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Short communication

Isolation and characterization of a potential process related impurity of phenazopyridine HCl by preparative HPLC followed by MS–MS and 2D-NMR spectroscopy

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ABSTRACT

During the process development of phenazopyridine HCl bulk drug, a potential impurity was detected in the routine impurity profiles by HPLC. Using MS–MS and multidimensional NMR techniques, the trace level impurity was unambiguously identified to be 3-phenyl-5-phenylazo-pyridine-2,6-diamine after its isolation from phenazopyridine HCl by semi-preparative HPLC. The formation of the impurity was discussed. To our knowledge, it is a novel impurity not reported elsewhere.

1. Introduction

The impurity profile of a drug substance is critical to its safety assessment and its manufacturing process. As per the guidelines of United States Pharmacopeia, the impurities that exceed 0.1% in a drug must be identified prior to clinical trials. 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^n) 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 [1–3]. 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 [4–6]. It is absorbed from the gastrointestinal tract and is excreted mainly from the urine [7]. During its production in large scale, an unknown impurity was detected by HPLC in all batches reducing the quality of the bulk drug.

A variety of analytical techniques including amperometry in a flowing stream at the glassy carbon electrode [8], UV spectrophotometry [9], and colorimetry [10,11] were used to assay PPH. Quantitation of PPH was carried out by spectrophotometric [12–14], HPLC [15], polarographic [16] and gravimetric [17] methods. The US pharmacopeia [18] adopts a spectrophotometric method for assay of PPH. Simultaneous determination of PPH and sulfonamides were also reported [19–21]. Iqbal et al. [22] have studied photo degradation of PPH and characterized its major degradation products. Sabry [23] has studied forced degradation of PPH by HPLC and spectrofluorimetric analyses. However, to the best of our knowledge, the impurity profiles of PPH particularly process related were not reported in the literature.

The present manuscript describes a comprehensive investigation on isolation and characterization of a major process related impurity of PPH by semi-preparative HPLC followed by ESI-MS–MS, ^{1}H NMR and 2D-NMR spectroscopy. The possible mechanism of its formation was proposed.

2. Experimental

2.1. Materials and reagents

All the reagents were of analytical-reagent grade unless stated otherwise. Glass-distilled and de-ionized water (Nanopure, Barnsted, USA), HPLC-grade acetonitrile and methanol (S.D. Fine Chem., Mumbai, India) were used. The investigated samples of PPH bulk drug materials, crude samples and impurities were synthesized in the laboratory.

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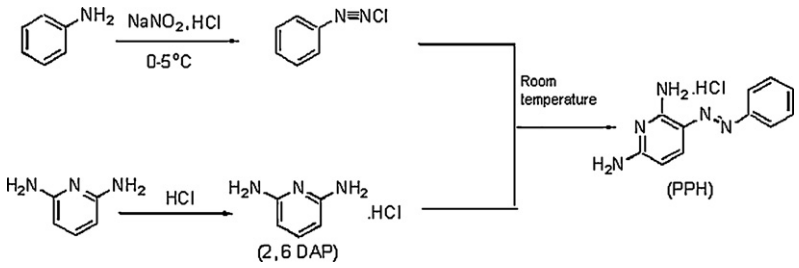

Fig. 1. The chemical process in synthesis of PPH.

2.2. High performance liquid chromatography (HPLC)

The HPLC system was composed of two LC-20AT pumps, an SPD-M20A diode array detector, a SIL-20AC auto sampler, a DGU-$20A_3$ degasser and CBM-20A communication bus module (all from Shimadzu, Kyoto, Japan) was used. The chromatographic and the integrated data were recorded using an Acer Power Entra (Acer India Ltd., Bangalore, India) computer system. Data processing was carried out using LC-Solution software (Shimadzu, Kyoto, Japan).

The analysis was carried out by HPLC on Inertsil ODS 3 V (250 mm × 4.6 mm i.d.; particle size 5 μm) column using H_2O and acetonitrile (25:75, v/v) as a mobile phase at a flow rate of 1.0 mL/min in an isocratic elution mode. Before delivering the mobile phase in to the system, it was degassed and filtered through 0.45 μm PTFE filter using vacuum. The injection volume was 10 μL and the detection was performed at 254 nm using a PDA detector.

2.3. Semi-preparative LC conditions

Separation and isolation of the process impurity was carried out on a semi-preparative Inertsil ODS 3 V (10 mm × 250 mm; particle size 5 μm) LC column using H_2O:acetonitrile (30:70, v/v) as a mobile phase at a flow rate of 5.0 mL/min and the detector was maintained at 254 nm.

2.4. ESI-mass spectrometry

The experiments were performed using a commercial LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA), equipped with an ESI source. The data was acquired using the Xcalibur software. The typical source conditions were: spray voltage, 4 kV; capillary voltage, 15–20 V; heated capillary temperature, 200 °C; tube lens offset voltage, 20 V; sheath gas (N_2) flow rate, 15 units; collision energies were 20–60 eV. Helium was used as a damping gas. MS measurements were performed in the full-scan mode over a mass range of m/z 50–800 with 0.21 scans/s. The mass spectra were recorded by scanning MS^1, and the collision-induced dissociation (CID) spectra were obtained by selecting the precursor ion of interest with MS^1 and scanning MS^2. All the spectra recorded were an average of 20–25 scans under identical experimental conditions.

2.5. FT-NMR spectrometry

Gemini 400 MHz, Fourier-transform (Varian, Switzerland) NMR spectrometer was used to record the ^{1}H NMR spectra. The operating conditions were: proton resonance frequency, 200 MHz; spectral width, 2929 Hz; pulse width, 18 μs; data points, 8192; spectral resolution, 0.2 Hz; probe temperature, 27 °C. Chemical shifts were referenced to tetramethyl silane (TMS) at δ = 0.0 ppm.

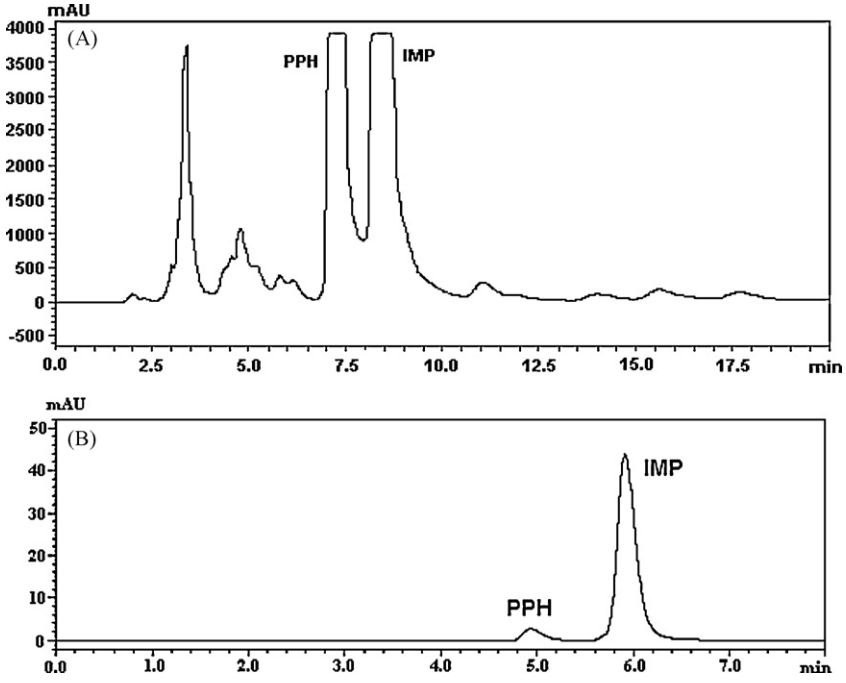

Fig. 2. HPLC chromatograms of (A) crude PPH and (B) impurity after isolation.

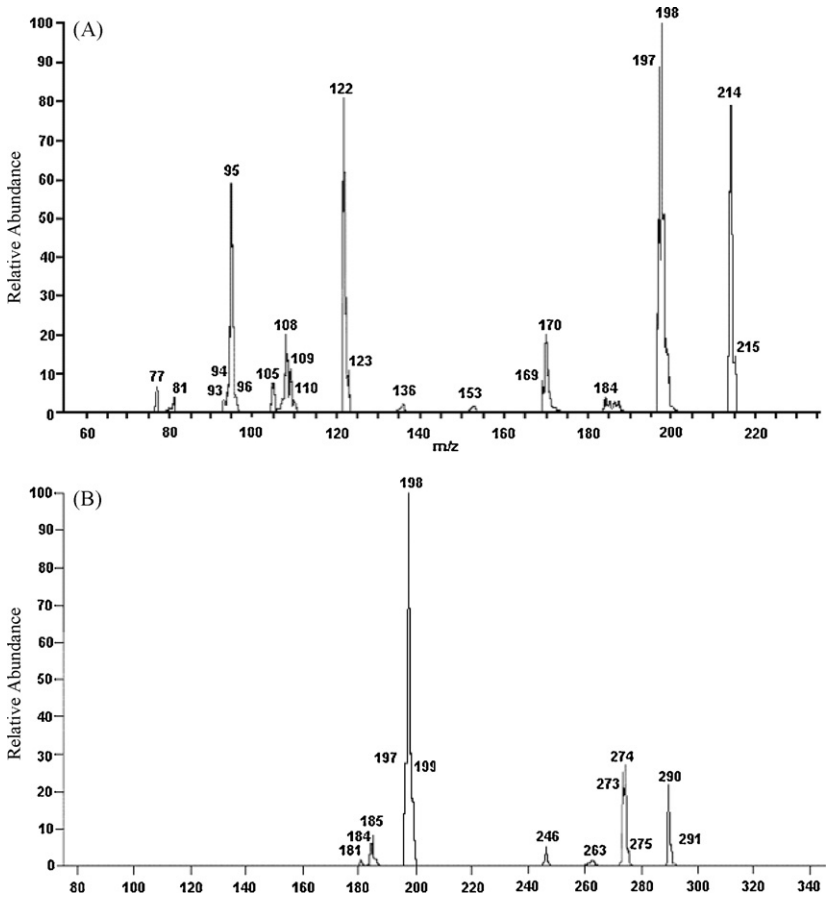

Fig. 3. ESI-MS/MS spectra of (A) PPH and (B) impurity.

3. Results and discussion

During the routine impurity profiling of PPH bulk drug by analytical HPLC, an unknown impurity in the range of 0.05–0.2% was consistently observed. The impurity was not matched with any of the raw materials or intermediates formed during the synthesis. As per ICH guidelines, any impurity which is ≥0.10% must be characterized chemically and pharmacologically. So MS–MS and 2D-NMR studies were undertaken to characterize the impurity.

3.1. Detection and isolation of impurity

During the synthesis of PPH (Fig. 1) the unknown impurity formed at a level 0.2% was enriched by column chromatography up to 5%. Then it was purified by semi-preparative LC, using the conditions described in Section 2.4 (Fig. 2).

The collected fractions of the impurity were concentrated separately under high vacuum on a rotavapor and the organic solvent was stripped off. The aqueous layer of impurity was subjected to solvent–solvent extraction with ethyl acetate and the compound was extracted into the organic layer. The ethyl acetate fractions were pooled together and concentrated on a rotavapor under vacuum. The chromatographic purity of the isolated impurity was >95%, indicating that the fractions were stable during isolation.

3.2. Structure elucidation of the process impurity

The ESI-MS (positive mode) spectrum of impurity showed a protonated molecular ion at *m*/*z* 290 and PPH showed molecular

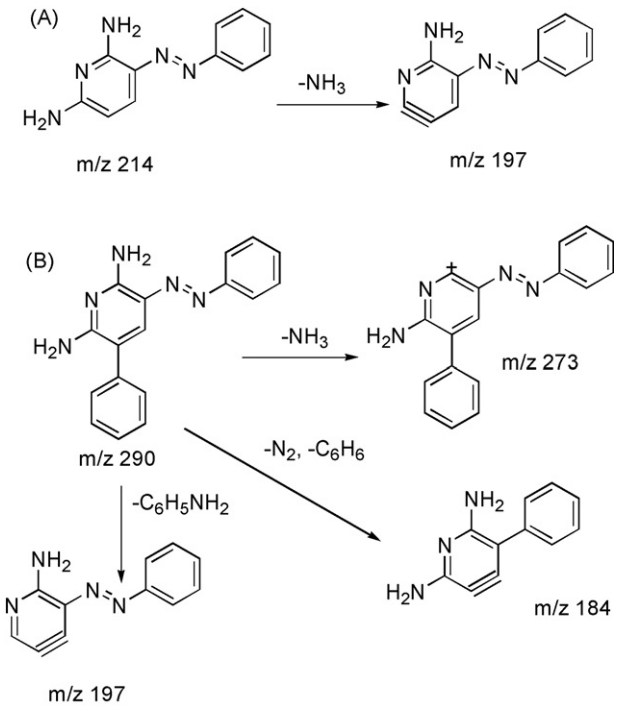

Fig. 4. ESI-MS/MS fragmentation patterns for (A) PPH and (B) impurity.

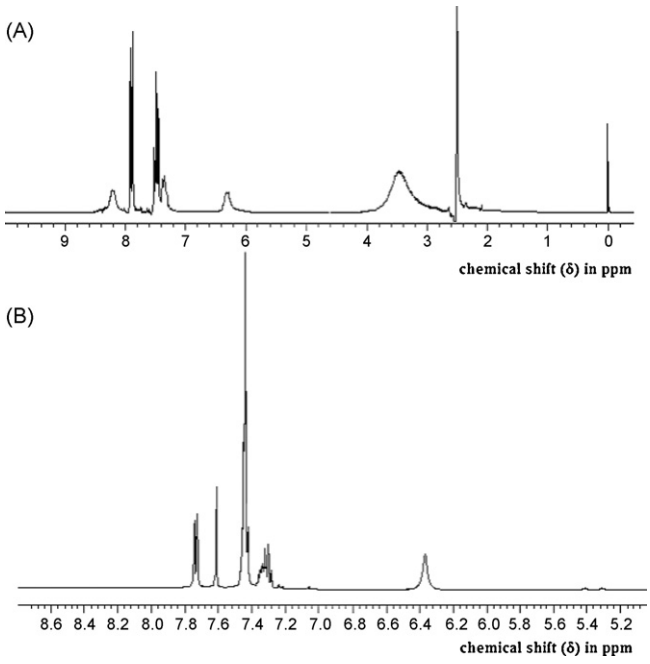

Fig. 5. The ^{1}H NMR spectra of (A) PPH and (B) impurity in DMSO-d_6.

ion at *m*/*z* 214. The difference of mass between PPH and impurity was 76 amu. This indicated the presence of an additional phenyl group in the impurity compared to PPH. The phenyl group could be attached at any of the two positions 3 and 4 in pyridine ring of PPH. To get structural information, mass fragmental studies were taken up for impurity and PPH simultaneously (Fig. 3A and B). The mass fragmentation path ways are given in Fig. 4 A and B, respectively.

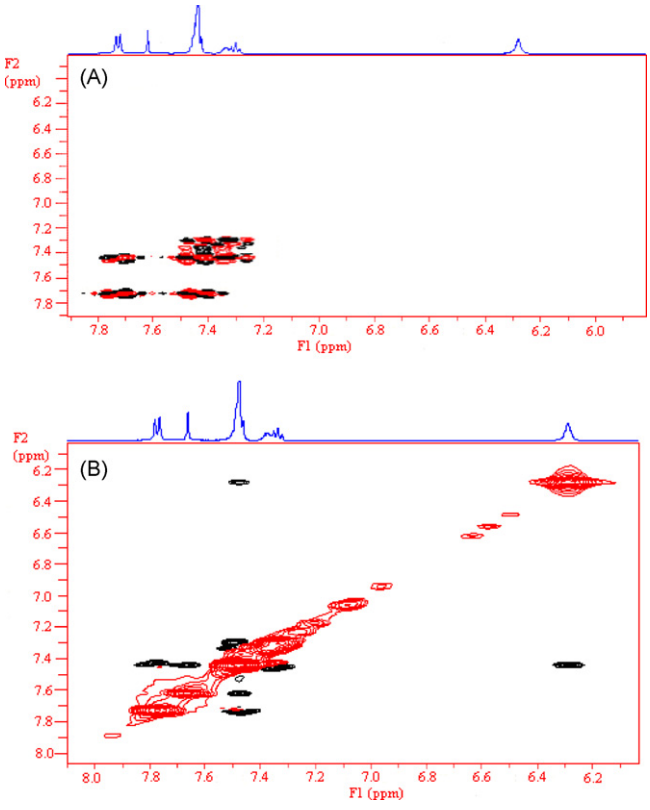

Fig. 6. (A) Cosy and (B) Nosy spectra of the impurity of PPH.

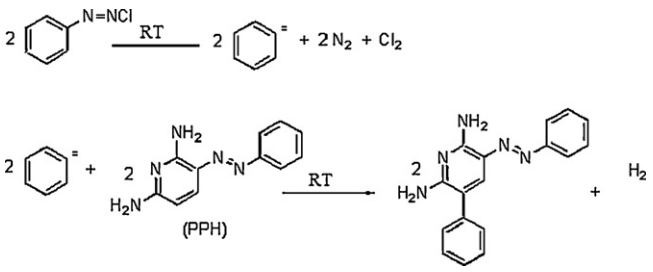

Fig. 7. The mechanism of formation of the impurity of PPH.

The MS/MS fragmentation pattern of the impurity could not give much information regarding the possible structure of the impurity. Thus NMR experiments were carried out. The ^{1}H NMR spectra of PPH and impurity are shown in Fig. 5. The ^{1}H NMR spectrum of impurity showed four additional aromatic protons compared to PPH indicating presence of an additional phenyl group in the impurity. To know the position of phenyl group, COSY and NOSY (Fig. 6) experiments were carried out. NOSY clearly showed the coupling between ortho protons of attached phenyl group and the amine protons at position 2 of pyridine ring. This could only be possible if phenyl group is attached near to amino group at position 3 in the pyridine ring. From the above spectral information, the structure of impurity was confirmed as 3-phenyl-5-phenylazo-pyridine-2,6-diamine.

3.3. Formation of impurity

The impurity could be formed as shown in Fig. 7. Since benzenediazonium chloride was added to 2,6 DAP at room temperature, benzenediazonium chloride undergoes free radical thermal degradation to form phenyl radicals. The phenyl radicals then attack PPH at position 3 of pyridine ring to form the impurity. The phenyl group may not attack at position 4 of pyridine ring as it is highly hindered by the diazo phenyl group of PPH.

4. Conclusions

During impurity profiling of PPH bulk drug by analytical HPLC, an unknown impurity in the range of 0.05–0.2% was observed consistently. The impurity was isolated and purified by semi-preparative HPLC and then characterized by ESI-MS/MS and 2D-NMR experiments. The formation of the impurity was discussed.

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