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Stability studies of ionised and non-ionised 3,4-diaminopyridine: Hypothesis of degradation pathways and chemical structure of degradation products

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Abstract

3,4-Diaminopyridine is used to treat some symptoms met in Lambert–Eaton myasthenia syndrome. It was shown efficient to reduce a form of variable muscle weakness and fatigability typical of the disease and correlated to a block of acetylcholine release. In France, 3,4-diaminopyridine is nowadays given to patients under capsules form and the status of hospital preparation. Whatever the diluant used in the formulation, the stability period could not exceed 12 months. Preliminary studies were made on a salt form in order to test the influence of various stress factors and determine if there is interaction between them. From this study, the most influent stress condition, presence of hydrogen peroxide, was selected and a comparative study was performed to compare the stability of molecular and salt species. Solutions of each species were exposed to 5 or 15% of hydrogen peroxide and analyzed at 8, 24, 72 and 216 h of degradation by HPLC-UV. Fractions of detected impurities were purified and collected by semi-preparative HPLC-UV and analyzed by HPLC-UV–ESI–MS and IR spectroscopy in order to determine their structure hypotheses. Theses experiments demonstrate that the salt species were more stable under oxidative stress condition than molecular species. The two main degradation products were collected and identified as 4-amino, 3-nitropyridine and 3,4-diaminopyridine-N-oxide when the molecular form was degraded whereas only 4-amino, 3-nitropyridine was found in less quantity in the salt solutions. Nitrogen pyridine and pyridine amine could not easily be oxidized by hydrogen peroxide in salt comparatively to molecular species due to the lone pair of electron engaged in a bound with hydrogen in the first case and by resonance change of the pyridine in the second case. This modification of structure promoted different pathways of degradation for the salt form which are more dependent of energy. Owing to the better stability of the salt species, a new pharmaceutical form containing it was developed to assess its stability under ICH standard conditions allowing an industrial manufacture of this drug.

Keywords: 3,4-Diaminopyridine; HPLC–MS; Stress condition; Stability studies; Impurities

1. Introduction

The Lambert–Eaton myasthenic syndrome is characterized by impaired neuromuscular transmission leading to muscle weakness, hyporeflexia and autonomic dysfunction. This pathology is rare and grave. The neuromuscular weakness is the consequence of the low release of acetylcholine in the synapse, due to a blockage of the pre-synaptic calcium channels. The most common cause of this blockage is an auto-immune attack of antibodies on these Ca^{2+} channels. Pyridine derivatives would improve neuromuscular transmission by enhancing the release of acetylcholine from the terminal nerve by promoting cellular calcium influx. Evidence has been made that 3,4-diaminopyridine (3,4-DAP) is an efficient drug to treat muscular weaknesses caused by multiple sclerosis and particularly by the Lambert–Eaton myasthenic syndrome [1–3]. Moreover, 3,4-DAP has fewer side effects than 4-aminopyridine and than pyridostigmine [1], which are the only alternative for an oral treatment. The 3,4-DAP molecule inactivates voltage-dependent K^+ channel on the pre-synaptic neurones [4]. This action lengthens the pre-synaptic stimulating potential because the repolarization is slowed down. The consequence is an increased time of acetylcholine liberation and an improvement of the muscles contractions [5,6]. This out-

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come confers short-term benefits for patients: restoration of the efficiency of the mobility muscles, with low toxicity [7]. Usually immunosuppressive and antineoplasic treatments are combined with 3,4-DAP for a long-term care [8].

Actually, 3,4-DAP is placed at disposal to patients under capsules form with the status of hospital preparation. Whatever the diluant used in the formulation, the stability period could not exceed 12 months. Nevertheless, we noted that 3,4-diaminopyridine under the ionic form in aqueous solutions is much more stable than its molecular form.

So the aim of this study is to test and compare the stabilities of the molecular and salted form of the 3,4-DAP, by performing degradations under stress conditions.

2. Experimental

2.1. Analytical standard and reagents

The 3,4-DAP salt was purchased from SERATEC (Courville sur Eure, France), hydrogen peroxide 30% (v/v) from VWR International (Fontenay sous Bois, France).

Methanol (VWR International, Fontenay sous Bois, France); acetonitrile (Sigma–Aldrich, Seelze, Germany), ultra-pure water prepared by a Milli-Q system (Molsheim, France); sodium octane sulfonate (Sigma, Steinheim, Germany); ammonium acetate (Merck, Darmstadt, Germany); glacial acetic acid (Sigma–Aldrich, Seelze, Germany); chloride acid (Prolabo, VWR, Fontenay sous bois, France); sodium hydroxide (Merck, Darmstadt, Germany); trifluoroacetic acid (Merk, Darmstadt, Germany).

All solvents were of HPLC grade.

2.2. Apparatus and chromatographic condition

2.2.1. Stress condition for preliminary studies

The UV irradiator was a TL-900 U (CAMAG, Muttenz, Switzerland).

2.2.2. HPLC system for solutions' analysis

Although other HPLC methods for assaying 3,4-DAP already exist [9,10], the following described method here employed, was established as a stability-indicating assay method for the determination of 3,4-DAP in the raw material and final product [11]. It was then used to follow the concentration as a function of time of 3,4-DAP in each solution and to determine the degradation profile and its evolution (retention time of impurities and relatives areas).

The obtained solutions were analyzed with an HPLC-UV system composed by a ThermoSepartionProducts (TSP) (Les Ulis, France) which includes helium degasser (SCM400), a quadratic pump (SpectraSystem P1000 XR) and an auto sampler (AS 3000) with a 100 μl loop and TSP Spectra system UV6000 LP UV spectrometer (Spectra system thermo Finnigan, San Jose, CA, USA). Analyses were made at a single wavelength of 262 nm. This system is piloted by the Chromquest software Version 2.51.

The chromatographic column was a Kromasil® C18: 250 mm × 4.6 mm ID, 5 μm dp and 100 Å porosity (Macherey Nagel, Düren, Germany).

The composition of the mobile phase was 9:1 solution A/acetonitrile. Solution A was prepared with 8 mmol of sodium octane sulfonate, 10 mmol of ammonium acetate for one litre of ultra-pure water. Solution A pH was adjusted at 2.5 by trifluoroacetic acid. Mobile phase flow rate was 1 ml min^{-1}.

2.2.3. Semi-preparative HPLC for degradation impurities collection

A scale up procedure was set up to isolate the major degradation products. It was based on the same dynamic ion exchange separation. A VWR semi-preparative apparatus (Knauer, Merck, Berlin, Germany) was used. It was composed by a preparative chromatography pump K1800, a six valves injector, a four wavelengths UV detector UV-K2600 and a twelve valves device for flow separation. This system was piloted by the EZ ChromElite v.3.1.3 software.

The stationary phase was a C18 modified silica column (Kromasil, VWR, Fontenay sous bois, France): 250 mm × 20 mm ID, particle size of 5 μm. The mobile phase composition was same as described above. To maintain the linearity of the separation we had to work at 20 ml min^{-1} flow rate. The injection volumes were between 0.4 and 1.0 ml in function of the sample.

2.2.4. HPLC-UV–MS and IR spectrometer system for degradation impurities analysis

2.2.4.1. HPLC-UV–MS. Samples of degradation products after semi-preparative HPLC purification were analyzed by mass spectrometry (Thermoquest LCQ Duo, Finnigan, San Jose, CA, USA) piloted by the X-Calibur software. Due to the presence of sodium octane sulfonate, the first analytical method was not usable with MS detection. That is the reason why another HPLC method was optimized to obtain sufficient retention of the compounds to analyze then. To eliminate the sodium octane sulfonate in each fraction a LC device was used before the spectrometer with electro spray ionization (ESI) interface. A similar TSP HPLC system as described above was used, with a short C18 modified silica column (5 mm × 4.6 mm ID, Kromasil, VWR, Fontenay sous bois, France). The mobile phase was composed by 200 ml methanol, 5 ml glacial acetic acid and 795 ml ultra-pure water. The flow rate was set at 1 ml min^{-1}. Analysis was performed at room temperature. An UV6000 detector (Spectra system thermo Finnigan, San Jose, CA, USA) was used to confirm the UV spectra of products before MS. The system is the LC–UV–ESI–MS.

2.2.4.2. IR spectrometer. The IR spectrometer used was an FT-IR spectrometer, Spectrum 1000 purchased from Perkin-Elmer (Beaconsfield bucks, England).

2.3. Method

2.3.1. Preliminary studies: stress factors selection

Eight conditions were designed to force the degradation of the 3,4-DAP (solution at 10 g l^{-1}) with factors such as pH (pH 1 with