Article

Main Degradation Products of Dabigatran Etexilate Evaluated by LC-UV and LC-ESI-MS, Degradation Kinetics and *in vitro* Cytotoxicity Studies

Raquel M. Bernardi,[a] Felipe B. D'Avila,[,a] Vítor Todeschini,[b] Juliana M. M. Andrade,[a] Pedro E. Fröehlich[a] and Ana M. Bergold[a]*

[a]Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, 90610-000 Porto Alegre-RS, Brazil

[b]Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Av. Aluísio da Silva Gomes 50, 27930-560 Macaé-RJ, Brazil

The present study reports the stability profile of an antithrombotic drug: dabigatran etexilate (DAB). The drug was subjected to thermal degradation at 60 °C and products formed were investigated by liquid chromatography-UV (LC-UV) and liquid chromatography-mass spectrometry (LC-ESI-MS). Chromatographic separation of the degradation products was performed on a GL Sciences Inc. Inertsil ODS-2 column (250 mm × 4.6 mm i.d., with a particle size of 5 µm and pore size of 110 Å) with mobile phase consisting of acetonitrile and ammonium acetate buffer (pH 5.5; 10 mmol L^{-1}) (65:35, v/v) pumped at 1.0 mL min^{-1} flow rate. Column temperature was set at 30 °C and detection at 225 nm using a UV detector. LC-UV method previously validated was extended to LC-ESI-MS for the characterization of the degradation products (DP-01 and DP-02) formed, without complicated isolation or purification processes, based on retention times and confirmation of molecular weight. Degradation kinetics of DAB was also evaluated and could be described as a first-order process ($R^2 = 0.9900$). Furthermore, no evidence of cytotoxicity in human mononuclear cells was observed for DAB degraded samples.

Keywords: cytotoxicity, dabigatran etexilate, degradation kinetics, degradation products, LC-ESI-MS

Introduction

Dabigatran is a non-peptidic, synthetic, oral, potent and specific agent, a direct inhibitor of free thrombin, fibrin-bound thrombin and thrombin-induced platelet aggregation with rapid onset of action, predictable pharmacodynamic effects and pharmacokinetic characteristics.[1-3] Dabigatran is available as a pro-drug dabigatran etexilate (ethyl *N*-{[2-({[4-((*E*)-amino{[hexyloxy)carbonyl]imino} methyl) phenyl]amino} methyl)-1-methyl-1*H*-benzimidazol-5-yl] carbonyl}-*N*-pyridin-2-yl-β-alaninate methanesulfonate (DAB) (Figure 1), converted into the active metabolite by non-specific plasma and liver esterases.

DAB is the first new oral anticoagulant to become available for the prevention of stroke and systemic embolism in patients with atrial fibrillation in over 50 years.[4] It has been approved for the prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation by
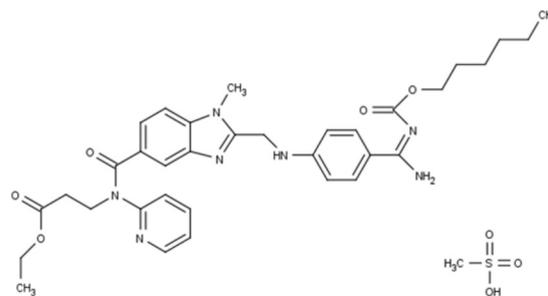

Figure 1. Chemical structure of dabigatran etexilate.

US Food and Drug Administration since October 2010 and by European Medicines Agency (EMA) since August 2011. EMA also approved it in March 2008 for the prevention of venous thromboembolic events after elective total hip replacement and/or total knee replacement.[5,6]

Venous and arterial thromboembolic diseases are still the most frequent cause of death and disability in high-income countries, and their incidence is dramatically increasing also in middle and low-income countries.[7,8] Since thrombin plays

*e-mail: felipebdavila@hotmail.com

a key role in fibrin formation and is very important for blood coagulation and platelet activation, it is a prime target for the development of anticoagulant agents for the prevention and treatment of thromboembolic disorders.[9,10]

It has been well documented that drug products will undergo physicochemical degradation during manufacturing processes and storage.[11] According to the International Conference on Harmonization (ICH), stress studies of pharmaceutical drugs can support the identification of probable degradation products. Therefore it is necessary to adopt stability-indicating methods.[12] The identification of degradation products in dosage formulations plays an important role in the drug development process.[11,13] Understanding drug degradation in formulated product is critical in pharmaceutical development because drug stability and degradation products could have a significant impact on formulation development, analytical method development, package development, storage conditions and shelf-life determination, safety and toxicology concerns. In formulation development, pharmaceutical stress testing has been widely used to predict drug stability problems, to develop stability-indicating analytical methods, and to identify degradation products and their reaction pathways.[14]

Traditional methods involving process scale-up, isolation, and purification of trace components such as degraded products and impurities are expensive and time-consuming. Besides, it is not possible to identify the chemical structure of the compounds by liquid chromatography (LC) alone.[15] Therefore, the use of hyphenated techniques, like the liquid chromatography-mass spectrometry (LC-ESI-MS) method is a versatile and attractive alternative to rapidly characterize degradation products and impurities.[16-19] Moreover, the chromatographic resolution of unresolved or co-eluting peaks detected by UV is not required to obtain product ion data for structural analysis, due to the high selectivity of MS.[15]

Most of the analytical aspects of the new anticoagulant remain unpublished. There are works describing the determination of DAB and metabolites in biological fluids by LC-MS-MS and radioactivity, but no information exists on its decomposition behavior.[4,20-23]

Previously, stress testing was performed to provide stability-indicating properties for analytical methods, and thus used to determine the analytes in the presence of degradation products by our group. The instability of DAB under thermal conditions was observed.[24] However, these components were not characterized.

The aim of the present study is to characterize the degradation products observed. Additionally, degradation kinetics was studied and a preliminary *in vitro* toxicity study was performed using mononuclear cells.

Experimental

Chemicals

Dabigatran etexilate (purity 99.5%) was purchased from Sequoia Research Products (Oxford, United Kingdom). The commercial Pradaxa® Boehringer Ingelheim do Brasil (São Paulo, Brazil) capsules containing 150 mg of dabigatran etexilate were obtained from commercial sources within their shelf life period. LC-grade acetonitrile was obtained from Tedia (Fairfield, USA). Ammonium acetate was purchased from Merck (Frankfurt, Germany). All chemicals used were of pharmaceutical grade. For all the analyses, ultrapure water was obtained using a Milli-Q Plus system Millipore (Bedford, USA).

Preparation of reference solution

Stock solution of DAB was prepared by weighing 10 mg of reference substance, transferred to individual 10 mL volumetric flasks and diluted to volume with methanol, obtaining a concentration of 1 mg mL^{-1}. The stock solution was stored at $-20\,°C$, protected from light, and daily diluted to an appropriate concentration in mobile phase to produce the final concentrations of 30 and 60 μg mL^{-1}.

LC-UV method

LC-UV method was developed on an Agilent® Infinity 1260 (Waldbronn, Germany) equipped with a 1260 quaternary pump, Standard degasser, 1260 TCC column oven, ALS 1260 autosampler and 1260 VWD detector. Peak areas were integrated automatically by computer using a ChemStation software program. UV detector was set at 225 nm. The experiments were performed on a reversed-phase GL Sciences Inc. Inertsil ODS-2 column (250 mm × 4.6 mm i.d., with a particle size of 5 μm and pore size of 110 Å) (Tokyo, Japan). LC system was operated isocratically at 30 °C, using a mobile phase consisting of acetonitrile and ammonium acetate buffer (pH 5.5; 10 mmol L^{-1}) adjusted with acetic acid 2 mol L^{-1} (65:35, v/v); filtered through a 0.45 μm Millipore membrane filter (Bedford, USA) with a flow rate of 1.0 mL min^{-1}. Injection volume was 20 μL for both standard and samples.

LC-UV method was previously validated, following the ICH guidelines.[24-26]

LC-ESI-MS method

LC-ESI-MS method was developed on the same equipment and under the same conditions as the LC-UV

method. Agilent quadrupole mass spectrometer (Waldbronn, Germany), model 6120, was equipped with an electrospray (ESI) source in positive mode. Studies were performed with capillary voltage, flow nebulizer, gas nebulizer pressure and dry temperature of 3000 V, 12 L min^{-1}, 55 psig and 300 °C, respectively. Degradation samples were injected and full scan spectra were acquired, then the selected ions were monitored in single-ion recording (SIR) mode by the LC-ESI-MS system. Data acquisition and analysis were performed using the ChemStation software.

Forced thermal degradation studies

Forced degradation protocol was performed in order to provide suitable analytical conditions for a stability study of the drug. Thermal evaluation of DAB was performed with solutions (60 μg mL^{-1}) exposed to 60 °C for 4 h (protected from light). Each time interval (0.25, 0.5, 0.75, 1, 1.5, 2, 3, and 4 h) was evaluated separately.

After thermal degradation treatment, samples were diluted with mobile phase to final theoretical concentrations of 30 μg mL^{-1} for LC-UV analysis. Subsequently, samples were diluted to 2 μg mL^{-1} with mobile phase and then analyzed by LC-ESI-MS. All samples were analyzed against a freshly prepared control sample (without degradation treatment).

Thermal degradation kinetics study

Degradation kinetics of DAB was analyzed by validated stability-indicating LC-UV[26] method and established using same experimental stress conditions described previously in section forced degradation studies.

DAB thermal degradation rate was determined by plotting the drug concentration (zero-order process), log (first-order process) and reciprocal (second-order process) concentration *versus* time. Determination coefficient (R^2) was obtained and the best observed fit indicated the reaction order. Kinetic parameter like apparent order degradation rate constant (k) and time at which 90% of original concentration of the drug is left (t_{90}) was also calculated.

Cytotoxicity assay

For cytotoxicity experiments, the peripheral blood of three healthy volunteers was collected and subsequently diluted with Hank's balanced salt solution. The human mononuclear cells were obtained by separation on Ficoll-Paque® gradient centrifuge, Sigma Aldrich (St. Louis, USA), washed and resuspended in Hank's medium to a concentration of 1.0×10^7 viable cells in

1.0 mL. The monitoring of total cells was performed using an Alphaphot-2 YS2 microscope from Nikon Instrument Inc. (New York, EUA).

All reagents, including cell suspension, commercial kit solutions Doles (Goiânia, Brazil) and samples before and after thermal degradation treatment (previously described) were distributed in a 96-well plate and maintained at 37 °C, for 30 min. Final analyzed concentrations were 10, 50, 100 and 200 μg mL^{-1} for DAB. Concurrently, a blank test (without sample addition) and positive control, which shows 100% cell death (Triton X-100 1%) were performed. Interference of methanol (final concentration of 5%) used to prepare the samples was also evaluated. All samples were performed in triplicate. Cellular injury (cytotoxicity) was determined by measuring lactate dehydrogenase (LDH) at 492 nm using a plate reader Envision, Perkin Elmer Inc. (Waltham, USA).

A one-way analysis of variance (ANOVA) followed by Dunnet's test was used for comparison of the means. Differences were considered statistically significant, if $p < 0.05$. Data analyses were performed using Prism 5.0, GraphPad Software Inc. (La Jolla, USA).

Results and Discussion

DAB degradation behavior

Stability testing is an essential part of any drug development because the quality of a drug product changes with time under the influence of environmental factors such as temperature, humidity and light.[12] The purpose of stability testing is to investigate those changes during the storage that are likely to influence quality, safety and efficacy, and to establish a shelf life for the drug product as well as recommend storage conditions. With ever increasing regulatory and compendial stringency on the control of degradation products in drug substances and finished pharmaceutical formulations, great emphasis is being laid on their characterization and analysis at trace levels.[27]

Chromatographic separation by time provided by an high performance liquid chromatography (HPLC) system combined with separation by mass achieved by a mass spectrometer often enable acquiring the structural information concerning a specific impurity or degradation product without the time-consuming isolation process. Drug degradation in formulations is a very complex and often unpredictable process. Depending on the structure of a drug molecule, its degradation process could be influenced by the composition of the formulation, the process by which the formulation is prepared, and storage conditions of the formulation including temperature, humidity, light, and

containers. In addition, the information on drug degradation products could be crucial for understanding potential toxicity or side effects associated with degradation. Therefore, a rapid and accurate elucidation of the drug degradation process has become essential to reduce drug development timelines.[13,27]

Following degradation behavior of DAB was investigated by the validated stability-indicating LC-UV[26] method and LC-ESI-MS studies. The overlay chromatograms of a DAB reference substance and DAB after thermal degradation are shown in Figure 2.

As previously mentioned, DAB was found to be labile under thermal conditions. After 4 h of exposure, approximately 75% reduction of concentration was observed (sufficient degradation to allow assessment of kinetics degradation with reliability). LC-UV chromatogram showed, besides significant decrease of peak area of DAB, rise of two signals detected by UV at 3.2 min and 5.5 min.

Based on their retention times, LC-ESI-MS studies indicated two major degradation peaks with *m/z* 500.2 and *m/z* 264.1, which were nominated DAB degradation products (DP). LC-ESI-MS chromatograms are shown in Figure 3.
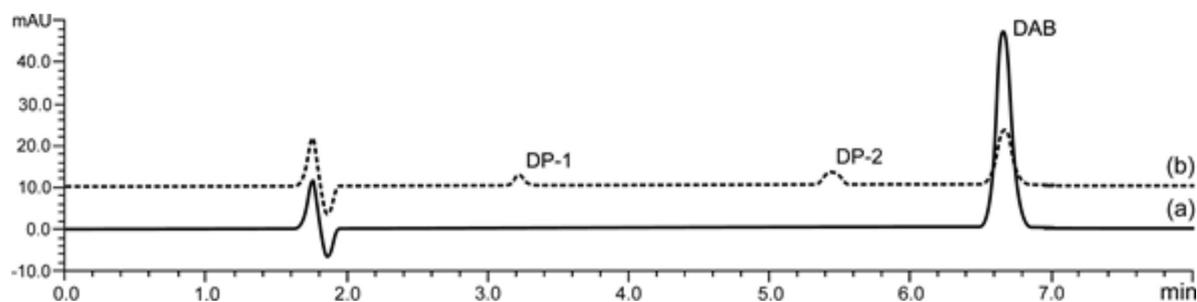

Figure 2. Overlapping LC-UV chromatograms of DAB (60 µg mL⁻¹): (a) DAB without exposure to stress; (b) DAB after thermal stress condition.
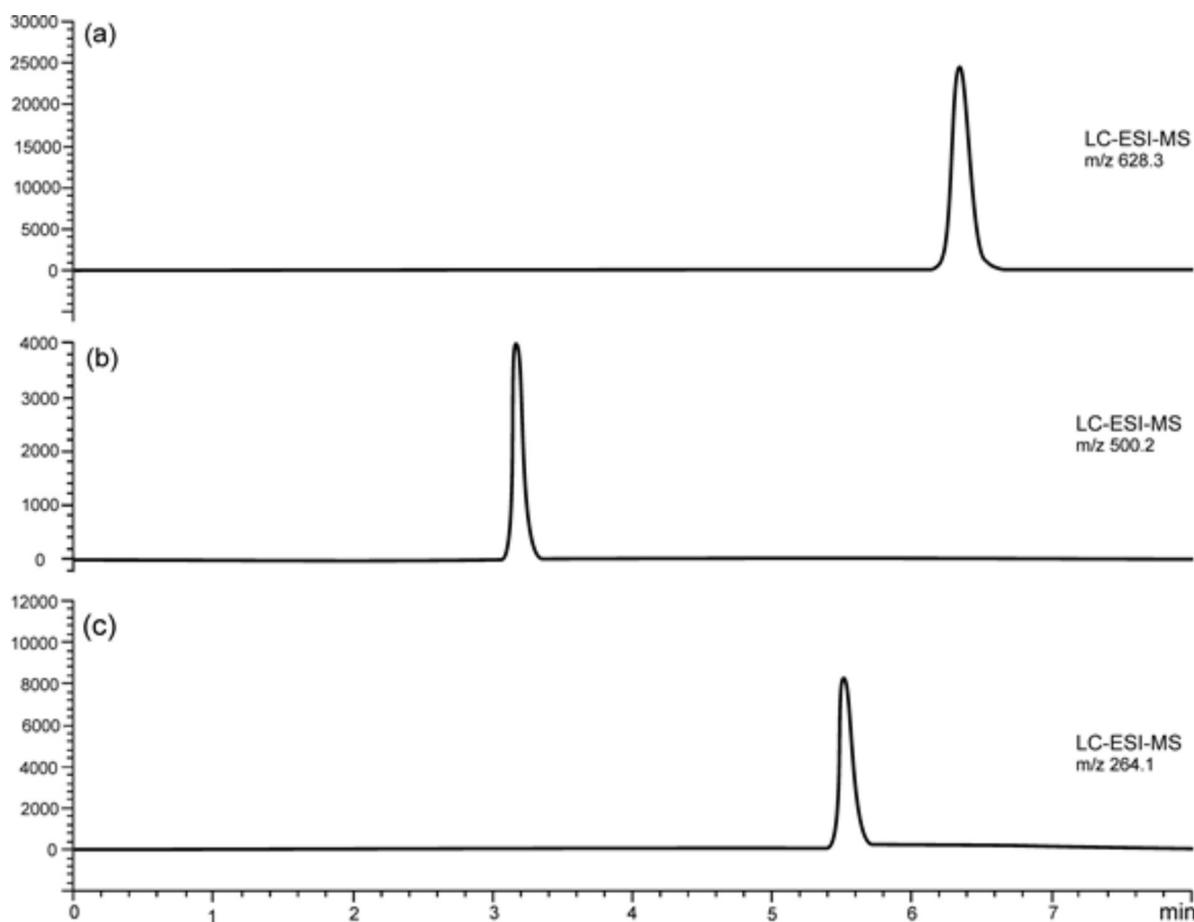

Figure 3. Representative chromatograms of DAB after thermal stress condition: LC-ESI-MS analysis: DAB at *m/z* 628.3 (a); DP-01 at *m/z* 500.2 (b); DP-02 at *m/z* 264.1 (c).

The product ion at *m/z* 500.2 (DP-01) is a carbamimidoylphenyl derivate formed by cleavage of carbamate, and was chemically characterized as ethyl 3-(2(((4-carbamimidoylphenyl)amino)methyl-*N*-(pyridine-2-yl)-1*H*-benzol[d]imidazole-5-carboxamide) propanoate. DP-01 is also identified as a DAB metabolite.[4]

The product ion at *m/z* 264.1 (DP-02) was chemically characterized as (*E*)-hexyl(amino(4-aminophenyl) methylene)carbamate and was formed by thermochemical cleavage of phenyl aminomethyl benzimidazolyl linkage to phenylamine.

Proposed structures of degradation products are shown in Figure 4.

Degradation kinetics studies

Degradation studies provide information on the quality of a drug under influence of different conditions and the degradation kinetics allows measuring speed of these processes.[28] In this study, thermal decomposition of DAB was carried out during different periods of time used for kinetic evaluation by LC-UV method.

The purpose of kinetics testing is to provide evidence on how quantity of a drug varies with time under the influence of stress factor. Furthermore, the information learned may be used to extrapolate rates of degradation and help to predict long-term stability under normal storage conditions.[29,30]

Thermal degradation kinetics was calculated through the decreasing concentration values of the remaining drug *versus* time. The remaining DAB concentration was calculated at each time interval for three replicates, comparing the mean drug concentration of the standard solution.

Through analysis of R^2 obtained by plotting drug concentration, log and the reciprocal concentration *versus* time, degradation kinetics of the drug could be better described as first-order. These findings indicate that DAB degradation speed is proportional to one component concentration.[29]

In this experimental protocol, the obtained degradation rate constants (*k*) and t_{90} were: 0.0028 min^{-1} and 37.49 min. Concentration, log and reciprocal concentration plots of remaining drug concentration *versus* time obtained during kinetics are shown in Figure 5. Additionally, ambient temperature sample was evaluated and no considerable variation was observed.

Cytotoxicity studies

Cytotoxicity assays are among the most used *in vitro* methods to predict potential toxicity of a substance in a cell culture.[31]

In this experimental protocol, non-significant differences ($p > 0.05$) were observed when DAB samples, before and after thermal degradation treatment, were compared with negative control, indicating that the presence of degradation products DP-01 and DP-02 did not reduce cell viability in all concentrations studied (Figure 6).
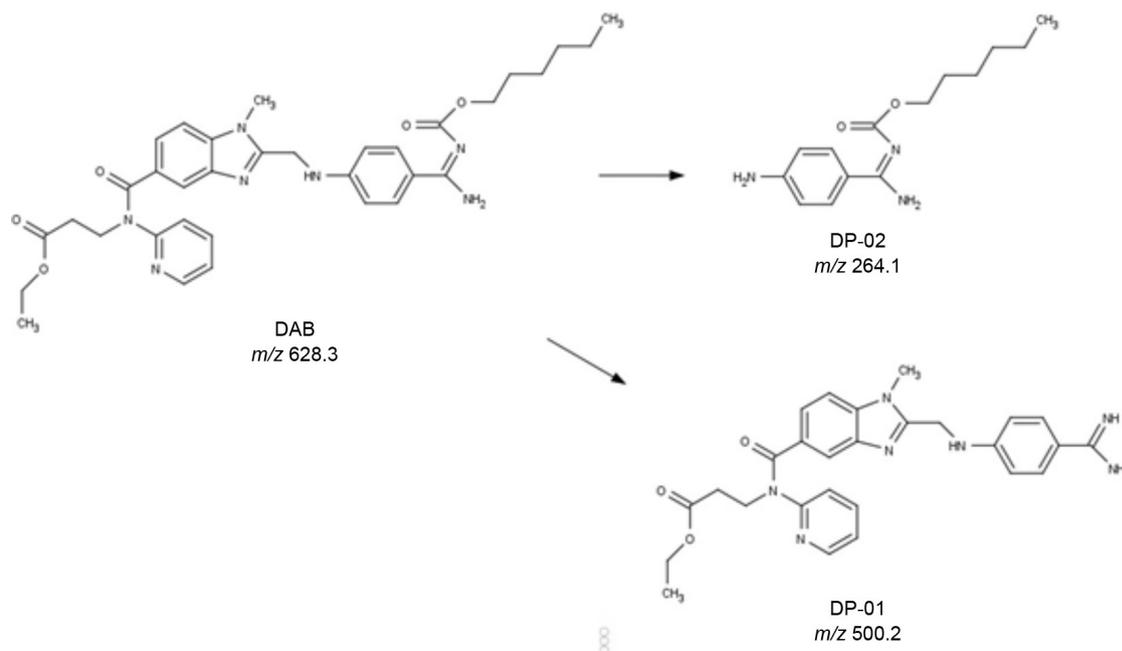

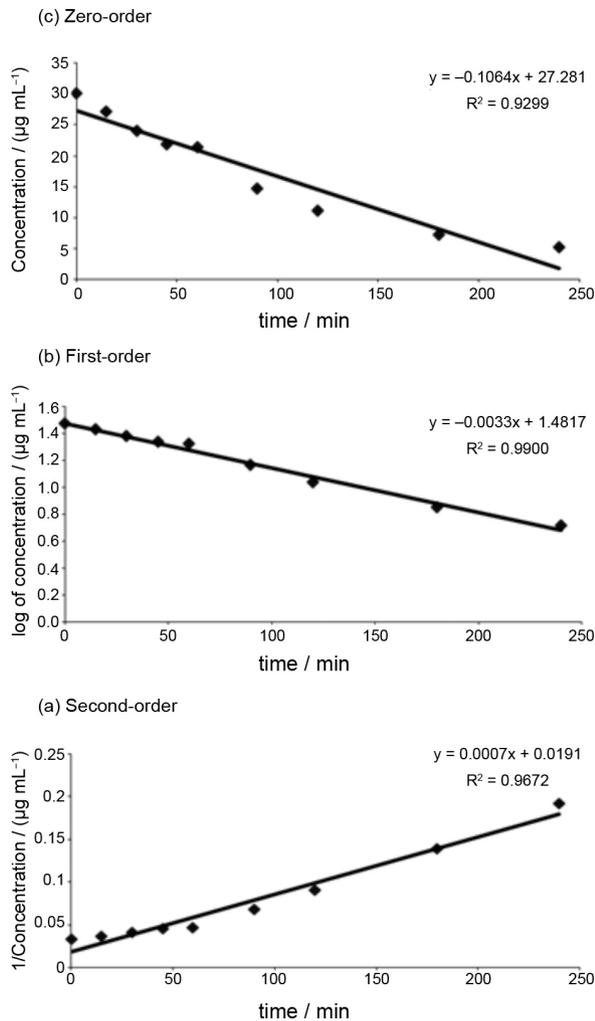**Figure 4.** Proposed structures of DP-01 and DP-02.

(c) Zero-order

$$y = -0.1064x + 27.281$$
$$R^2 = 0.9299$$

(b) First-order

$$y = -0.0033x + 1.4817$$
$$R^2 = 0.9900$$

(a) Second-order

$$y = 0.0007x + 0.0191$$
$$R^2 = 0.9672$$

Figure 5. Degradation kinetics assay and plots obtained for first-order reaction of DAB after thermal degradation.
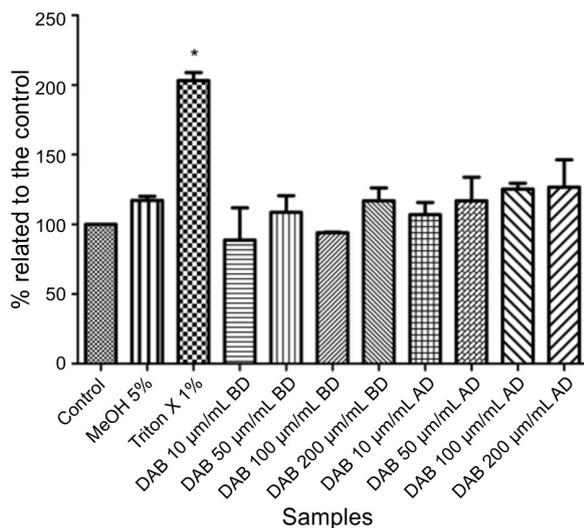
Figure 6. Cytotoxicity study of DAB, before and after degradation treatment. Values of 100% indicate total cellular viability. Triton X-100 1% represents cell injury (positive control). BD: before degradation (without degradation treatment); AD: after degradation (with degradation treatment); *significant differences between means ($p < 0.05$).

Conclusion

Evaluation of degradation products is essential for future biological and toxicological investigations, aiming at maintenance of safety and efficacy of pharmaceutical products. In this study, DAB stability study was performed using thermal stress conditions. The main degradation products formed were analyzed by LC-UV and LC-ESI-MS methods and structures were proposed. Additionally, degradation kinetics of the drug and cytotoxicity of the degraded samples were also studied, contributing to the improvement of the quality control of this drug.

Acknowledgements

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