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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

Table 1
Preliminary studies: stress conditions chosen for 3,4-DAP degradation

Degradation conditions	Irradiation during 6 h/day	pH	Hydrogen peroxide concentration (%, v/v)	Temperature	Duration (h)
A	254 nm		Nitrogen stream	25 °C	24
B	Visible		Nitrogen stream	25 °C	24
C		1	3%	25 °C	24
D	254 nm	1	3%	25 °C	24
E	Visible	1	3%	25 °C	24
F		1	3%	80 °C/6 h	24
G	254 nm	1	3%	80 °C/6 h	24
H		1	30%	25 °C	24
I	Visible	13	Nitrogen stream	25 °C	24

chloride acid and pH 13 with NaOH 0.1 mol l^{-1}), temperature (25, 80 and 121 °C), UV irradiation (visible versus 254 nm), and oxidation agent (hydrogen peroxide 3%) (Table 1). The aim of this part was to test the influence of each factor and determine if there is interaction between them.

The testing conditions are described in Table 1; they were all led on a solution of the salt of 3,4-diaminopyridine in water. Three replicates were made at each time.

The recovery rate of 3,4-DAP was calculated at the end of the degradation by area normalization on chromatograms. The numbers of degradation products formed during the experiments were also determined.

2.3.2. Comparative study

The objectives of the study were to compare stability of the salt form *versus* the molecular form of 3,4-DAP in solution and to elucidate the main degradation pathways for each species. The two products were dissolved in water at the concentration of 5 g of 3,4-DAP and put under various stress conditions determined from the results of the previous part.

The most influent stress factor from the previous work, hydrogen peroxide, was considered. 3,4-DAP solution were exposed to 5 or 15% (v/v) of hydrogen peroxide under a temperature of 25 °C. Solutions of 3,4-DAP (5 g l^{-1}) were prepared in brown flask in order to avoid light interaction. Three replicates were made for each level. Each solution was analyzed at 8, 24, 72 and 216 h of degradation.

The objectives of this part were to analyze and trying to determine the structures of the main degradation products of each 3,4-DAP species in order to appreciate their specific degradation profile.

2.4. Results and discussions

2.4.1. Preliminary studies

The main objective of these preliminary studies was to determine the most influent stress factors on the stability of the salt of 3,4-DAP. The selection of the most influent factor would enable to compare the stability of the salt form versus the molecular form in aqueous solutions.

Experiments A and B showed that when irradiation was intensified, a very limited impact on the stability of the salt was observed. A deep examination of these results (Fig. 1) revealed that UV-radiations had more effects on 3,4-DAP than visible irradiation. This is coherent because UV-radiations are more energetic than visible radiations. By the way, introduction of hydrogen peroxide (H_2O_2), at pH 1 (condition C) had also a low impact on 3,4-DAP degradation. This first set of experiments demonstrates that 3,4-DAP under ionic form is stable in water solution when opposed to a single stress factor.

However, when H_2O_2 was combined to another stress factor, such as light irradiation or high temperature, the degradation of the molecule increased. These significant degradations were particularly observed with conditions D and F. For these two experiments the recovery rates of 3,4-DAP were quite similar, around 80%. Thus, the temperature and UV irradiation seemed to provide sufficient energy for quantitative degradation of 3,4-DAP in the presence of H_2O_2. This is strengthened by the experiment G. On the contrary, the experiment E revealed that the visible irradiation at about 2000 klx/h was not enough energetic to enhance the degradation of 3,4-DAP when combined to H_2O_2.

The analysis of the results obtained when the salt of 3,4-DAP was in presence of 30% (v/v) of H_2O_2 in the water solution (stress condition H) showed that this oxidative agent may have a real impact on the stability of the product. Its concentration was indeed very important, but the final degradation was quantitatively comparable to those obtained when combining two stress agents. Nevertheless, on a qualitative side, the number and the retention times of the degradation products were different which tended to demonstrate that the degradation pathways in these experiments (D, F and H) were not the same. It is then highly probable that precise degradation reactions occur when a specific stress agent is used. Results of experiment H showed that,

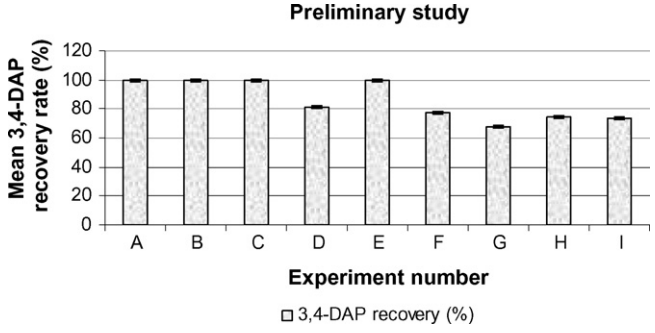

Fig. 1. Preliminary studies: number of degradation products formed after 3,4-DAP salt exposure to various stress conditions.

a large part of these degradation reactions could be oxidation reactions.

Finally the experiment I was interesting because these conditions were made in order to have a solution of the molecular form of the 3,4-DAP: a pH value of 13 is above the two pK_a of the 3,4-DAP. Thus, experiment I could be compared to the experiment B, the first showed the behavior of the molecular form of the 3,4-DAP and the second the one of the salt of 3,4-DAP. This little comparison exhibited the poor stability of the molecular form: nearly 27% of this form was lost whereas only 0.1% of the salt was degraded. The comparison will be more detailed in the next section.

This study demonstrated the necessity of an accumulation of stress factors to obtain degradation products of the salt. The most influent factor seemed to be the hydrogen peroxide used as an oxidative agent. This factor was used to perform the comparative study between the molecular and the salted form of the 3,4-DAP.

2.4.2. Comparative study

These results show that, in the studied experimental domain, the two species of 3,4-DAP exhibit different behaviors depending on the degradation conditions (Fig. 2). The oxidative agent was much more active on the molecular form than on the salt. For example, the comparison of the values obtained at 25 °C showed that the degradation was in both cases more advanced for the molecular form and that the more hydrogen peroxide was introduced, the more the 3,4-DAP was degraded. This difference was probably due to the presence of the lone pair of electrons on the pyridine nitrogen. This lone pair is not engaged in the resonance system and then would represent a target for the oxidative agent or at least seems to be the cause of the greater sensibility to oxidation of the molecular form. On the contrary, this lone pair of electrons is engaged in a bound with hydrogen in the case of the salt of 3,4-DAP. This bound creates a positive charge on the pyridine nitrogen and thus modifies the mesomeric system. This modification seems to favor the formation of a state of the molecule less sensible to oxidation. This difference could explain the better stability of the salt towards oxidation at room temperature.

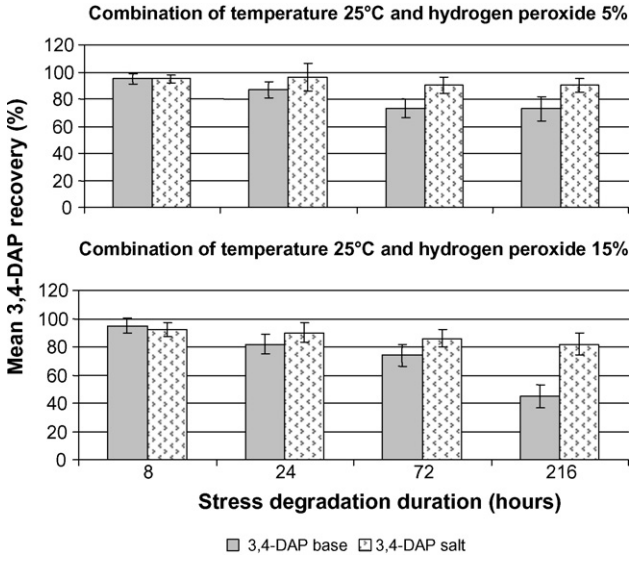

Fig. 2. 3,4-DAP recovery rates obtained after exposition to 5 or 15% (v/v) of hydrogen peroxide.

By the way, different pathways for the degradation of the two forms of the 3,4-DAP can be envisaged which means that the chemical functions attacked by the oxidation agent would not necessarily be the same. Besides it seems that the salt of the 3,4-DAP would be more stable than the molecular form in pharmaceutical compositions in standard conditions of storage.

2.4.2.1. Major degradation products. Chromatograms for each 3,4-DAP species obtained after exposition to stress conditions were compared to determine the major degradation products and the conditions that enhanced their production. Main impurities were isolated by a semi-preparative chromatographic system and submitted to structure analysis in order to formulate chemical structure hypotheses.

Concerning the molecular form, two peaks were spotted giving high response signals. Both were eluted after 3,4-DAP (RT: 19.9 min) as shown in Fig. 3.

The first product (RT: 27.7 min) was formed under 5 and 15% (v/v) of hydrogen peroxide. At the end of the experiment the amount of impurity, eluted at 27.7 min, was larger with 15% of H_2O_2 than with 5% of H_2O_2, whereas the peak area of the second product (RT: 31.1 min) was related to initial concentration of hydrogen peroxide.

These observations let think that these two degradation products are oxidation products of the 3,4-DAP and that the reactive path to produce the molecule eluted at 31.1 min presents a higher activation energy because its formation required high temperature. In this latter case, the molecular site oxidized is less sensible than the site involved in the formation of the first product.

Results obtained were then compared with chromatograms of the salt. In every stress conditions the degradation product eluted at 27.7 min was observed at the end of the study. The UV spectra obtained on the range 200–700 nm were similar at the retention time of 27.7 min for the two forms of 3,4-DAP. This peak could be detected at the first analyzing time under 15% of hydrogen peroxide. It seems that 5% of hydrogen peroxide at 25 °C during 8 h were not sufficient conditions to degrade the salt of 3,4-DAP (Fig. 4). The peak areas for this product were always smaller than corresponding peak areas for the molecular form of the 3,4-DAP. This was most probably due to the fact that all the H_2O_2 was not completely consumed at the end of the study, which fit with the previous conclusion that oxidative agent is more efficient on the molecular 3,4-DAP than on the salt.

The impurity eluted at 31.1 min has never been detected during the analysis of the solutions of the salt of 3,4-DAP.

2.4.2.2. Structure hypothesis. Impurities eluted at 27.7 min after the exposition of the salt (**1a**) and the molecular species (**1b**) to a various concentration of H_2O_2 were collected by semi-preparative HPLC-UV and analyzed by HPLC-UV–ESI–MS.

Analysis by mass spectrometry gave us a protonated ion characterized by a *m/z* of 140 amu for the molecular protonated ion

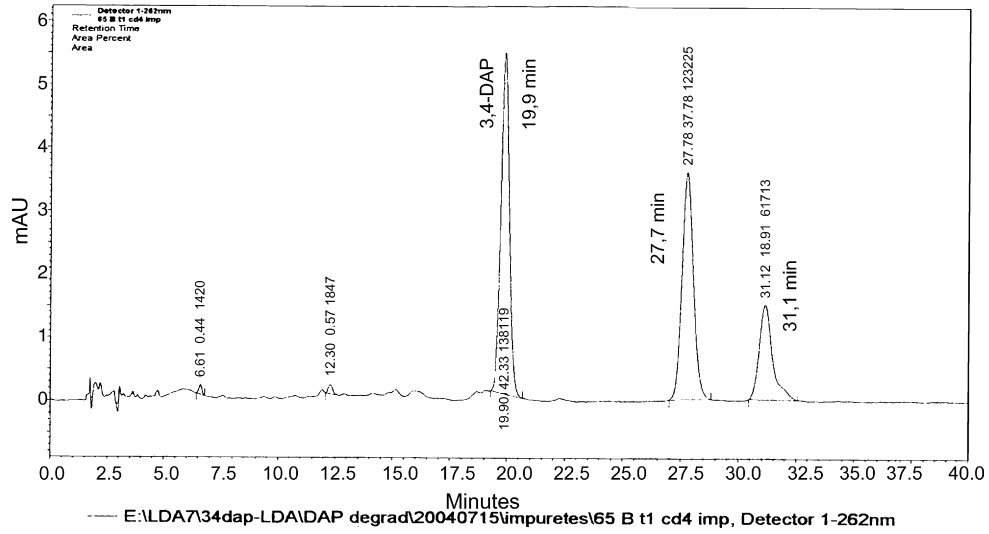

Fig. 3. Analysis of the solution of 3,4-DAP after 8 h of degradation in stress condition ([H_2O_2] = 15%, T = 25 °C), by the dynamic ion exchange chromatography.

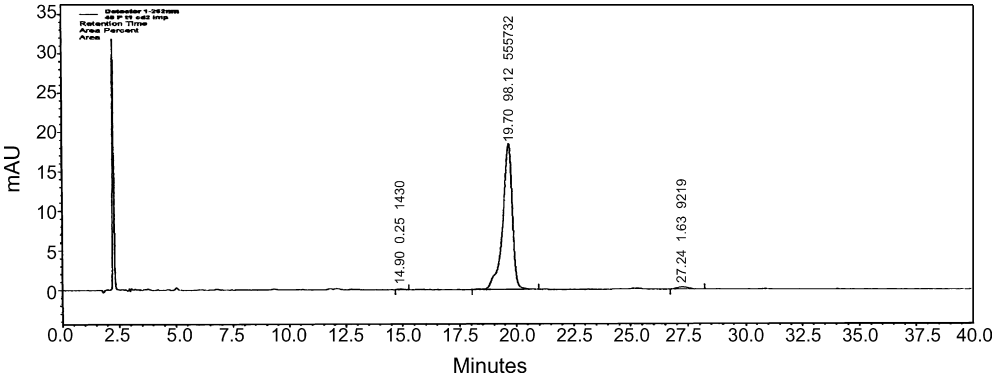

Fig. 4. Analysis of the solution of 3,4-DAP after 8 h of degradation in stress condition ([H_2O_2] = 5%, T = 25 °C), by the dynamic ion exchange chromatography.

$(MH)^+$ for the products **1a** and **1b**. UV and IR spectra were also similar for these two products.

UV spectrum showed three relative maxima at 228, 258 and 328 nm. Comparison of this spectrum to the one of the 3,4-DAP, revealed a bathochrom effect on the last maximum: it shifted from 290 to 328 nm. It is usually related to an augmentation of the number of π-electrons in the mesomeric system.

IR spectra showed two bands which were not present on the spectrum of the 3,4-DAP at 1553 and 1354 cm^{-1}. They could be attributed to valence vibration of the nitro group, respectively, asymmetric and symmetric vibrations.

These data suggested that products **1a** and **1b** were the same product which is the 4-amino-3-nitropyridine ($C_5H_5O_2N_3$). The developed chemical formula is given in Fig. 5.

This structure was confirmed by the analysis of a commercial sample of this product.

This molecule responds to the criteria previously given: it is an oxidation product of the 3,4-DAP and it could be obtained from the salt as well as from the molecular form, the oxidation site is not protected in both case. However, the presence of the charge seems to interfere with the oxidation. Indeed the resonance system would be modified and the amino group would be then not as reactive as it is in the molecular form.

It is worth noting that the activation energy to transform an amino group in a nitro group is very high so that the transformation should not arise in our degradation conditions. The chemical path which leads to the formation of the nitro group is not direct and should probably need the formation of hydroxylamine and nitroso intermediates. This hypothesis is in agreement with the results published by Panigrahi and co-workers [12].

The impurity eluted at 31.1 min after the exposition of the molecular form to H_2O_2 was detected in mass spectrometry, which likely is the molecular protonated ion $(MH)^+$, characterized by a *m/z* of 126 amu. The different of 16 towards the *m/z*

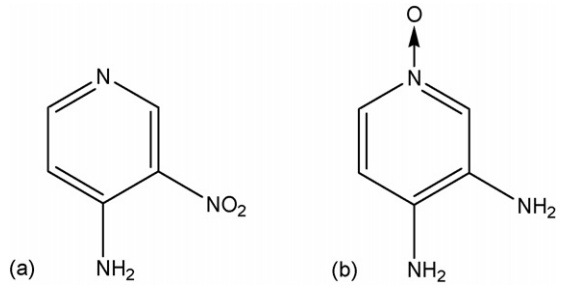

Fig. 5. Structure of 4-amino-3-nitropyridine and 3,4-diaminopyridine-*N*-oxide.

of the $(MH)^+$ of 3,4-DAP would most probably means that an oxygen atom was added to the molecular 3,4-DAP.

UV spectrum gave two maxima at 224 and 290 nm which is very similar to the spectrum of the 3,4-DAP. The modification due to oxidation has then nearly no effect on the mesomeric system.

With these results and as this product is only obtained with the molecular form of 3,4-DAP the hypothesis was formulated that the 3,4-diaminopyridine-*N*-oxide ($C_5H_7ON_3$) was isolated. The chemical formula is given in Fig. 5.

More over, IR spectrum of this collected impurity presented a supplementary absorption at 960 cm^{-1}, comparatively to 3,4-DAP one's, which is characteristic of *N*-oxide function. That kind of molecule has already been observed when peracid like metachloroperbenzoic acid reacts on aminopyridines [13].

3. Conclusion

The engagement of the lone pair of electron of nitrogen pyridine in a bound with hydrogen in the salt form reduced the possible oxidation of 3,4-DAP under oxidative stress conditions comparatively to the molecular species and promoted different pathways of degradation for the salt form which are more dependent of energy.

By the way, due 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. However, this new pharmaceutical form has to demonstrate the same efficacy and safety by assessing its bioequivalence than the previous one containing the molecular form which is already given to treat patients affected by Lambert–Eaton myasthenic.

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