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Identification, characterization and cytotoxicity *in vitro* assay of nitazoxanide major degradation product

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ABSTRACT

Stress studies of the broad-spectrum antiparasitic nitazoxanide were conducted in order to isolate and elucidate the major degradation product involved in thermal, acid, alkaline, oxidative and photolytic decomposition of the drug in solution and solid state. The major degradation product was identified and characterized using techniques namely LC-DAD, ^{1}H NMR, ^{13}C NMR, IR, and MS/MS. The stability of nitazoxanide raw material and nitazoxanide in tablets and in suspension powder was studied under different conditions and the results suggest the formation of the same deacetylated degradation product occur in all cases. This product was also studied in order to determine the preliminary cytotoxicity *in vitro* with mononuclear cells. Compared with nitazoxanide, the degradation product showed a higher cytotoxicity at a concentration of 40 μg mL^{-1} after 48 h of incubation, under tested conditions. Therefore, stress studies showed that special care must be taken during the preparation, manufacture, and storage of this pharmaceutical drug.

1. Introduction

Nitazoxanide (NTZ, Fig. 1) is a new nitrothiazole benzamide compound (2-acetyloxyl-N-(5-nitro-2-thiazolyl) benzamide) notable for its activity in treating both intestinal protozoal and helminthic. NTZ has been reported to be effective against a broad range of parasites, including *Giardia lamblia*, *Entamoeba histolytica*, *Cryptosporidium parvum*, and *Ascaris lumbricoides* [1]. The US Food and Drug Administration (FDA) approved the oral suspension of nitazoxanide for the treatment of diarrhea caused by Cryptosporidium species and *G. lamblia* in pediatric patients in December 2002 [2]. It is the first agent with proven efficacy for the treatment of cryptosporidiosis and giardiasis in children and adults. Its precise mechanism of action is unknown, but studies have shown that NTZ inhibits pyruvate ferredoxin oxireductase (PFOR) enzyme-dependent electron transfer reactions essential to anaerobic energy metabolism in these organisms [1,2].

NTZ is currently available in coated tablets (500 mg) and oral suspension powder (100 mg/5 mL). An official method for determination of this drug in oral formulation has not been described yet. Literature concerning the quantitative determination of NTZ is relatively limited. However, there are studies describing the determination of NTZ and metabolites in biological fluids by liquid chromatographic (LC) [3] and MS/MS [4,5]. Recently, visible spectrophotometrics [6], high-performance thin-layer chromatographic (HPTLC) [7] and LC methods for the estimation of NTZ in bulk and pharmaceuticals formulations were developed [8–10].

In these studies, some authors have developed stability-indicating methods evaluating the forced degradation of the drug. These studies also evaluated the methods capability (specificity/selectivity) for drug determination in the presence of degradation products, which were formed under oxidative, acidic, basic and photolytic conditions. In all cases, isolation and characterization of the degradation products were not performed. The study of degradation products formation in pharmaceutical formulations, their isolation, and characterization is a very important area, because it can help to understand the decomposition patterns of drug molecules, what is a valuable information about its stability [11–14]. Many factors can affect the stability of a pharmaceutical product; some of them include the stability of the active ingredient, the manufacturing process, the environmental conditions (such as heat, light and moisture during storage), as well as some chemical reactions like oxidation, reduction, hydrolysis and racemization that might occur [15,16]. Such information is used to determine storage and packaging conditions of raw material and the drug in the pharmaceutical product. According to ICH, the stress testing of

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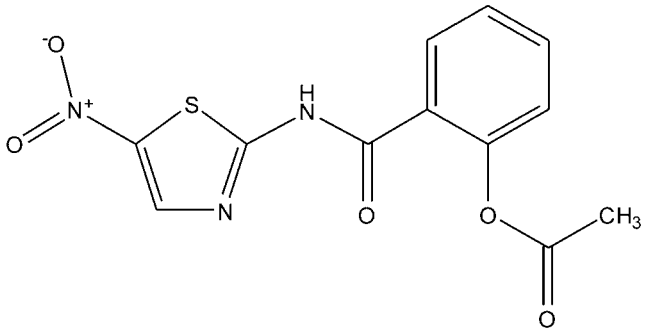


Fig. 1. Chemical structure of nitazoxanide.

drug substance can help to identify the likely degradation products, which helps to establish the degradation pathways [17].

In preliminary forced stress testing, we observed the instability of the drug under thermal, oxidative, acid, basic and photolytic conditions [9,18]. Considering the extensive degradation of NTZ in different conditions and the possibility to identify the major degradation product, the aim of the present study was to evaluate the NTZ stability under forced degradation conditions and to isolate the majoritary degradation product, employing ^{1}H NMR, ^{13}C NMR, IR, MS/MS and LC-DAD for its characterization. The isolated degradation product was also evaluated to determine the preliminary cytotoxicity *in vitro* using mononuclear cells. In agreement with ICH, it is important to evaluate the biological safety of an individual impurity or a given impurity profile, including degradation products [19].

2. Experimental

2.1. Chemicals and reagents

NTZ applied as reference substance (assigned purity, 99.53%) and raw material were kindly supplied by Shin Yang–Hangzhou Shinyang Samwoo Fine Chemical Co. (Ningbo, China).

Annita® (manufactured by Farmoquímica, Rio de Janeiro, Brazil) coated tablets for oral administration (500 mg per tablet, excipients: maize starch, pregelatinized maize starch, talc, hydroxypropyl methylcellulose, magnesium stearate, granulated sugar, primogel, purified water, eudragit, eudracolor yellow, isopropyl alcohol and acetone) and oral suspension powder (20 mg mL^{-1}, excipients: sodium benzoate, granulated sugar, xanthan gum, sodium citrate dihydrate, strawberry flavoring, citric acid, avicel RC-591 and red corant no. 33) were purchased in the local market. All chemicals were of pharmaceutical or analytical grade.

2.2. Instrumentation

The analysis of the degraded samples was carried out by using the LC method previously validated by our research group [9]. The applied column was C_{18} (250 mm × 4.6 mm, i.d., 4 μm particle size) coupled to a C_{18} guard column (4.0 mm × 3.0 mm, i.d., 4 μm). The system was operated isocratically at 25 °C using a mobile phase composed of *o*-phosphoric acid (0.1%, v/v; pH 6.0, adjusted by addition of triethylamine)-acetonitrile (45:55, v/v), at a flow-rate of 1.0 mL min^{-1}. The detection wavelength was 240 nm (DAD), and the injection volume was 20 μL.

Nuclear magnetic resonance spectra were recorded on two spectrometers: a Bruker DPX 400 MHz and a Bruker 200 MHz (Karlsruhe, Germany). Mass spectrometry analyses were conducted using a triple quadrupole mass spectrometer (Micromass, Manchester, UK), model Quattro LC, equipped with an electrospray (ESI) source operating in negative ion mode. The infrared

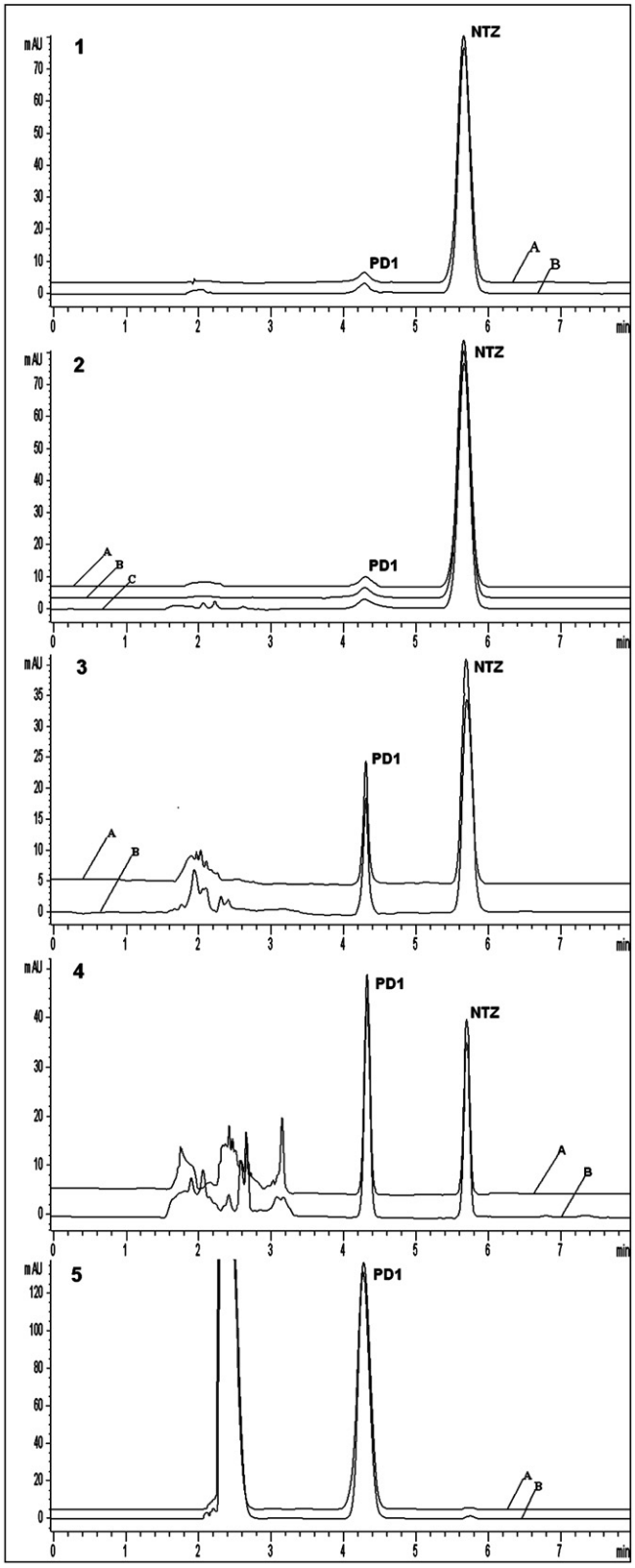


Fig. 2. Chromatograms showing decomposition of nitazoxanide in the stability studies. *Key*: (A) tablets powder, (B) oral suspension powder, and (C) oral suspension reconstituted with water. Tested experimental conditions: (1) photolytic, (2) thermal, (3) alkaline, (4) acid, and (5) oxidative.

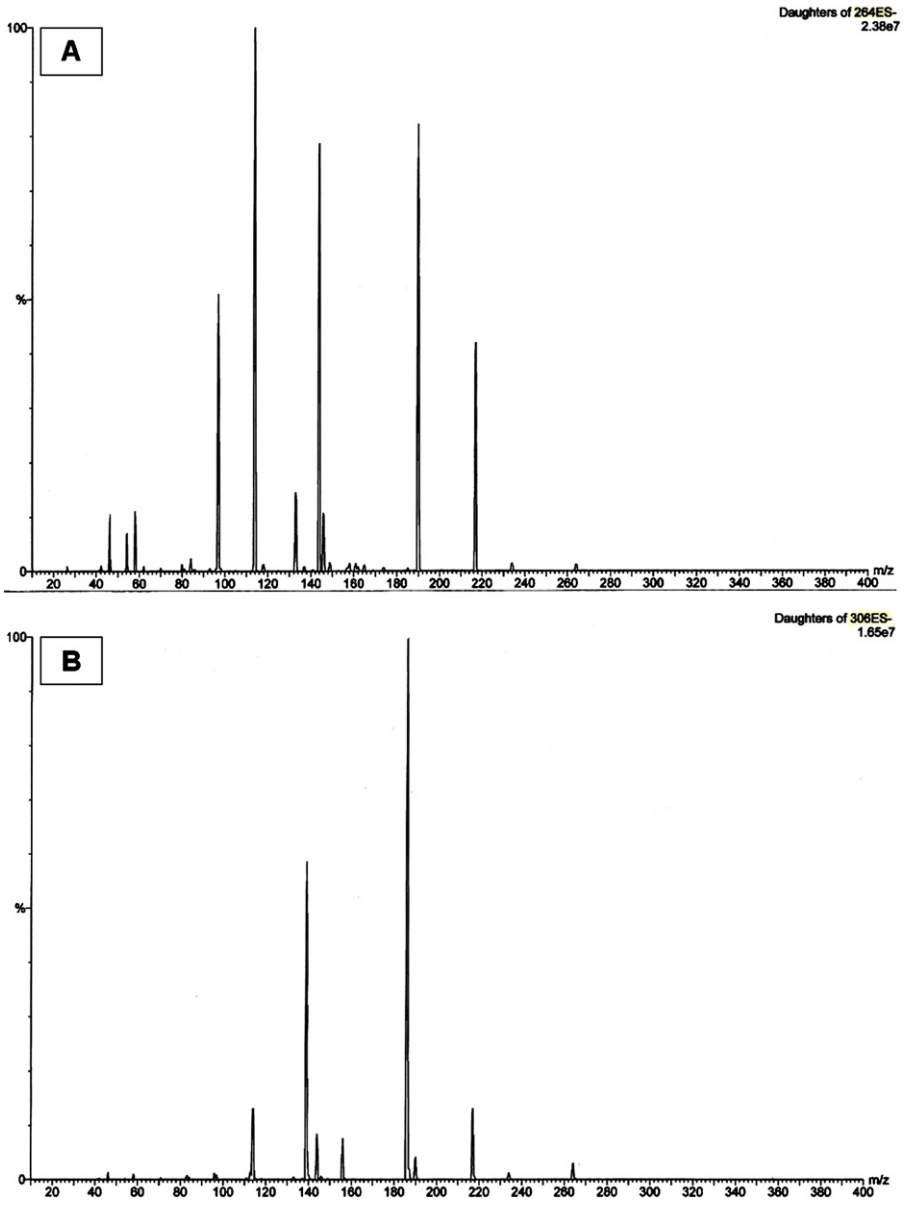


Fig. 3. MS/MS fragmentation of PD1 (A) and nitazoxanide (B).

spectroscopy was recorded in Perkin-Elmer instrument using universal ATR sampling accessory (Beanconsfield, UK). Ficoll-Paque gradient centrifuge (Uppsala, Sweden) and a FACScalibur cytometer equipped with 488 nm argon laser (San Diego, USA) were used for cytotoxic assay.

2.3. Stress degradation

The thermal degradation was conducted by exposing samples of oral suspension powder, oral suspension reconstituted with water and tablets powder in an oven at a temperature of 60 °C for 5 months. After, the samples were maintained at room temperature and aliquots were diluted with acetonitrile to achieve a final concentration of 30 $\mu g\,mL^{-1}$.

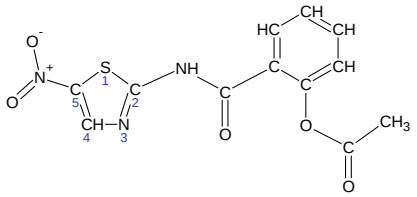
The drug is poorly soluble in ethanol and practically insoluble in water, but freely soluble in acetonitrile. The NTZ solutions for acid hydrolysis were prepared dissolving the drug (tablets powder and oral suspension powder) in small volume of acetonitrile (5%, v/v) and then diluted with aqueous hydrochloric acid to achieve a concentration of 1 $mg\,mL^{-1}$. The acid hydrolysis was performed in 0.1 $mol\,L^{-1}$ HCl at 70 °C for 2 h, after the sample was maintained at room temperature and neutralized. The study in alkaline condition was carried out in 0.01 $mol\,L^{-1}$ NaOH at room temperature (25 ± 2 °C) for 2 h and neutralized. An aliquot of each solution was diluted with acetonitrile to a final concentration of 30 $\mu g\,mL^{-1}$.

The oxidative reaction was performed dissolving the powders in a small volume of acetonitrile (5%, v/v) and then diluted with 3% H_2O_2 (1 $mg\,mL^{-1}$), at room temperature for 30 min. After, an aliquot of this solution was diluted in acetonitrile to a final concentration of 30 $\mu g\,mL^{-1}$ of NTZ.

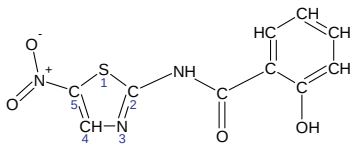
The stress degradation study in direct UV radiation (UVC – 254 nm) was performed exposing the NTZ solutions in acetonitrile (1 $mg\,mL^{-1}$) for 15 min at room temperature in a photostability chamber provided with mirrors. The distance between the lamp

Table 1
^{1}H and ^{13}C NMR chemical shift assignments for nitazoxanide and its degradation product – PD1.

Nitazoxanide



PD1



Assignments	Chemical Shifts (ppm)	
	Nitazoxanide (DMSO-d_6)	PD1 (DMSO-d_6)
^{1}H	13.56 (s.a.,1H,NH), 8.57 (s,1H,H4), 7.86-7.82 (m,1H,Ph), 7.69-7.62 (m,1H,Ph), 7.45-7.38 (m,1H,Ph), 7.29-7.25 (m,1H,Ph), 2.27 (s,3H,CH_3)	12.15 (s.a.,1H,NH), 8.67 (s,1H,H4), 7.91-7.88 (m,1H,Ph), 7.53-7.47 (m,1H,Ph), 7.06-6.97 (m,2H,Ph + 1H,OH)
^{13}C	168.57 (OCO), 164.99 (C2), 161.85 (NCO), 148.69 (C4), 142.15 (C5), 141.66 (Ph), 133.20 (Ph), 129.57 (Ph), 125.62 (Ph),125.25 (Ph), 123.21 (Ph), 20.60 (CH_3)	165.5 (C2), 161.5 (NCO), 152.3 (C4), 142.3 (C5), 141.9 (Ph), 135.05 (Ph), 130.5 (Ph), 119.82 (Ph), 117.2 (Ph), 116.5 (Ph)

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Assignments	Chemical shifts (ppm)	
	Nitazoxanide (DMSO-d_6)	PD1 (DMSO-d_6)
^{1}H	13.56 (s.a., 1H, NH), 8.57 (s, 1H, H4), 7.86–7.82 (m, 1H, Ph), 7.69–7.62 (m, 1H, Ph), 7.45–7.38 (m, 1H, Ph), 7.29–7.25 (m, 1H, Ph), 2.27 (s, 3H, CH_3)	12.15 (s.a., 1H, NH), 8.67 (s, 1H, H4), 7.91–7.88 (m, 1H, Ph), 7.53–7.47 (m, 1H, Ph), 7.06–6.97 (m, 2H, Ph + 1H, OH)
^{13}C	168.57 (OCO), 164.99 (C2), 161.85 (NCO), 148.69 (C4), 142.15 (C5), 141.66 (Ph), 133.20 (Ph), 129.57 (Ph), 125.62 (Ph), 125.25 (Ph), 123.21 (Ph), 20.60 (CH_3)	165.5 (C2), 161.5 (NCO), 152.3 (C4), 142.3 (C5), 141.9 (Ph), 135.05 (Ph), 130.5 (Ph), 119.82 (Ph), 117.2 (Ph), 116.5 (Ph)

and the samples was 10 cm. After that, this solution was diluted to 20 μg mL^{-1} of NTZ in acetonitrile.

2.4. Identification of the degradation product

The identification of the isolated degradation product was carried out by LC-DAD, ^{1}H NMR, ^{13}C NMR, IR and MS/MS spectroscopy analysis. The same analysis was also done for the nitazoxanide reference substance.

2.5. Cytotoxicity study

Human mononuclear cells were separated from the peripheral blood of three healthy donors, after receiving their written informed consent. Heparinized venous blood was diluted 4:3 with Hank's balanced salt solution (Sigma). Mononuclear cells were isolated by centrifugation on Ficoll-Paque (Amersham) gradient centrifuge and washed twice in Hank's medium. Viable cells were counted in Neubauer chamber by tripan blue exclusion. Mononuclear cells were washed and resuspended in RPMI to a concentration of 10^6 viable cells in 1.0 mL.

The isolated degradation product and NTZ reference substance were prepared in dimethyl sulfoxide (DMSO – Merck) (10.0 mg mL^{-1}) and diluted in RPMI 1640 medium (Sigma) until the concentrations of 2.0, 20.0, 40.0, 80.0, 160.0 and 200.0 μg mL^{-1}.

The cell suspensions were dispensed in two 96-well plates (100 μL in each well), and the samples were immediately added (100 μL in each well). In the first plate, the final analyzed concentration of each sample was 1.0, 10.0 and 100.0 μg mL^{-1}, in each well. In the second plate, the final analyzed concentration of each sample was 20.0, 40.0 and 80.0 μg mL^{-1}. Both assays were prepared in triplicate. Controls with the sample diluent (DMSO, 0.01%, v/v) and mononuclear cells were included. After the addition of the samples, the cells were cultivated in a humidified 5% CO_2 incubator at 37 °C for 48 h. Cell viability was determined by flow cytometry after addition of propidium iodide. Analyses were conducted on a FACScalibur cytometer equipped with 488 nm argon laser using the CellQuest Software. At least 5.000 events were collected, and the WinMDI 2.8 software was used to obtain the final results.

Table 2
Results of cell viability obtained by *in vitro* cytotoxic assay for NTZ and degraded product (PD1 – tizoxanide) against mononuclear cells.

Concentration (μg mL^{-1})	Cell viability (%) ± SD (n = 3)			
	NTZ	PD1	Control	Diluent (DMSO)
1.0	99.26 ± 0.19	98.74 ± 0.64	98.89 ± 0.27	99.13 ± 0.49
10.0	98.7 ± 0.73	97.47 ± 1.18		
20.0	90.45 ± 3.33	88.95 ± 2.43		
40.0	51.02 ± 4.03	24.76 ± 10.14		
80.0	0.33 ± 0.11	0.28 ± 0.17		
100.0	0.80 ± 0.48	0.57 ± 0.27		

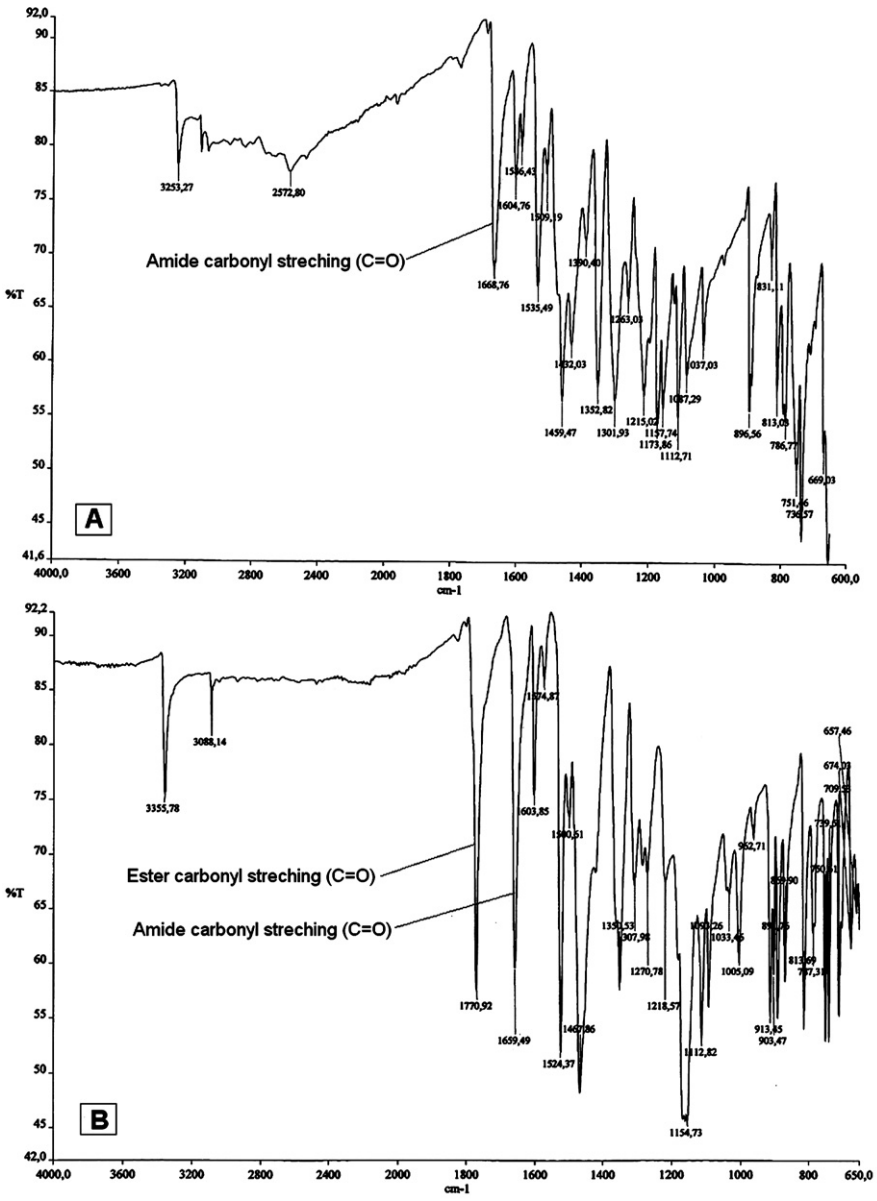

Fig. 4. Infrared transmission spectrum of PD1 (A) and nitazoxanide (B).

3. Results and discussion

The stability studies have received considerable attention in recent years because of their importance in development and quality control of pharmaceutical products. The stress testing is the first part of the stability evaluation and can help to identify the likely degradation products, establish the degradation pathways and the intrinsic stability of the molecule. In general, these goals achieved in forced degradation studies should be conducted, in most cases, under conditions that induce thermal, alkaline, acid, oxidative and photolytic drug decomposition [11–14].

In thermal decomposition study, the drug percentage present in the samples, stored at 60 °C, decreased approximately 20%. When submitted to acid and alkaline conditions the area of NTZ decreased about 70% and 60% after 2 h, respectively. In the oxidative reaction, it was observed a total drug degradation after 30 min. In the photolysis procedure, the area of NTZ decreased approximately 10% after 15 min. The different samples tested (oral suspension powder, oral suspension reconstituted with water and tablets powder) showed similar drug residual levels in all conditions tested, as shown in Fig. 2. The chromatograms obtained showed a decrease of the NTZ area and one additional majoritary peak (PD1) at 4.3 min, in all cases (Fig. 2). Analyzing the diode-array spectrum (200–500 nm) of each condition degradation products (at 4.3 min), it was verified that absorbance spectrum was the same. In all degradation studies, a yellow crystalline precipitate was formed. During oxidative degradation, it was observed only this degradation product and its concentration increased proportionally to the degradation of NTZ. This precipitate was isolated by filtration, washed with acetonitrile and dried under reduced pressure. Several solvents were tested, but the product has high solubility only in DMSO. Chromatograms, obtained from LC analysis, showed that the precipitate had the same retention time and absorption spectrum of the degradation product PD1. Also, it was observed that the product was pure. This study was also conducted with the raw material and found that the degradation profile was maintained, with the formation of the same product. Thus, we decided to perform the raw material product isolation and identification, removing any possibility of pharmaceutical excipients interference. Considering the obtained results, it is suggested the formation of the same degradated product when

nitazoxanide is exposed to thermal, alkaline, acid, oxidative and photolytic conditions.

The structure of majoritary product – PD1 was proposed according to the results obtained by ^{1}H NMR, ^{13}C NMR, MS/MS, and IR techniques.

The ^{1}H NMR and ^{13}C NMR spectra were interpreted by comparing the chemical shifts of NTZ standard with those of degradation product, as shown in Table 1.

The chemical shifts are in good agreement for the proposed structure. The decomposition applied in this study allowed a small molecular modification. The NMR preliminary analysis spectra suggest the deacetylation of NTZ molecule, due to the missing of the ester signals of carbons and hydrogens, as shown in Table 1.

The mass spectral properties of the degradation product confirm the NMR results and PD1 structure. The analyses were conducted using the direct insertion technique, without coupling with LC method. Full scan mass spectral analysis of nitazoxanide and PD1 showed deprotonated ($M-H^{+}$) molecular ions of m/z 306 and 264, respectively. Fragmentation of deprotonated molecular ions of nitazoxanide in the mass spectrometer lead to seven main product ions m/z: 264, 217, 190, 186, 139 and 114. The MS/MS spectrum of PD1 generated series of fragment ions at 217, 190, 144 and 114 (Fig. 3). The fragments obtained are in agreement with the literature [3–5,20].

According to the IR spectrum, DP1 showed a similar absorption pattern to NTZ. The largest change was the loss of the band corresponding to the ester carbonyl stretching, shown in Fig. 4.

The complete examination of the MS, NMR and IR spectra of the degradation product suggest the deacetylation of NTZ and formation of PD1 – tizoxanide (2-hydroxy-N-(5-nitro-2-thiazolyl)benzamide). According to some authors, the tizoxanide was first reported as active metabolite of nitazoxanide in plasma and its formation occur when the nitazoxanide is hydrolyzed by plasma esterases into its deacetyl derivative [3–5,20]. However, at this moment, the tizoxanide had not been reported in the literature as a degradation product from a stability study.

The cytotoxicity assay with mononuclear cells was performed to evaluate the effect of the PD1 in relation to the intact molecule, to foresee possible undesirable effects resulting from the degradation products. In this study, samples were considered cytotoxic when they presented a reduction of 50% in cell viability. The results obtained indicate that the degraded product at 40.0 $\mu g\,mL^{-1}$, after 48 h of incubation, showed cytotoxic effects with a reduction in cell viability of 75.24% (Table 2). This value is approximately 27% higher than that obtained with NTZ at the same concentration, showing a higher cytotoxic potential of the PD1 compared with the NTZ, in the tested conditions. According to some authors, following oral administration of 500 mg nitazoxanide tablet, the drug is partially absorved from the gastrointestinal tract and rapidly hydrolyzed in plasma to form its active circulating metabolite, tizoxanide. Maximum serum concentrations of tizoxanide reach approximately 10.0 $\mu g\,mL^{-1}$, and its elimination half-life from plasma is approximately 1.5 h [3–5,20]. Therefore, common dose administration has no potential to reach cytotoxic concentrations. These results corroborate the importance of conducting these biological assays in stability studies.

4. Conclusion

Nitazoxanide demonstrated to be unstable in solid form and solution for different factors, such as heat, oxidation, acid, basic and light. The major degradation product, isolated by filtration and identified by LC-DAD, MS/MS, ^{1}H NMR, ^{13}C NMR and IR, was tizoxanide originated from the deacetylation of nitazoxanide. In this study, we observed the formation of this degradation product in all tested stress conditions. In the tested conditions, the in vitro cytotoxicity assay with mononuclear cells demonstrated that the degradation product has a higher citotoxicity when compared to nitazoxanide in high concentrations. Considering the obtained results, special care must be taken during the manipulation, manufacture and storage for the pharmaceutical preparations.

Acknowledgments

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