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Investigation Of The Oxidative Degradation Pathway Of Amidoximes By LC/MS/MS

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Abstract

The oxidative degradation pathway for a newly prepared amidoxime prodrug has been studied using LC/MS/MS. The amidoxime was subjected to oxidation with $K_3Fe(CN)_6$/NaOH for different time periods and at different temperatures. The chromatographic separation was performed on RP amide column and 0.1% aqueous formic acid; acetonitrile containing 0.1% formic acid (50:50 v/v) as a mobile phase. The elution in Ultra-performance liquid chromatography (UPLC) was carried out in the isocratic mode. The normal LC/MS/MS showed that the cleavage occurs through amidoxime ester. At room temperature, no important oxidation products were obtained. However, upon heating the parent amidoxime was disappeared with the concomitant appearance of a new compound which is the most stable peak (base peak) at m/z = 333.80. The formed degradation product was suggested to be the disodium salt of benzaldoxime peroxide. These results give indication about the mechanism of release of nitric oxide from amidoxime and ensure that amidoximes are important nitric oxide donors.

Amidoximes; Potassium Ferricyanide; Oxidative degradation, LC/MS/MS

1. Introduction

Amidoximes has gained a great interest in the last decades [1]. Besides their important biological activity such as antituberculous [2], antiarrhythmic [2], antimicrobial [3]; they have a great importance in coordination chemistry [4]. Recently, researchers have interested in the nitric oxide (NO) donor ability of amidoximes [5]. It is known that the *in vivo* nitric oxide is biosynthesized through oxidation of the amidoxime containing *N*-hydroxy-L-arginine (NOHA) [6]. The released NO has important biochemical and physiological functions in the biological system [7]. Due to the short half-life of NO, it was important to find suitable extrinsic NO donor that release NO in sustained release manner. From this point of view, it is important to study the degradation pathways of amidoximes both in vivo and *in vitro*. Several studies have been carried out for oxidation of amidoximes *in vivo* and *in vitro*. Oxidation of amidoximes with Cytochromes P_{450} (CYP_{245}) or horseradish peroxidase resulted in its conversion into the corresponding amide and/nitrile [8]. *In vitro*, several reagents have been used for oxidation of amidoximes including 2-iodobenzoic acid (IBX) [9], IBX associated with tetraethylammonium bromide (TEBA) [10], or $K_3Fe(CN)_6$ at pH 12 [11].

The aim of the present work is to demonstrate an approach that involves the use of liquid chromatography (LC) and liquid chromatography mass spectrometer (LC-MS) to separate, and identify the oxidation product of newly prepared amidoxime **1** [12] without prior separation from reaction mixtures. The selected compound was subjected to oxidation using $K_3Fe(CN)_6$/NaOH under various conditions and the behaviour of its oxidation was studied. The degradation of the selected amidoxime was characterized with the aid of the fragmentation pattern and the masses obtained from LC–MS/MS analysis and a proposal of the degradation pathway was presented.

2 Experimental

An HPLC system (Agilent Corporation, SPD-M20A, Kyoto, Japan) equipped with LC Solution software was used for LC studies equipped with a photo-diode array (PDA) detector, sample injector with 20 mL loop, and on-line degasser containing binary pump. The chromatograph was controlled by LC Solution software and LC–MS system was controlled by Xcalibur software (version 2.0) consisted of LCQ Fleet and TSQ Quantum Access with Surveyor Plus HPLC System (Thermo, San Jose, USA). The

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chromatographic separation was performed on RP-Amide column (50 mm x 3.0 mm i.d.; 2.7 µm particles) Ascentis Express (Supelco™ Analytical, Sigma-Aldrich Chemie Gmbh Munich, Germany). Water bath (Biotech Company. For Medical & Lab. Equip., Cairo, Egypt) controlled by a thermostat to control the temperature was used for heating purpose. Sonicator used is with Operating frequency 33 - 3KHz, Input Voltage range of 170V AC- 270V AC, 50 Hz, Single phase Micro Controller based timer range 0 to 30/99 minutes. Thermostatic controlled temperature controller.

2.2 Chemical and reagents

Sodium hydroxide (El-Nasr Pharmaceutical Chemicals company, Abou Zabaal, Cairo, Egypt), naproxen (Sigma-Aldrich), Potassium ferricyanide (Sigma-Aldrich), methanol (HPLC grade, Sigma-Aldrich, Steinheim, Germany) N'-hydroxy-4-methoxybenzimidamide were purchased from (Alfa Aeser ™, Massachusetts, USA). De-ionized water was purchased from Otsoka pharmaceuticals, Cairo, Egypt. The amidoxime pro-drug 1 was prepared as reported [12].

2.3 Synthesis of amidoxime

The amidoxime **1** (**scheme 1**) was prepared using a reported procedure by coupling naproxen with *N*'-hydroxy-4-methoxybenzimidamide using carbonyl di-imidazole (CDI) at room temperature [12].

2.4 Analysis method

2.4.1 Preparation of $K_3Fe(CN)_6$/NaOH (0.4 M)

A mixture of potassium ferricyanide (329 mg, 0.4 M) and sodium hydroxide (131.7 mg, 0.4 M) was completed to 100 mL with deionized water [13].

2.4.2 Preparation of amidoxime 1 stock solution

Into 25 mL volumetric flask, 2.5 mg of amidoxime **1** was dissolved in methanol (HPLC grade), the mixture was sonicated for 30 min at 25 °C using a sonicator with operating power about 400 Watt, and then completed to 25 mL with the same solvent.

2.4.3 Oxidation of amidoxime 1 and $K_3Fe(CN)_6$/NaOH (0.4M)

One millilitre of amidoxime stock solution was mixed well with 1 mL $K_3Fe(CN)_6$/NaOH (0.4 M). The reaction mixture was allowed to stand at room temperature for; 1 hr, one day and one week. After the specified time, samples were subjected to analysis by LC/MS/MS. The same solution was heated in water bath at 80 °C for 2 hr. The obtained solution was cooled and was subjected to analysis by LC/MS/MS.

2.4.4 Chromatographic conditions

The HPLC instrument was operated under isocratic elution using mobile phase was composed of 0.1% aqueous formic acid: acetonitrile containing 0.1% formic acid (50:50 v/v) and the flow rate was 0.7 mL/min, the column temperature was adjusted to 35 °C and the injection volume was 2 µL.

2.4.5 Mass spectral studies on amidoxime 1

Mass spectral studies were performed in positive electrospray ionization (ESI) mode in the mass range of 50–1000 Da to establish the fragmentation pattern of the drug. In order to get clear mass spectrum without any background noise, the drug at a concentration of 5 µg/mL in methanol: water (50:50, v/v) was directly infused using a syringe pump into the mass spectrometer. The mass parameters were appropriately tuned to get clear molecular ion peak of the drug. High purity nitrogen was used as the nebulizer and auxiliary gas. The drug was further subjected to MS/MS analysis in positive ESI mode to explore the origin of each individual fragment.

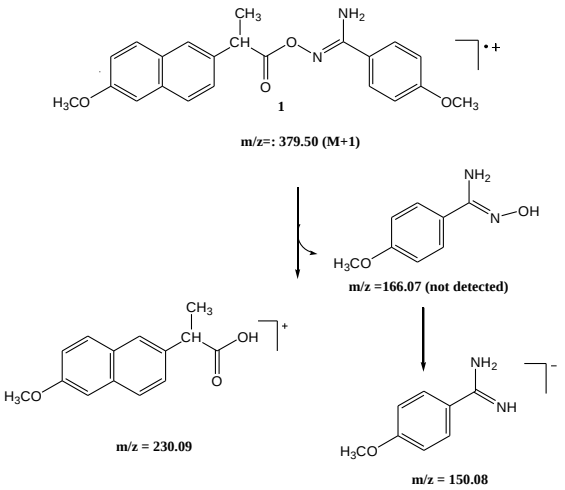
2.4.6 LC–MS/MS studies

The degraded drug samples were subjected to LC–MS/MS analysis in positive ESI mode using the previously mentioned isocratic elution system. A satisfactory separation of degradation products was achieved by using RP amide column. For structure elucidation of the degradation product, the samples with maximum degradation were subjected to LC–MS analysis. The structural identity of each DP was performed with the LC–MS fragmentation analysis.

3 Results and Discussion

3.1 Fragmentation pathway

The MS spectrum of the parent amidoxime **1** showed 3 main fragments as shown in **Figure 1**. The spectrum showed a base peak at m/z 379.50 representing the parent compound as (M+1) peak. In addition, two important fragments appeared at m/z 231(3%) and m/z 149.20 (75 %). The first was the naproxen scaffold, while the other was **4-methoxybenzamidine** scaffold which was mediated by the undetected amidoxime. This result shows that the main fragmentation pathway is through cleavage of the amidoxime ester moiety and this may be the key degradation pathway, **Scheme 1**.



Scheme 1: Proposed fragmentation pathway of amidoxime 1.

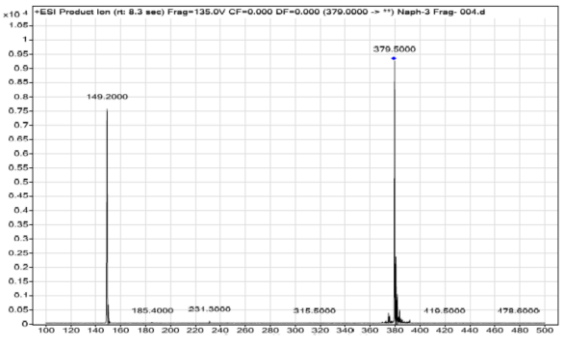


Fig. 1: LC/MS/MS Spectrum of amidoxime 1.

3.2 LC–MS/MS studies on oxidized samples

The parent amidoxime was mixed with the oxidizing mixture at room temperature, and samples were taken at different time intervals. The spectrum of the sample taken after 20 min showed the parent amidoxime **1** at m/z = 378.90 (60 %) and M+Na of compound **1** at m/z = 400.90 (100 %). In addition, other important peaks appeared at m/z = 353, 314, 274, 233, 148 and 118, **Figure 2**. Analyzing another sample taken after one hour from reactants mixing, showed peaks at m/z 401, 381, 353, 321, 267, 223 and 156, **Figure 3**. It is obvious that parent amidoxime and M+ sodium still present and no important oxidative degradation fragments has been happened. Also, after one day and one week mixing at room temperature there is no important oxidation products appeared, the sample showed the parent peak of **1** and additional peaks at m/z 333.10, 241.100, 189.10 and 149.200, **Figure 4**.

On the other hand, heating amidoxime **1** with the oxidizing mixture for 2 hr in water bath (80 ^{0}C) showed disappearance of the parent amidoxime and appearance of the most stable peak (base peak) at m/z = 333.800 as important peak that may be the main oxidation product. In addition, other new peaks with low prevalence appeared at m/z = 272.700 and 156.600, **Figure 5**.

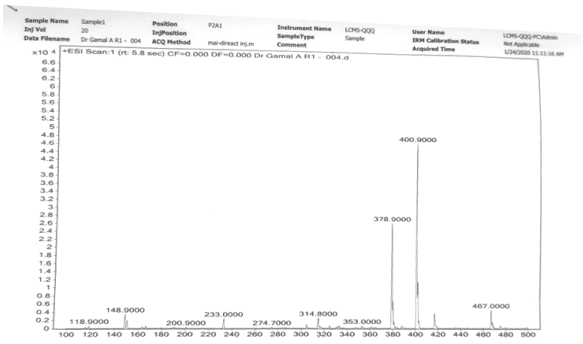


Fig. 2**:** LC/MS/MS Spectrum of amidoxime 1 oxidation after 20 min.

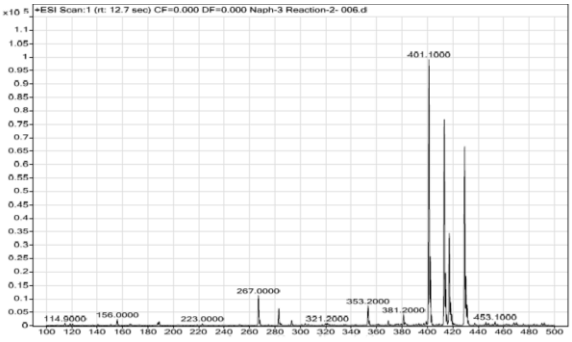


Fig.3: LC/MS/MS Spectrum of amidoxime 1 oxidation after 1hr.

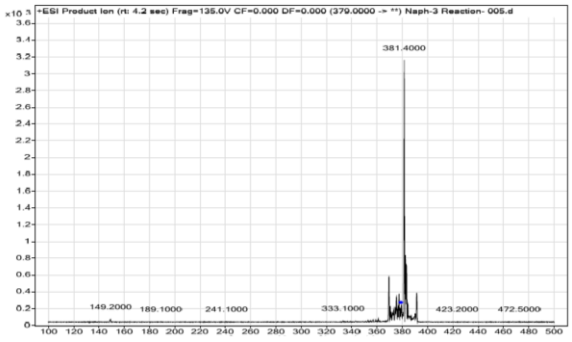


Fig. 4**:** LC/MS/MS Spectrum of amidoxime1oxidation after one week.

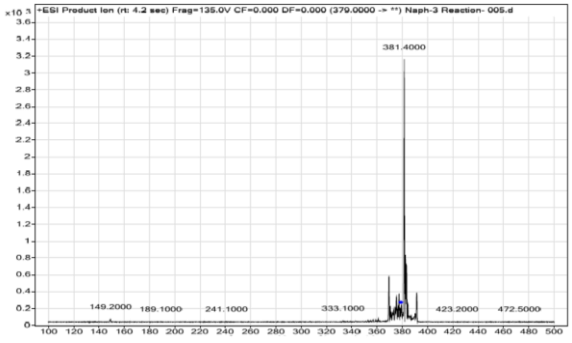


Fig. 5**:** LC/MS/MS Spectrum of amidoxime oxidative degradation after heating for 2 hr.

From the above results, it is clear that the parent amidoxime still resist oxidation and appeared at all spectra as in **figures (2-4)** as the parent and base peak. Some new oxidation product appeared in case of oxidation at room temperature specially after one week stirring namely where peaks appeared at m/z = 149, 333, 241, 253 and 267, **Figure. 4 or 5**. After heating, the parent peak appeared at m/z = 379 (2 %). Surprisingly, a fragment appeared at m/z = 333.80 as the base peak (100 %) which is considered the main degradation product of amidoxime **1**. It is important to propose structure of this new fragment; which will give important information about the oxidative degradation pathway of amidoximes.

Some literature studies about oxidation cleavage of amidoximes proved that the main oxidation products are either, amides and/or nitriles [14], dimer product [8], oxadiazoles [15], aminohydrooxadiazol [17], furoxan [16], benzaldoxime anhydride *N*-oxide [16, 17], 2-nitrosoazomethine [17], and benzaldoxime peroxide [18]. According to aforementioned literature studies, the expected structure of the main oxidation product lies among those proposed in **Figure 6**. Hence, it is expected cleavage occurs that amidoxime ester occurs unmasking the ester prodrug to the unstable amidoxime. The formed amidoxime may be not stable under the reaction conditions and can release nitric oxide to form the more stable amidine at m/z = 150 or amide at m/z = 151. Under these conditions, no dimer was found, it is expected that the oxidative dealkylation would occur forming the dealkylated dimer at m/ z = 272. The base peak appeared at m/z = 333 is expected to be the disodium salt of benzaldoxime peroxide II, the benzaldoxime peroxide itself appeared at m/z = 317, **Figure 7**, **Scheme 2**.

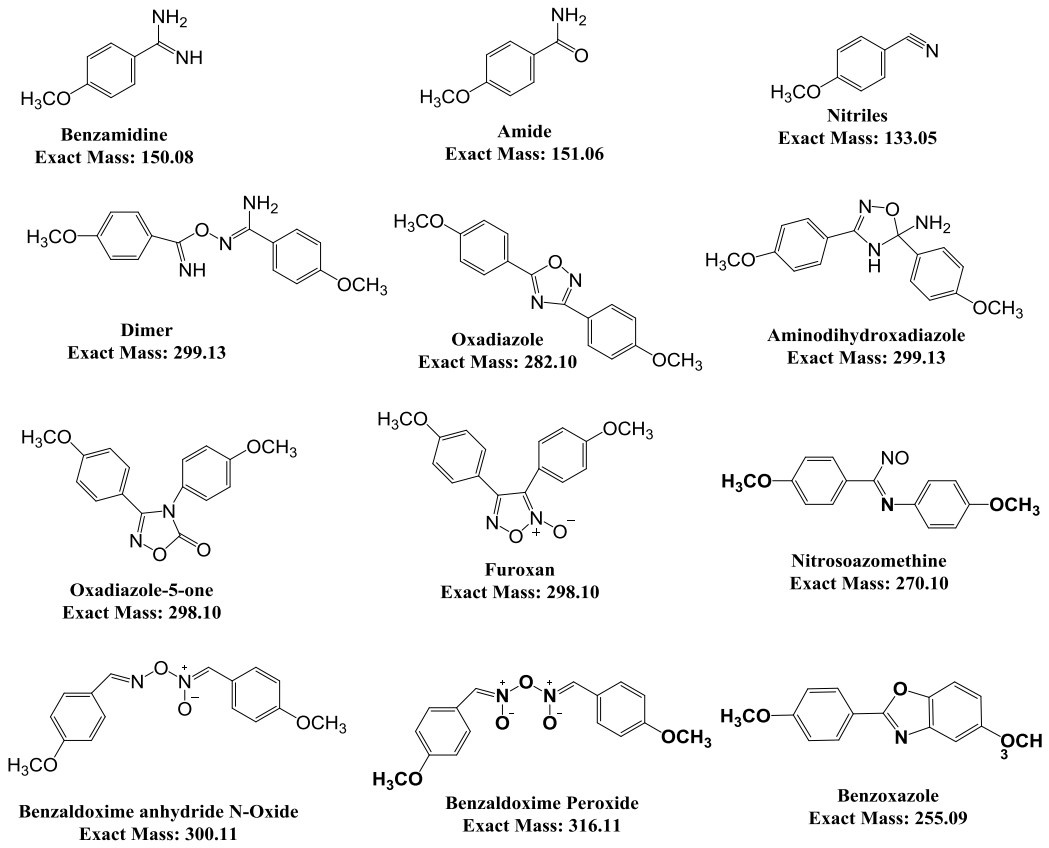


Fig. 6**:** The expected oxidation products of amidoxime according the literature

Based on the above results, it is clear that dimer formation and benzaldoxime peroxide formation are the main oxidation products in case of oxidation of amidoxime in the presence of sodium hydroxide and potassium ferricyanide mixture.

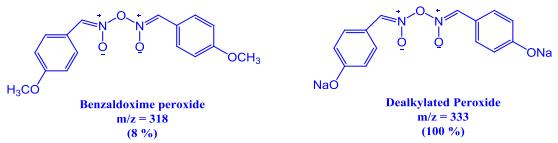
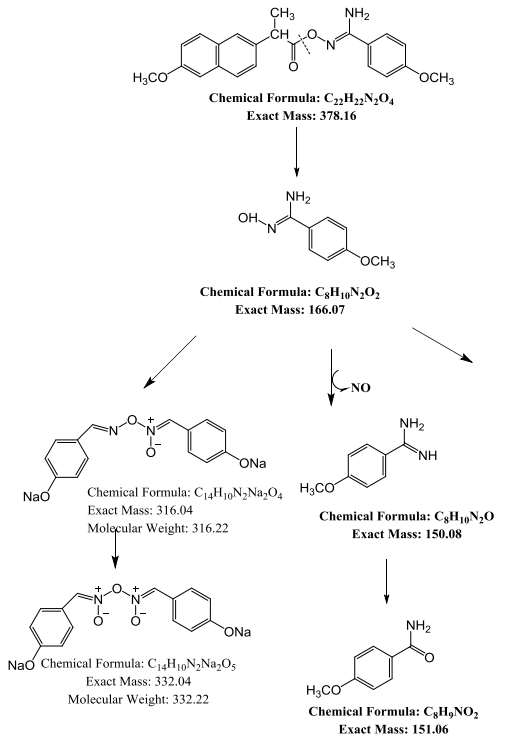


Fig. 7: The proposed degradation product



Scheme 2: Proposed degradation pathway of amidoxime prodrug 1 in sodium hydroxide

3.3 LC/MS/MS of Chromatogram at m/ = 333

Separation of the peak at 333, **Figure 8** was carried out under the following conditions

Column:	Ascentis Express RP-Amide, 5cm x 3.0 mm I.D., 2.7 mum particles.
Column Temp.	35°C
Mobile phase	[A] water with 0.1% formic acid; [B] acetonitrile with 0.1% formic acid 50:50 (A:B)
Flow rate	0.7 mL/min
Pressure	3900 psi (269 bar)
Sample	500 ng/mL in mobile phase
Injection	2µL
Detector	MS,SIM,ESI^+

Description

Analysis notes:	The application demonstrates the suitability of Ascentis express RP-Amide for the separation of androsterone and trans-androsterone. The polar embedded group provides good selectivity for these compounds based interaction between the amide carbonyl and the steroid phenol group.
Categories:	Analytical Chromatography, Steroids
Featured industry:	Clinical Pharmaceutical (small molecule)
Legal information:	Ascentis is registered trademark of sigma-Aldrich Co.LLC
Suitability:	Application for LC-MS

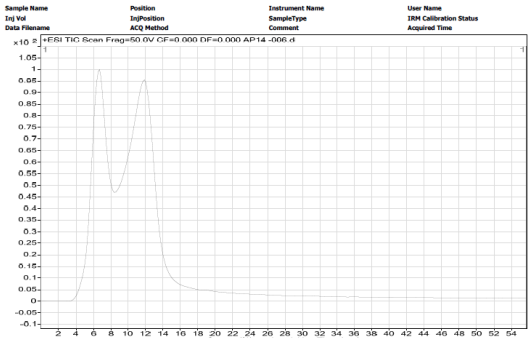


Fig. 8: The LC-MS chromatogram of benzaldoxime peroxide

2. Conclusions

Investigation of the oxidative degradation of amidoxime prodrug has been carried out using 0.4M $K_3Fe(CN)_6$/NaOH at different time and temperatures. LC-MS/MS detection for different fragments was studied. No important degradation products were detected on oxidation at room temperature but surprisingly, a characteristic base peak appeared at m/z = 333 was formed on heating the amidoxime prodrug with the used oxidizing system for 2hr. The formed stable fragment at m/z = 333 is suggested to be the disodium salt of benzaldoxime peroxide. Hence, it is clear that the ester prodrug is hydrolyzed into the corresponding amidoxime which is unstable enough to be detected by LC-MS/MS and release nitric oxide spontaneously to form the corresponding benzamidine at m/z = 150. Another pathway is suggested to be dimerization and oxidative dealkylation of the methoxy groups to form the stable disodium salt of benzaldoxime peroxide

3. Declaration

Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported; This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

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