for Phenazopyridine began with the discovery by the reaction of aniline with Sodium nitrite to get benzene diazonium chloride intermediate. Further 2, 6-diaminopyridine Diazo coupled with benzene diazonium chloride to get phenazopyridine hydrochloride as shown in reaction scheme 1. Initially it was reported that the diazo coupling results in the formation of compound as the major product along with unknown impurities i.e. Impurities at RRT 0.70 (majorly) and RRT 0.56



Scheme 1

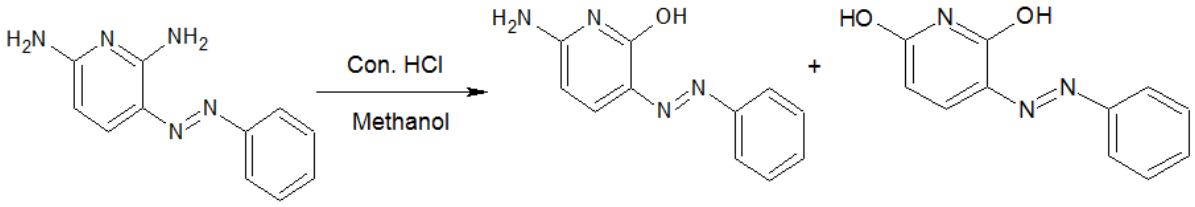


0.56 RRT Imp



0.70 RRT Imp

1.1 Synthesis and characterization



Phenazopyridine **0.70 RRT Imp** **0.56 RRT Imp**

Scheme 2

Charge phenazopyridine (20 gm) in water 350 mL and Con. HCl. 250 ml. Heat the reaction mass to 90-95°C and stir till complete formation of desired impurities (~5hr). The reaction was monitored by TLC. Cool the reaction mass to 20-25°c & filter the solid & wash with water 20 ml. Charge above wet sample in to flask, charge 10 vol. Methanol 200ml & stire the reaction mass at 40-45°c for one hour. Cool the reaction mass at 20-25° & maintain the one hour at same temperature Filter the reaction mass & wash with Methanol (20 ml). Unload the wet material & dry in tray dryer at 60-65°C. The impurities were further purified by column chromatography (Ethyl acetate: Methanol: TEA).

The purified impurity was characterized by IR, mass, NMR and used to develop related substances method by HPLC. The method is developed and made suitable for specified impurities 2,6-diamino-5-phenyl-3-Phenazopyridine, 3,5–Bisphenylazo-2,6-diaminopyridine, 6-Amino-2-hydroxy-3-phenyldiazenylpyridine and potential degradants.

2, 6-Diamino pyridine is a key starting material of Phenazopyridine hydrochloride which is extremely polar impurity. It is not retaining in the column and eluting in the void volume so the separate method is developed for quantification of 2, 6-Diamino pyridine impurity.

2.0 Related substances by HPLC: (Method-I)

Shimadzu HPLC system LC-2010 CHT with UV detector with LC solutions software or its equivalent was used. The analysis was carried out on Cosmosil C-18 column 250 mm x 4.6 mm, 5.0 µm with Guard cartridge: Make; Phenomenex C18, 4.0 mm x 3.0 mm ID. Separation was achieved with the mixture of mobile phase-A and mobile phase-B in gradient elution with timed programme T_{min}/A:B: T_0/70:30; T_{40}/20:80; T_{50}/20:80; T_{51}/70:30; T_{60}/70:30. The flow rate was 1.0 mL/min and sample injection volume was 20 µL.

Detector wavelength is 240 nm and column temperature 30^0C.

2.1 Buffer preparation: Weigh accurately and transfer 1.36g Potassium dihydrogen orthophosphate in 1000 mL water dissolve, add 1.0 mL Triethylamine and mix well and adjust to pH 5.8 with Orthophosphoric Acid filter through 0.45µ filter and degas.

Mobile Phase-A: Buffer: Acetonitrile (980: 20) v/v
Mobile Phase-B: Acetonitrile
Diluent: Methanol: Acetonitrile (80:20) v/v

2.2 Solution preparation
Standard stock solution: Weigh and transfer accurately about 10 mg of 2,6-diamino-5-phenyl- 3-Phenazopyridine reference standard, 10 mg of 3,5 –Bis phenylazo-2-6 Diaminopyridine reference standard, 5 mg of Phenazopyridine HCl reference standard and 10 mg of 6-Amino-2-hydroxy-3-phenyldiazenylpyridine into 100 mL volumetric flask, add diluent sonicate to dissolve and dilute up to mark with diluent.

Standard solution-A: Further transfer 5.0 mL of Standard stock solution in to 50 mL volumetric flask and dilute up to mark with diluent.

Standard Solution: Further transfer 10.0 mL of standard solution-A in to 100 mL volumetric flask and dilute up to mark with diluent.

System suitability solution: Weigh accurately and transfer 25.0 mg of Phenazopyridine hydrochloride reference standard into 50 mL volumetric flask, add Standard solution, sonicate to dissolve dilute up to the mark with standard solution.

Sample solution: Weigh and transfer accurately about 25 mg of sample in to 50mL volumetric flask, add diluent, sonicate to dissolve and make up to the mark with diluent

2.3 Procedure: Inject Blank (diluent) in duplicate, System suitability solution, standard solution six times, blank (diluent) and test solution two preparations. Phenazopyridine peak is eluting at retention time of about 16 minutes. The relative retention times of all components are as follows (for information purpose).

Name of component	Relative retention time (RRT)
Phenazopyridine	1.00
6-Amino-2-hydroxy-3-phenyldiazenylpyridine.	0.70
2,6-diamino-5-phenyl-3-Phenazopyridine	1.91
3,5 –Bis phenylazo-2-6 Diaminopyridine	2.45

2.4 System suitability Criteria: The tailing factor for the phenazopyridine peak in system suitability solution should be not more than 2.0% RSD for Area of each component in six replicate injections of standard solution should not be more than 5.0%.

2.5 Calculations: Disregard the peaks due to blank and peaks due to 2,6 diamino pyridine at retention time 2.7 and 3.01 and calculate known impurities and unknown impurities by following formula

% of 2,6-Diamino-5-phenyl-3-phenazopyridine

$$= \frac{\text{Area of impurity in sample X impurity Std Wt X5 X 10 X50 X Potency of Impurity Standard}}{\text{Avg. impurity area in Standard X 100 X 50 X100 X Sample Weight}}$$

% of 3,5-Bis Phenylazo-2-6 Diaminopyridne

$$= \frac{\text{Area of impurity in sample X impurity Std Wt X5 X 10 X50 X Potency of Impurity Standard}}{\text{Avg. impurity area in Standard X 100 X 50 X100 X Sample Weight}}$$

% of 6-Amino-2-hydroxy-3-phenyldiazenylpyridine

$$= \frac{\text{Area of impurity in sample X impurity Std Wt X5 X 10 X50 X Potency of Impurity Standard}}{\text{Avg. impurity area in Standard X 100 X 50 X100 X Sample Weight}}$$

% of any other individual impurity

$$= \frac{\text{Area of impurity in sample X PPH Std Wt X5 X 10 X50 X Potency of PPH Standard}}{\text{Avg. PPH area in Standard X 100 X 50 X100 X Sample Weight}}$$

% of Total unknown impurities

$$= \frac{\text{Sum of areas of total unknown impurities in sample X Std Wt X5 X 10 X50 X Potency of PPH Standard}}{\text{Avg. PPH area in Standard X 100 X 50 X100 X Sample Weight}}$$

Total impurities=Total known impurities (Method-1+Method-2)+Total unknown impurities

3.0 Related substances by HPLC: (Method-II)
2,6 Diaminopyridine Content: Shimadzu HPLC system LC-2010 CHT with UV detector with LC solutions software or its equivalent was used. The analysis was carried out on Cosmosil C-18 column 250 mm x 4.6 mm, 5.0 µm with Guard cartridge: Make; Phenomenex C18, 4.0 mm x 3.0 mm ID. Separation was achieved with the mixture of mobile phase-A and mobile phase-B in gradient elution with timed programme T_{min}/A:B: T_0/95:05; T_5/95:05; T_{15}/20:80; T_{25}/20:80; T_{26}/95:05; T_{40}/95:05.The flow rate was 1.0 ml/min and sample injection volume was 10 µL. Detector wavelength is 240 nm and column temperature 30^0C.

3.1 Buffer preparation: Weigh accurately and transfer 1.36g Potassium dihydrogen orthophosphate in 1000 mL water dissolve, add 1.0 mL Triethylamine and mix well and adjust to pH 5.8 with Orthophosphoric Acid filter through 0.45µ filter and degas.

Mobile Phase-A: Buffer: Acetonitrile (980: 20) v/v
Mobile Phase-B: Acetonitrile
Diluent: Water: Acetonitrile (90:10) v/v

3.2 Solution preparation
Standard stock solution: Weigh and transfer accurately about 5.0 mg of 2,6-Diaminopyridine reference standard into 50 mL volumetric flask, add diluent sonicate to dissolve and dilute up to mark with diluent.

Standard Solution: Further transfer 1.0mL of Standard stock solution into 100-mL volumetric flask and dilute up to mark with diluent.

Sample solution: Weigh and transfer accurately about 25 mg of sample in to 50mL volumetric flask, add diluent, sonicate to dissolve and make up to the mark with diluent.

3.3 Procedure: Inject Blank (diluent) in duplicate, standard solution six times, blank (diluent) and test solution two preparations. Phenazopyridine peak is eluting at retention time of about 16.0 minutes. The relative retention times of all components are as follows.

Name of component	Relative retention time (RRT)
2,6-Diaminopyridine	0.27
Phenazopyridine	1.00

3.4 System suitability Criteria: %RSD for Area of 2, 6-Diamino pyridine in six replicate injections of standard solutions should not be more than 5.0%.

3.5 Calculations: Integrate peak due to 2, 6-Diminopyridine and Phenazopyridine only.
Calculate 2, 6-Diaminopyridine by following formula.

% of 2,6-Diamino pyridine

$$= \frac{\text{Area of 2,6-DAP in sample X Std Wt X1 X 50 X P}}{\text{Avg.peak area of Standard X 50 X 100 X Sample Weight}}$$

Where,
P=Potency of 2,6Diamino pyridine standard.

4.0 ANALYTICAL METHOD VALIDATION
Method validation is closely related to method development. When a new method is being developed, some parameters are already being evaluated during the "development stage," while in fact, this forms part of the "validation stage." Related substances method-I and method-II both methods are validated as per ICH guideline.[9]

4.1 Specificity and force degradation
The ability of the method to determine accurately and specifically the analyte of interest in the presence of other components in a sample matrix that may be expected to be present in the sample matrix under the stated conditions.
Specificity of the method was evidenced by comparing blank, phenzaopyridine and all specified impurities separate injections as well as spiking all impurities into Phenazopyridine hydrochloride test solution.

Force degradation study is performed by exposing the sample to heat at 105^0C for 24 hours, sample treated with base 1 N sodium hydroxide for 24 hour and with acid 1N hydrochloric acid for 6 hours. Sample was exposed to 15% hydrogen peroxide solution and humidity at 75% relative humidity. Photo stability study was carried out by exposing the sample to 1.2 million lux hours of white Fluorescent light and 200 watts hour/square meter of UV light in photo stability chamber. After exposure samples were tested using the proposed related substances method with photo diode array detector.

4.2 Solution stability
Drug stability in Active Pharmaceutical Ingredient is a function of storage conditions and chemical properties of the drug and its impurities. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. Stability data are required to show that the concentration and purity of analyte in the sample at the time of analysis corresponds to the concentration and purity of analyte at the time of sampling.

The solution stability till fifteen hours of Phenazopyridine hydrochloride API had been checked by injecting test solution and spiked solution. Test solution was prepared fresh before injection and immediately injected and same solution was injected after fifteen hours.

4.3 Linearity
The ability of the method to obtain test results proportional to the concentration of the analyte within a given range. It was evaluated by linear regression

analysis, which was calculated by the least square regression method.

4.4 Limit of detection

The limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected but not necessary quantified. The obtained LOD values of specified impurities and API is discussed.

$LOD = 3.3 \times \sigma / S$

Where, σ = the standard deviation of the response and S= slope of the calibration curve

4.5 Limit of quantitation

The limit of quantitation is the lowest concentration or amount of analyte that can be determined quantitatively within an acceptable level of repeatability precision and trueness.

Limit of quantitation (LOQ) = $10.0 \times \times \sigma / S$.

Where, σ = the standard deviation of the response and S= slope of the calibration curve

Precision at LOQ is confirmed by six replicate analyses of impurities at LOQ level.

4.6 Accuracy: Accuracy can be defined as the closeness of agreement between a test result and the accepted reference value. Accuracy of the method was determined by recovery study.

Analytical method may be considered validated in terms of accuracy if the mean value is within ± 20% of the actual value. During recovery study Phenazopyridine hydrochloride API batch was analyzed and then all specified impurities of known concentration is spiked in the API at LOQ level, 50%, 100% and 150% with respect to the limit of specified impurity.

4.7 Ruggedness

The (intra-laboratory tested) behavior of an analytical process when small changes in environment and/or operating condition are made.

The ruggedness of the method was evaluated by estimating % RSD of spiked solution tested by two different analysts using different HPLC instrument and columns on different days. Six spiked solutions were prepared by each analyst separately. % RSD of each impurity of twelve preparations of both analysts should not be more than 10%.

4.8 Robustness

Robustness is a measure of the capacity of the analytical procedure to remain unaffected by small but deliberate variations in method–performance parameters, which provides an indication of its reliability during normal usage.

Robustness of the method was determined by analyzing the system suitability solution and batch analysis with deliberate change in the parameters like (a) flow rate of mobile phase ± 0.1 ml/min, (b) column temperature ± 5°C, (c) mobile phase pH ±0.2 and (d) mobile phase ratio ±30% of acetonitrile.

5.0 Method validation of Related substances by HPLC Method-II for quantification of 2,6-Diaminopyridine is performed similar to method-I as per ICH guideline.

RESULTS AND DISCUSSION

During impurity profiling of Phenazopyridine hydrochloride drug substance by HPLC, an unknown impurity at RRT 0.70 and RRT 0.56 was observed about 11.9% and 2.9% respectively in acid degraded sample during force degradation study. Both impurities are synthesized and characterized and structure elucidation data is discussed below. The impurity at RRT 0.70 is consistently detected about 0.10% in commercial batches so this impurity is included in the related substances method for routine analysis.

H1 NMR in CDCL$_3$: (RRT 0.70)
6-Amino-2-hydroxy-3-phenyldiazenylpyridine (figure I)

5.93-5.96 (d,1H), 7.28-7.32 (t,1H), 7.41-7.44 (t,2H), 7.66-7.67 (d,2H), 7.93-7.95 (d, 1H) 11-12 (s,2H)

Mass peak at m/e 215.3 (M+1) (calculated for $C_{11}H_{10}N_4O$)

H1 NMR in CDCL$_3$: (RRT 0.56)
2,6-Dihydroxy-3-phenyldiazenylpyridine (figure II)

6.21-6.23 (d,1H), 7.23-7.24 (t,1H), 7.41-7.44 (d,5H), 7.66-7.67 (d,2H), 8.91 (s, 1H) 14.26 (s,1H)

Mass peak at m/e 216.1 (M+1) (calculated for C11H9N3O2).

From the experimental data of method validation following are observations.

Specificity: There are no interfering peaks at the retention times of phenazopyridine and specified impurities are observed from the chromatogram. All specified impurities are well resolved without any interference is observed from the spiked chromatogram. Peak purity of all peaks are checked with photo diode array detector (PDA). It is found that purity angle is less than purity threshold which proves that all peaks are pure without any interference. Refer Table-I and Table-II for retention time (RT) and peak purity which is found passing. Refer figure III, IV and V of Blank, system suitability solution and test solution chromatograms.

Table I- Specificity of individual and spiked solution

Name of the compound	RT Obtained in individual solutions	RT Obtained in Spiked solution	
		Retention time	RRT
Phenazopyridine	14.7	14.8	1.00
2,6 Diaminopyridine	3.0	3.0	0.21
6-Amino-2-hydroxy-3-phenyldiazenylpyridine	10.1	10.2	0.69
2,6 Diamino-5-phenyl-3-phenazopyridine	29.1	29.3	1.98
3,5-Bis phenylazo-2-6-diaminopyridine	38.0	38.1	2.58

Table II- Peak purity information (For spiked solution)

Name of the compound	Purity angle	Purity Threshold	Peak purity
Phenazopyridine	3.858	5.025	Pass
6-Amino-2-hydroxy-3-phenyldiazenylpyridine	0.610	0.663	Pass
2,6 Diamino-5-phenyl-3-phenazopyridine	0.513	0.778	Pass
3,5-Bis phenylazo-2-6-diaminopyridine	0.471	0.864	Pass

Force degradation

It was observed that Phenazopyridine hydrochloride is stable when exposed to heat, UV light, base degradation and humidity. Impurity profile is matching with freshly injected test solution and mass balance is also found within 98.0% to 102.0%. In oxidized sample 4 to 5 impurities are observed but no major degradant had been formed. In acid degradation two degradants were observed at relative retention time (RRT) 0.56 is about 2.9% and RRT 0.70 is about 11.9% (refer figure VI). Both impurities were synthesized and characterized with mass, NMR and IR. Impurity of RRT 0.56 is 2,6-Dihydroxy-3-phenyldiazenylpyridine and impurity at RRT 0.70 is 6-Amino-2-hydroxy-3-phenyldiazenylpyridine. The impurity at RRT 0.70 i.e. 6-Amino-2-hydroxy-3-phenyldiazenylpyridine is a major degradant and observed consistently in all commercial batches so this impurity had been included in the proposed related substances method. All degradant impurities are well resolved and peak purity of all peaks is passing.

Solution stability

The solution stability till fifteen hours of Phenazopyridine hydrochloride API had been checked by injecting test solution and spiked solution. Phenazopyridine as well as all specified impurities was not significantly changed after fifteen hours. So it can be concluded that API is stable in solution state more than fifteen hours in given diluent.

LOD and LOQ

Limit of detection and limit quantification of 6-Amino-2-hydroxy-3-phenyldiazenylpyridine, Phenazopyridine hydrochloride, 2,6-diamino-5-phenyl-3-Phenazopyridine and 3,5 –Bis phenylazo-2-6 Diaminopyridine are determined by slope method and tabulated in table-III.

Table III- LOD and LOQ Values

Sr. No	Name of the compound	LOD in %	LOQ in %	LOQ precision (%RSD of six replicate)
1	6-Amino impurity	0.007	0.021	0.2
2	Phenazopyridine	0.007	0.021	0.7
3	5-Phenyl impurity	0.006	0.017	0.2
4	3,5-Bis impurity	0.005	0.014	0.2

Linearity: Under the experimental conditions, the peak area vs. concentration plot for the proposed method was found to be linear over the range of LOQ, 50% to 150% of the specified limit with a regression coefficient as tabulated in Table-IV. The regression coefficient (r^2) is > 0.998 is generally considered as evidence of accept-able fit of the data to the regression line.

Table IV- Linearity

Sr. No	Name of the compound	Regression coefficient (R^2)
1	6-Amino impurity	0.9990
2	Phenazopyridine	0.9997
3	5-Phenyl impurity	0.9997
4	3,5-Bis impurity	0.9990

Accuracy: Analytical method may be considered validated in terms of accuracy if the mean value is within ± 20% of the actual value. Recovery of specified impurities was found in the range of 80.0% to 120.0%, which was well within the acceptance criteria. During recovery study Phenazopyridine hydrochloride API batch was analyzed and then all specified impurities of known concentration is spiked in the API at LOQ level, 50%, 100% and 150% with respect to the limit of specified impurity. Refer Table-V.

Table V- Recovery study

Sr. No	Name of the compound	LOQ level	50% level	100% level	150% level
1	6-Amino impurity	111.2%	100.7%	99.2%	101.6%
2	5-Phenyl impurity	107.0%	114.1%	108.9%	109.0%
3	3,5-Bis impurity	86.7%	101.0%	101.5%	105.1%

Ruggedness study: Experiment was performed by two different analysts using different HPLC instrument and columns on different days. Six spiked solutions were prepared by each analyst separately. % RSD of each impurity of twelve preparations of both analysts was observed less than 10%. Refer Table-VI.

Table VI- Overall RSD for method precision and intermediate precision

Results	6-Amino impurity (% w/w)	5-Phenyl impurity (% w/w)	3,5-Bis impurity (% w/w)
Method precision-1	0.19	0.21	0.18
Method precision-2	0.19	0.21	0.18
Method precision-3	0.19	0.20	0.18
Method precision-4	0.19	0.21	0.18
Method precision-5	0.19	0.20	0.18
Method precision-6	0.19	0.20	0.18
Intermediate precision-1	0.20	0.21	0.19
Intermediate precision-2	0.20	0.21	0.19
Intermediate precision-3	0.20	0.21	0.19
Intermediate precision-4	0.20	0.21	0.19
Intermediate precision-5	0.20	0.21	0.19
Intermediate precision-6	0.20	0.21	0.19
Mean	0.20	0.21	0.19
stdev	0.006	0.003	0.005
%RSD	2.9	1.4	2.6

Robustness: Robustness of the method was determined by analyzing the system suitability solution and batch analysis with deliberate change in the parameters of flow rate, column temperature, mobile phase pH and mobile phase ratio of acetonitrile. Results are discussed in table VII. RSD of all impurities are found within limit below 10.0%.

Table VII-Robustness study with deliberate changes in method parameters

FLOW (0.9 mL/min) And (1.0 mL/min)	%RSD for Results of spiked sample in actual condition and changed condition is NMT:10%	**High flow**	**Low flow**
		Complies	complies
pH (5.6 and 6.0)	% RSD for Results of spiked sample in actual condition and changed condition is NMT:10%	**High pH**	**Low pH**
		Complies	complies
TEMP (25°c and 35°C)	% RSD for Results of spiked sample in actual condition and changed condition is NMT:10%	**35°C**	**25°C**
		Complies	complies
Solvent ratio (980:20) (Buffer :ACN) v/v	%RSD for Results of spiked sample in actual condition and changed condition is NMT:10%	**-30% CAN**	**+30% ACN**
		Complies	Complies

Validation of Related substances by HPLC Method-II
Method validation of Related substances by HPLC Method-II for quantification of 2,6-Diaminopyridine is performed as per ICH guideline. The method satisfies all validation parameters and results are well within limit which proves that the method is robust, rugged and delivers accurate and reliable results. Refer system suitability chromatogram figure-VII and results are summarized in table VIII.

Table VIII-Summary of validation results of related substances method II

S.NO	PARAMETER	ACCEPTANCE CRITERIA	RESULTS			
1	SPECIFICITY	All known and unknown peaks should be well separate from 2,6-Diaminopyridine peak	Complies. Method is selective. 2,6-Diaminopyridine peak is pure			
2	SOLUTION TABILITY	Report Result	Solutions are stable up to 24hrs			
3	LOD&LOQ	Report Result	Component name	LOD in %	LOQ in ppm	
			2,6-DAP	0.005	0.015	
4	LOQ PRECISION	%RSD for six replicates of LOQ level standard solutions is NMT:10.0%	2.1			
5	LINEARITY	Regression coefficient: NLT 0.99	R^2 =0.9999			
6	ACCURACY	80% to 120% for LOQ to 150%accuracy	LOQ	50%	100%	150%
			100.8	101.0	98.4	100.7
7	METHOD PRECISION	%RSD for results of six replicates of spiked samples is NMT:10%	%RSD=0.8			
8	INTERMEDIATE PRECISION	1.%RSD for results of six replicates of spiked samples is NMT:10% 2. %RSD for results of twelve spiked samples(Method precision and Intermediate precision) is NMT:10%	1) %RSD= 0.9 2) %RSD=1.0			
9	ROBUSTNESS					
9.1	FLOW (0.9 mL/min) And (1.0 mL/min)	%RSD for Results of spiked sample in actual condition and changed condition is NMT:10%	**High flow**		**Low flow**	
			1.1		5.2	
9.2	pH (5.6 and 6.0)	% RSD for Results of spiked sample in actual condition and changed condition is NMT:10%	**High pH**		**Low pH**	
			1.5		0.4	
9.3	TEMP (25°c and 35°C)	% RSD for Results of spiked sample in actual condition and changed condition is NMT:10%	**35°C**		**25°C**	
			4.8		1.8	
9.4	Solvent ratio (980:20) (Buffer : ACN) v/v	%RSD for Results of spiked sample in actual condition and changed condition is NMT:10%	**-30% ACN**		**+30% CAN**	
			6.4		6.4	

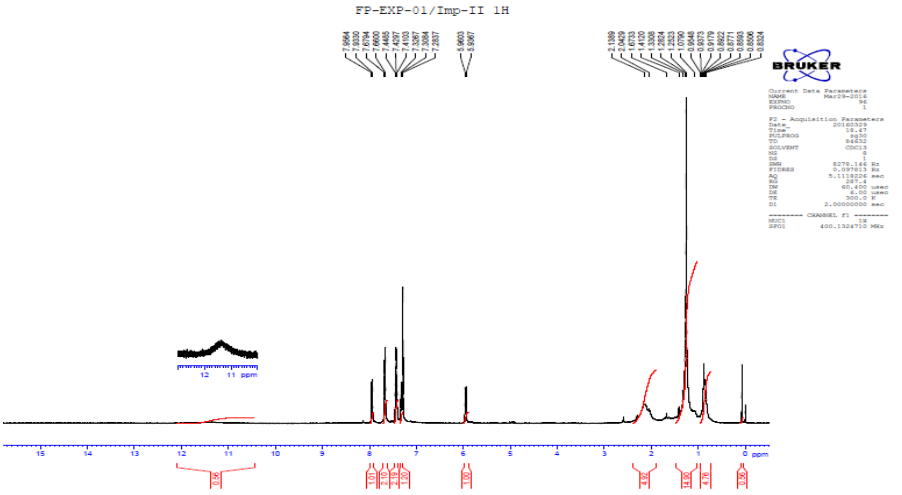


Figure I-NMR of (RRT 0.70) 6-Amino-2-hydroxy-3-phenyldiazenylpyridine

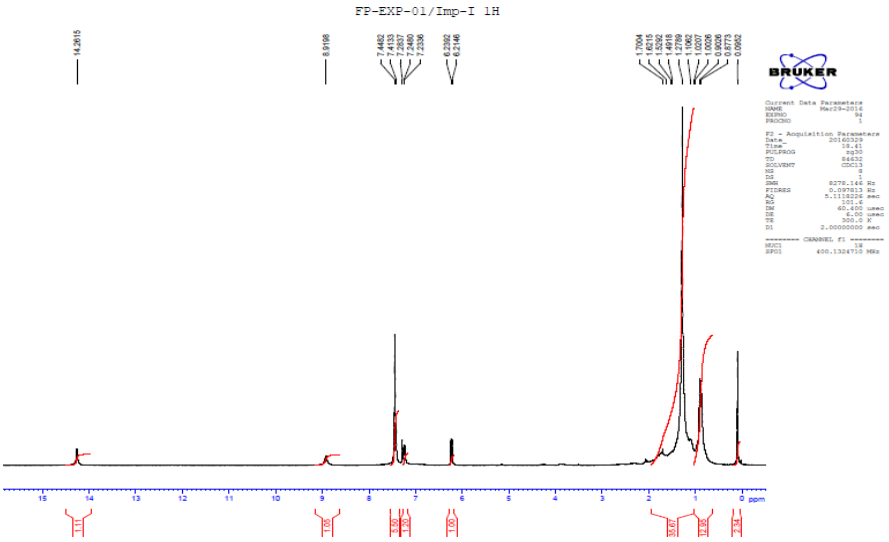


Figure II-NMR of (RRT 0.56) 2,6-Dihydroxy-3-phenyldiazenylpyridine

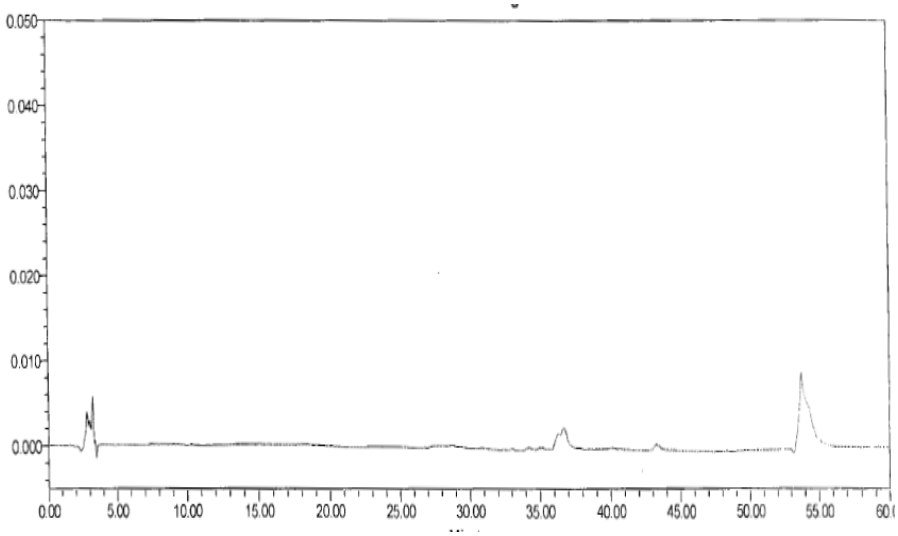


Figure III-Blank chromatogram of related substances method I

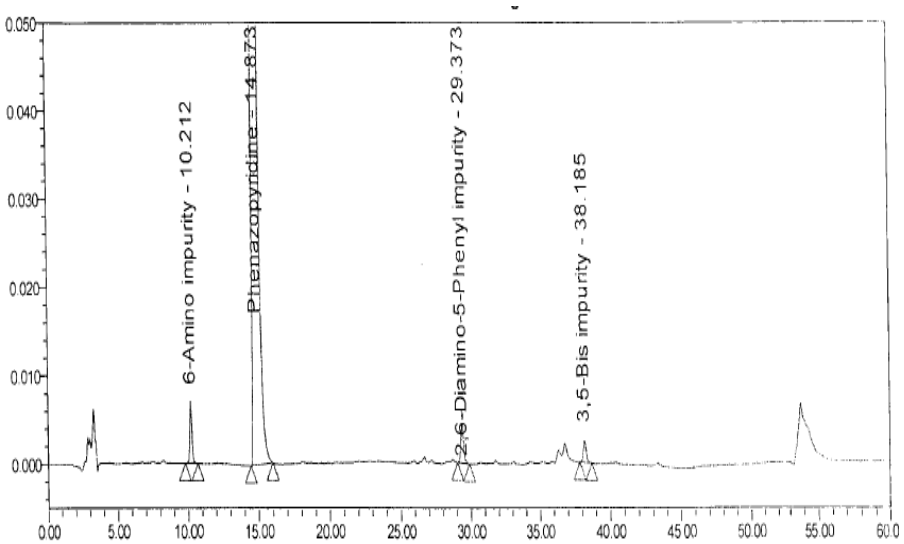


Figure IV-System suitability chromatogram of related substances method I where RT 10.2 min is 6-Amino-2-hydroxy-3-phenyldiazenylpyridine, 14.8 min is Phenazopyridine, 29.3 min is 2,6 Diamino-5-phenyl-3-phenazopyridine and 38.1 min is 3,5-Bis phenylazo-2-6-diaminopyridine

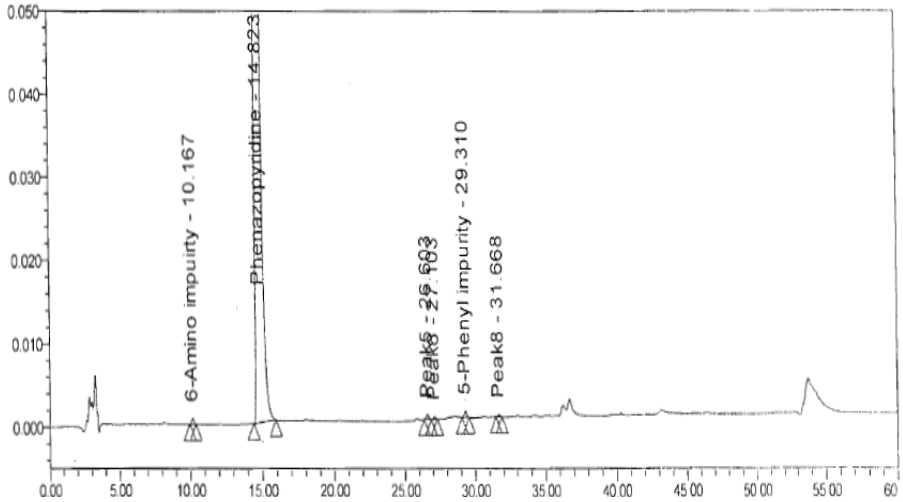


Figure V-Test solution chromatogram of related substances method I

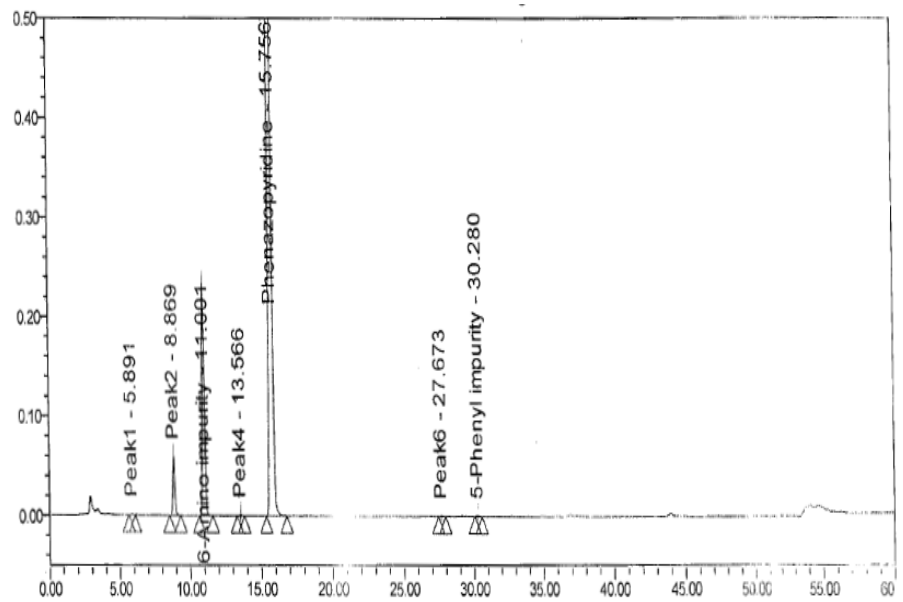


Figure VI-Acid degraded Phenazopyridine hydrochloride sample of force degradation study of related substances method I where degradant observed at RRT 0.56 and 0.70

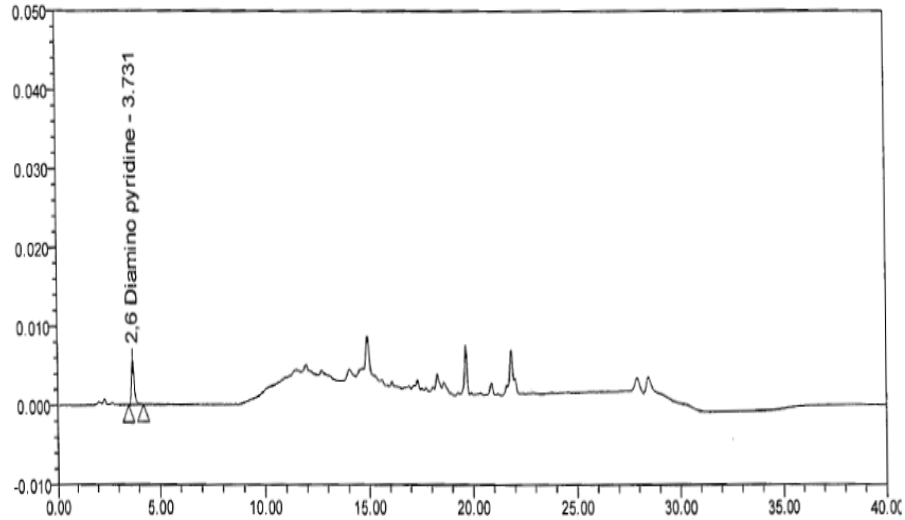


Figure VII-System suitability chromatogram of related substances method II where RT 3.7 min is 2,6-Diaminopyridine

CONCLUSION

In conclusion, a process related impurities of Phenazopyridine hydrochloride, produced according to the synthetic route given in scheme 1, was identified, synthesized and characterized. Structural elucidations of all synthesized compounds were done by using NMR, IR and MS spectral data. Impurity of RRT 0.56 is 2,6-Dihydroxy-3-phenyldiazenylpyridine and impurity at RRT 0.70 is 6-Amino-2-hydroxy-3-phenyldiazenylpyridine. The impurity 6-Amino-2-hydroxy-3-phenyldiazenylpyridine is a major degradant and observed consistently in all commercial batches so this impurity had been included in the proposed related substances method. Thus, the regulatory requirement was fulfilled by characterizing this impurity and the prepared impurity standard was used during analytical method validation studies. The above RP-HPLC analytical methods satisfies all validation parameters like system suitability, precision, specificity, accuracy, linearity of detector response, ruggedness (Analyst to analyst, system to system and column to column variability, solution stability) and robustness (variation in flow rate, column temperature variation, pH, mobile phase concentration). At the same time the method satisfies the forced degradation study. It indicates that the method is stable and suitable for the Phenazopyridine hydrochloride and its related substances determination. Hence, the validated method can be used for routine analysis of related substances in Phenazopyridine hydrochloride in quality control laboratories in the pharmaceutical industry.

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