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Structural elucidation of degradation products of a benzopyridooxathiazepine under stress conditions using electrospray orbitrap mass spectrometry – Study of degradation kinetic

Marie Lecoeur *, Valérie Vérones, Claude Vaccher, Jean-Paul Bonte, Nicolas Lebegue, Jean-François Goossens

Univ Lille Nord de France, UDSL, UFR Pharmacie, EA 4481, 3 rue du Pr. Laguesse, BP 83, 59006 Lille Cedex, France

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ABSTRACT

1-(4-Methoxyphenylethyl)-11*H*-benzo[f]-1,2-dihydro-pyrido[3,2,c][1,2,5]oxathiazepine 5,5 dioxide (BZN) is a cytotoxic derivative with very promising *in vitro* activity. Regulatory authority for registration of pharmaceuticals for human use requires to evaluate the stability of active compound under various stress conditions. Forced degradation of BZN was investigated under hydrolytic (0.1 M NaOH, 0.1 M HCl, neutral), oxidative (3.3% H_2O_2), photolytic (visible light) and thermal (25 °C, 70 °C) settings. Relevant degradation took place under thermal acidic (0.1 M HCl, 70 °C) and oxidative (3.3% H_2O_2) conditions. Liquid chromatography-mass spectrometry (LC–MS) analyses revealed the presence of ten degradation products whose structures were characterized by electrospray ionization-orbitrap mass spectrometry. The full scan accurate mass analysis of degradation products was confirmed or refuted using three tools furnished by the MS software: (1) predictive chemical formula and corresponding mass error; (2) double bond equivalent (DBE) calculation; and (3) accurate mass product ion spectra of degradation products. The structural elucidation showed that the tricycle moiety was unstable under thermal acidic and oxidative conditions since four degradation products possess an opened oxathiazepine ring. Then, a simple and fast HPLC–UV method was developed and validated for the determination of the degradation kinetic of BZN under acidic and oxidative conditions. The method was linear in the 5–100 $\mu g\,mL^{-1}$ concentration range with a good precision (RSD = 2.2% and 2.7% for the repeatability and the intermediate precision, respectively) and a bias which never exceeded 1.6%, whatever the quality control level. With regards to the BZN concentration, a first-order degradation process was determined, with $t_{1/2}$ = 703 h and 1140 h, under oxidative and acidic conditions, respectively.

1. Introduction

Benzopyridooxathiazepine derivatives are novel and potent cytotoxic compounds (Gallet et al., 2004). They belong to the class of antimitotic agents and were reported to inhibit tubulin polymerization leading to cell cycle arrest and apoptosis. Among the series of synthesized products (Gallet et al., 2009), 1-(4-methoxyphenylethyl)-11*H*-benzo[f]-1,2-dihydro-pyrido[3,2,c][1,2,5]oxathiazepine 5,5 dioxide (BZN) is a potent candidate for the treatment of cancer. Indeed, first results of *in vitro* cytotoxicity toward the L1210 leukemia cell line were very promising with IC_{50} value in the submicromolar range (IC_{50} = 9.5 nM). Nevertheless, this compound revealed poor *in vivo* activity. These results led us to investigate its chemical stability.

Stability study is essential in the development of a new drug. Indeed, drug decomposition may result in a loss of activity and the advent of possible adverse effects due to the formation of degradation products (Gómez et al., 2008). Moreover, drug instability problem leads to the choice of packaging and storage conditions required to avoid or to slow down the degradation process. Depending on the structure of the drug, degradation process may be influenced by temperature, humidity, light and occurs from oxidation conditions, hydrolysis, dehydration, dimerization, rearrangement. Hence, the International Conference on Harmonisation (ICH) guideline Q1A(R2) on Stability Testing of New Drug Substances and Products (ICH, 2003) recommends to lead decomposition studies under various stressed conditions. The susceptibility of the substance towards light, heat, humidity, hydrolysis or oxidation is currently investigated.

Owing to its selectivity, sensitivity and speed, liquid chromatography tandem mass spectrometry (LC–MS/MS) has become the method of choice for the degradation product identification

* Corresponding author. Tel.: +33 3 20 96 40 40; fax: +33 3 20 95 90 09.
E-mail address: marie.lecoeur@univ-lille2.fr (M. Lecoeur).

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(Lee and Kerns, 1999; Wu, 2000). While time-of-flight (TOF) instrumentation is often used to acquire accurate mass information (Wu, 2000; Piccinelli et al., 2009), the structural elucidation of degradation products is also possible by means of ion trap instrument. Indeed, MS^n experiments provide useful information about fragmentation mechanism (Piccinelli et al., 2009; Meetani et al., 2010). Nevertheless, this technology does not reach to the high resolution and therefore to the elemental composition of degradation products.

Since its introduction in 2005 (Makarov et al., 2006), orbitrap mass analyzer has become a mainstream mass spectrometer technique. The instrument is featured by the use of a C-trap which allows the storage of a significant ion population and then its injection into the orbitrap analyzer. Apparatus equipped with an HCD collision cell can provide some structural information. Indeed, when HCD collision cell is activated, all ions entering in the cell are fragmented and an algorithm allows yielding the fragmentation patterns similar to those of triple quadrupole mass spectrometers. The main advantage of orbitrap mass analyzer compared to triple quadripole one is to access to the high resolution leading to the knowledge of elemental composition for structural elucidation. Coupled to liquid chromatography, orbitrap mass spectrometry is a powerful combination for degradation studies.

In the present work, the chemical degradation pathways of BZN were established through a forced degradation study. LC/ESI orbitrap MS was used to characterize the degradation products. An accurate HPLC–UV method was also developed and validated to determine its degradation kinetic.

2. Experimental

2.1. Instrumentation

2.1.1. Chemicals and reagents

The benzopyridooxathiazepine derivative (BZN) was synthesized according to the procedure described in the literature (Gallet et al., 2009). Acetonitrile and formic acid were of analytical grade and were purchased from VWR – Prolabo (Fontenay sous Bois, France). Hydrochloric acid (0.1 M) was obtained from J.T. Baker (Deventer, The Netherlands), 5 M sodium hydroxide solution from Panreac (Barcelona, Spain) and hydrogen peroxide (33%) from Sigma–Aldrich (Saint-Quentin Fallavier, France). Ultra pure water (18.2 MΩ cm) was obtained by means of MilliQ apparatus from Millipore (Milford, MA, USA).

2.2. Instrumentation and chromatographic conditions

2.2.1. LC–MS analyses

2.2.1.1. HPLC–orbitrap FTMS. An UPLC–ESI-MS system including an Accela Autosampler, an Accela LC pump and an Exactive FTMS mass spectrometer (Thermo Fisher Scientific, San Jose, USA) was used for the characterization of degradation products of BZN formed under forced degradation conditions. Chromatographic separations were performed on a Hypersil Gold C18 (150 × 2.1 mm i.d., 3 μm) from Thermo Fisher Scientific. The mobile phase was a mixture of acetonitrile and water (60:40 v/v) containing 0.1% formic acid. The analytes were eluted under isocratic conditions at a flow rate of 0.2 μL min^{-1}. The temperature was set at 25 °C and the volume of injection was 5 μL. All ESI-MS experiments were acquired in the positive mode and the ESI-source parameters were as follows: sheath and auxiliary gas flow rate 60 and 10 (nitrogen, arbitrary units), respectively, spray voltage 4.5 kV, capillary temperature 350 °C, capillary voltage 42.5 V, tube lens voltage 95 V and skimmer voltage 20 V. The scan range was *m/z* 50–600. For fragmentation study, the voltage of the HCD collision cell was set at 30 eV. Prior to analysis, the orbitrap mass analyzer was externally calibrated, in the scan range *m/z* 70–650, to obtain mass accuracy with ±5 ppm. UPLC–ESI/MS system was controlled with Xcalibur software version 2.1. All data were processed using the same software which provides accurate mass from raw data. In addition, chemical formula calculator, included in Xcalibur software was used to provide chemical formula and saturation values (double bond equivalent (DBE)).

2.2.1.2. UPLC–MS/MS (QqQ). LC analysis was carried out by an UPLC system (Waters, Saint-Quentin en Yvelines, France) constituted of a quaternary pump, a degasser, an autosampler and a column oven. The chromatographic separation was performed on a reversed phase HPLC column Acquity HSS T3 C18 Waters (50 mm × 2.1 mm; 1.7 μm). The mobile phase consisted of (A) H_2O + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. The gradient program was 1% (B) for 0.5 min, from 1% to 95% (B) for 6 min then 95% (B) for 1 min. The flow rate was 500 μL min^{-1}, the injection volume was 2 μL and the column oven was maintained at 40 °C. The LC system was coupled to a DAD detector and a Xevo TQ triple quadrupole mass spectrometer (Waters, Saint-Quentin en Yvelines, France) equipped with an electrospray ionization source (ESI) through a Z-spray interface and controlled by Masslynx software (version 4.5). The mass spectrometer was operated in positive ESI mode. The source conditions were as follows: desolvation temperature: 650 °C, source temperature: 150 °C, cone gas flow (N_2): 50 L h^{-1}, desolvation gas flow (N_2): 850 L h^{-1}, capillary voltage 3 kV, extractor 3 V. The analyzer parameters were as follows: LM 1 and LM 2 resolution 3, HM 1 and HM 2 resolution 15. Argon was used as the collision gas at a pressure of 3.5×10^{-3} mbar.

2.2.2. HPLC–UV analyses

Chromatographic analyses of the forced degradation studies were performed on a Waters system equipped with a gradient quaternary 600E pump model, an on-line degasser apparatus, a 7125 Rheodyne injector (20 μL sample loop) and a 996 photodiode array detector (Milford, MA, USA). Separations were carried out on a reversed-phase Symmetry C18 (150 × 4.6 mm i.d., 5 μm) column from Waters, kept at 25 °C. The mobile phase composed of acetonitrile:water:formic acid (60:40:0.1 v/v/v) was eluted at a flow rate of 1 mL min^{-1}. Detection was performed at 318 nm. Data were

Table 1
Hydrolytic, oxidizing and photolytic stress conditions applied for BZN.

Stress condition	Solvent	Time (days)	Temperature (°C)	Decomposition products
Hydrolytic				
Neutral	H_2O	60	RT	Stable
	H_2O	60	70	Stable
Acidic	0.1 M HCl	60	RT	Stable
	0.1 M HCl	60	70	A1, A2, A3, A4
Basic	0.1 M NaOH	60	RT	Stable
	0.1 M NaOH	60	70	Stable
Oxidizing	3.3% H_2O_2	60	RT	O5, O6, O7, O8, O9, O10
Photolytic				
Visible-light	H_2O	60	RT	Stable
Solid state				
Photolytic visible light	–	60	RT	Stable
Dry heat	–	60	70 °C	Stable

RT: room temperature.