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# A simple and sensitive RP-HPLC method for trace-level quantification of 2-Amino pyridine in antifungal drug products

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**Abstract**

A highly sensitive simple analytical method for quantification of trace level impurity of 2-Aminopyridine in the antifungal pharmaceutical product has been developed. 2-Amino pyridine is toxic impurity causes headache, dizziness, heaviness and convulsion. Continuous exposure to this impurity leads to death. The analysis was accomplished on an Inertsil poroshell C18 column (50mm x 4.6mm, 3μ) using acetate buffer and methanol as the organic solvent. The flow rate was set at 1.0 ml/minute. The method was validated for the analytical parameters such as system suitability, specificity, linearity and range, accuracy, limit of detection (LOD), limit of quantification (LOQ), precision, and solution stability. The limit of detection and limit of quantification were found to be 0.06μg/ml and 0.2μg/ml respectively with respect to Drug Product sample concentration (140mg/ml).

**Keyword:** 2-Aminopyridine, antifungal, Ciclopirox, convulsion, impurity.

**Introduction**: Ciclopirox is a synthetic, broad-spectrum antifungal[1] agent with additional antibacterial[2] and anti-inflammatory[3] activities. It exerts action by binding to and chelating trivalent cations, such as $Fe^{3+}$ and $Al^{3+}$, thereby inhibiting the availability of essential co-factors for enzymes[4]. This may lead to loss of activity of enzymes that are essential for cellular metabolism, organization of cell wall structure and other crucial cell functions[5]. 2-Amino pyridine is primarily used in the synthesis of various drugs[6].2-Aminopyridine is also useful precursor for the synthesis of a variety of heterocyclic compounds possessing medicinal value[7]. Ciclopirox ethanolamine can be prepared by reaction of 6-cyclohexyl-4-methyl-2-pyron (A) with hydroxylamine hydrochloride in hot 2-Aminopyridine to get intermediate B. The intermediate B is reacted with ethanolamine to get Ciclopirox ethanolamine[8].


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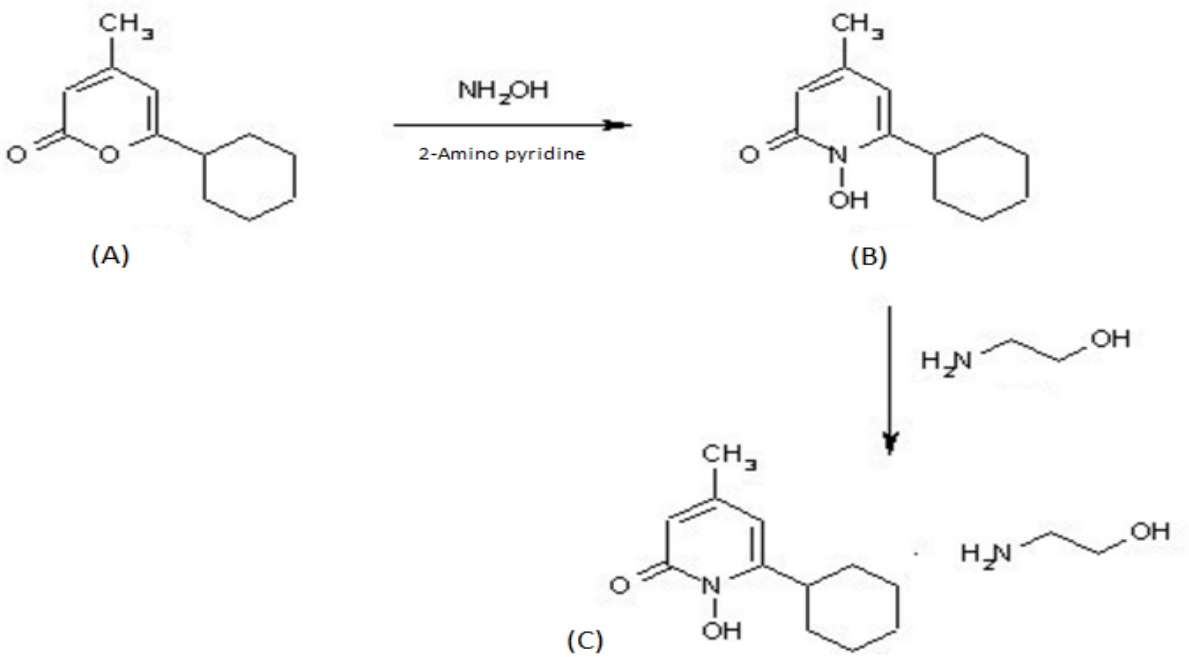


Aminopyridines are widely used starting materials in the production of pharmaceutical compounds and may potentially be present as toxic impurities[9] at trace levels in drug and drug products.[10]2-Aminopyridine is anticonvulsant in humans and is moderately irritating to the skin and eye[11]. The lowest concentration and duration of exposure reported to be toxic in human is 0.5 ppm and 8 hr respectively. Ingestion of as little as 60 mg of 2-Aminopyridine in adult human has resulted in severe poisoning, which include such signs and symptoms as profuse sweating, burning of throat, weakness, nausea, psychotic like behavior, tremor, dyspnea and convulsion. Daily exposure of 2-Aminopyridine causes headache, difficulty in breathing, dizziness and convulsion[12].

## 2. Experimental

### 2.1 Chemical and reagent

Ciclopirox Olamine Liquid (1.5%w/w) manufactured by ZYG PHARMA PVT. LTD. and Fluocinolone Acetonide and Ciclopirox Olamine cream USP (1.0%w/w) manufactured by Glenmark pharmaceutical was procured from market. 2-Amino Pyridine was procured from Sigma Aldrich. While MeOH (spectroscopic grade) was obtained from Fisher-Scientific and Ammonium Acetate (A.R. grade) from Merck. Water (spectroscopic grade) was used as received from Rankem.


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**2.2 Instrumentation**

The analysis was performed on Agilent 1260 High Performance Liquid Chromatography[13] (HPLC) system with an auto sampler and binary solvent system interfaced to an Agilent DAD detector and Chemstation Software. The detection was carried out at 295 nm using an Inertsil Poroshell C18 column (50mm x 3.5mm, 2.7μ).

**2.3 Chromatographic condition**

The HPLC method was developed with mobile phase consisting of Solution A (0.02M Ammonium Acetate) and Solvent B (Methanol) as gradient mixture, which was pumped at a flow rate of 1.0 mL/min. The temperature of the column[14] was maintained at 25°C whilewavelength selected was 295 nm. The injection volume was 5 μl and Methanol (100%)was used as diluent.

Pump Gradient:-

| Time | (0.02M Ammonium Acetate) | Methanol (100%) |
|---|---|---|
| **0.01** | 90 | 10 |
| **4.0** | 90 | 10 |
| **6.0** | 20 | 80 |
| **12.0** | 20 | 80 |
| **12.1** | 90 | 10 |
| **15.0** | 90 | 10 |

**2.4 Preparation of standard and sample**

Standard preparation: Standard solution containing 1μg/mL of 2-Aminopyridine was prepared in diluent.

Sample preparation: Sample Solution was prepared by dissolving 140 mg of sample per mL of diluent.

**3. Results and discussion**

**3.1. Method development**

Selection of the HPLC column has played a critical role in achieving the separation of 2-Aminopyridine, placebo and unknown impurities. Method development was initiated by using water and Methanol (1:1 v/v) at a flow rate of 1.0 mL/min. The column used was prontosil C18, 150 mm in length having internal diameter 4.6 mm and 5μm particle sized Stationary phase[15]. The peak shapes of 2-amino pyridine were not appreciable and not retained on column. To improve peak shape various mobile phases and columns were tried. Finally acetate buffer[16] and poroshell RP-18 column was selected and gradient program applied.


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Typical chromatograms of standard 2-Aminopyridine, sample and sample with spiked impurity (2-Aminopyridine) are shown in (Fig. 4,Fig. 5 and Fig. 6) respectively. System suitability data is as shown in Table 1

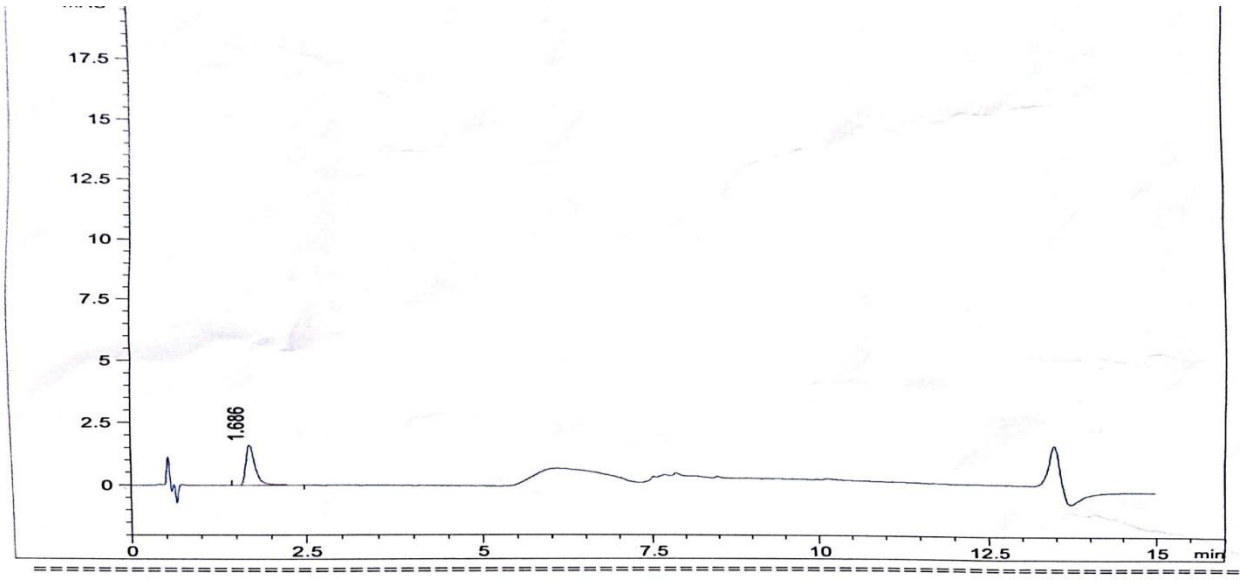


**Fig. 4** Standard chromatogram of 2-Amino Pyridine


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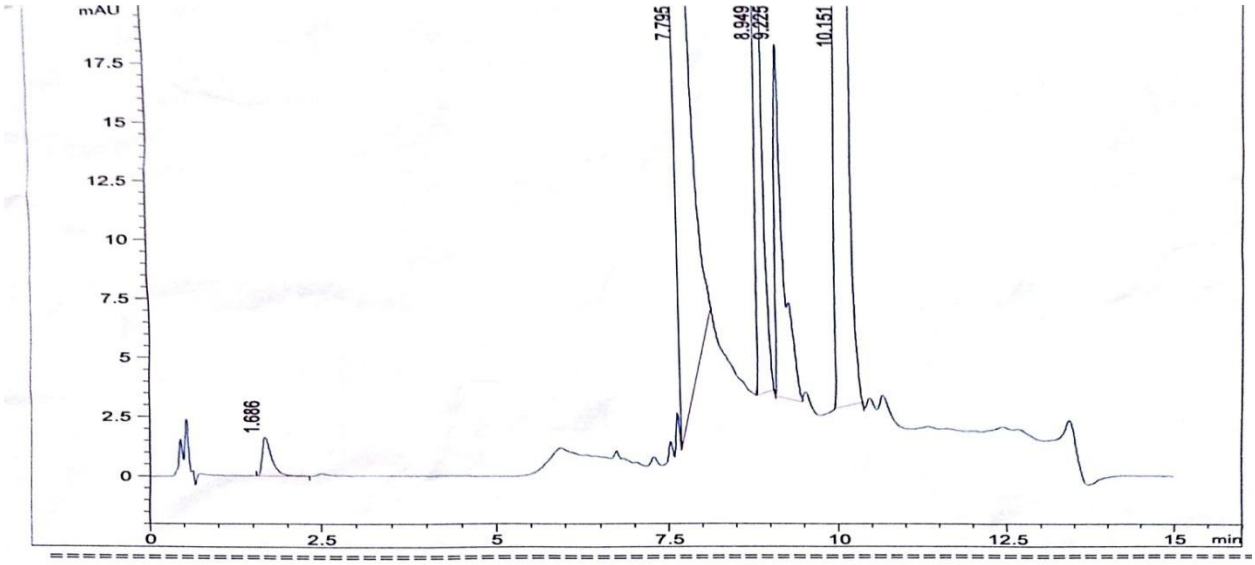


**Fig.5** Sample chromatogram.

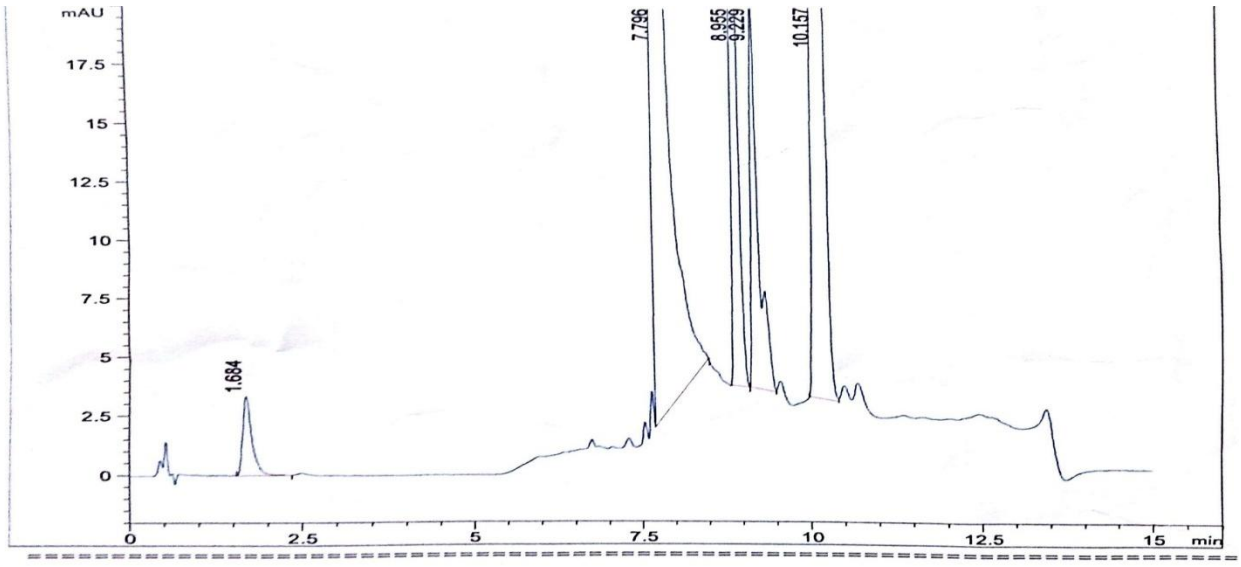


**Fig.6** Standard spike chromatogram of Sample


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**Table 1** System Suitability data

| Parameter | Limit | 2-Amino Pyridine |
|---|---|---|
| **Theoretical plate** | 2000 | 5890 |
| **Symmetry** | 0.8-2.0 | 1.3 |

### 3.2 Validation of method

#### 3.2.1. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of component which may be expected to be present. Typically these might include impurities, degradants, matrix etc.[17]The retention time of 2-Aminopyridine in the standard solution was compared with the ones in the sample solution. Moreover, the diluent was injected to see whether there was any interference at the retention time of 2-amino pyridine. It was checked that there was no interference at retention time 1.68 min in diluent.

#### 3.2.2. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.[17] Linearity of the method was checked by determining mean responses at different dilutions. Hence solutions ranging from of 0.2 ppm to 10.0 ppm were prepared and injected in triplicate. The mean response for 2-amino pyridine was plotted against Concentration. The Correlation Coefficient was found to be 0.999, which indicates good linearity (Table 2).

**Table 2** Linearity data

| Peak Name | Slope | Y intercept | Correlation Coefficient |
|---|---|---|---|
| **2-amino pyridine** | 22.15 | -0.16 | 0.999 |

#### 3.2.3. 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 value found.[18]Sample solution of 140 mg/ml was spiked with 2-amino pyridine at different concentrations i.e. 0.2µg/mL, 0.5µg/mL, 1.0µg/mL, and 1.5µg/mL. Each solution was injected in duplicate. The recovery percentage was calculated. Results of recovery are shown in Table 3. % Recovery for solutions of all concentration was found between 90 to 110.


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**Table 3** Accuracy results

| Amount added (µg/mL) | Amount obtained (µg/mL) | Recovery (%) |
|---|---|---|
| **0.211** | 0.203 | 95.81 |
| **0.529** | 0.516 | 97.41 |
| **1.059** | 1.075 | 101.47 |
| **1.589** | 1.567 | 98.61 |

### 3.2.4. Limit of detection (LOD)

The Detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.[17] A signal to noise (S/N) ratio between 3 to 10 is generally considered to be acceptable for estimating the detection limit. The S/N ratio of the individual peak was determined at different concentrations to estimate LOD. Based on S/N ratio data, estimated LOD is 0.06µg/mL. The results are shown in the Table 4.

### 3.2.5. Limit of quantitation (LOQ)

The quantitation or limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.[17] S/N ratio of more than 10 is generally considered to be acceptable for estimating the limit of quantification, however the S/N ratio obtained in present study was 25. The LOQ calculated is 0.2µg/ml. The results are listed Table 4.

**Table 4:** LOD and LOQ data

| Parameters | Concentration(µg/mL) | S/N ratio |
|---|---|---|
| **LOD** | 0.06 | 8 |
| **LOQ** | 0.20 | 25 |

### 3.2.6. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurement obtained from multiple sampling of the same homogeneous sample under the prescribed condition.[17] The system for 2-Aminopyridine impurity was checked for repeatability.[18] In order to determine System precision, the sample was prepared by spiking sample with the 2-Aminopyridine (0.0007%) and injected six times. The %RSD (Relative Standard Deviation) so found out was less than 5.0% indicating good system precision.

To determine the Method Precision, six independent solutions of sample were prepared by spiking with the impurity as mentioned for system precision. Each solution was injected once. The variations in the result are expressed in terms of % RSD. The values calculated are found to be below 5.0%, indicating satisfactory Method Precision.


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### 3.2.7. Solution stability

A solution of sample containing impurity was prepared and stored at ambient temperature. This solution was injected at intervals of 0, 4, 8 and 12 hr. Areas of 2-Aminopyridine were nearly identical to that obtained at 0 h and absence of additional peaks indicate good solution stability[19].

## 4. Conclusion

This study describes a trace-level method for determination of 2-Aminopyridine which is a potential toxic impurity in drug products. The analytical method described in present study is cost effective, simple, accurate, linear and precise convenient quality control tool for determination of this impurity in Drug products. The advantage of this method lies in its improved sensitivity, shorter run-time and simple sample preparation technique. The method is validated as per requirements of ICH guidelines.

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