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pH Effect on Stability and Kinetics Degradation of Nitazoxanide in Solution

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Stability studies correspond to a set of tests designed to assess changes in the quality of a given drug over time and under the influence of a number of factors. Among these factors, pH plays an important role, due to the catalytic effect that hydronium and hydroxide ions can play in several reactions. In the present study, the degradation kinetics of nitazoxanide was evaluated over a wide pH range, and the main degradation product generated was identified by LC-MS/MS. Nitazoxanide showed first-order degradation kinetics in the pH range of 0.01 to 10.0 showing greater stability between pH 1.0 and 4.0. The degradation rate constant calculated for these pH was 0.0885 x 10^{-2} min^{-1} and 0.0689 x 10^{-2} min^{-1}, respectively. The highest degradation rate constant value was observed at pH 10.0 (0.7418 x 10^{-2} min^{-1}) followed by pH 0.01 (0.5882 x 10^{-2} min^{-1}). A major degradation product (DP-1) was observed in all conditions tested. Through LC-MS/MS analysis, DP-1 was identified as a product of nitazoxanide deacetylation. The effect of pH on the stability of nitazoxanide and the kinetic data obtained contribute to a better understanding of the intrinsic stability characteristics of nitazoxanide.

Keywords: Nitazoxanide, sability, kinetic, degradation products.
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Introduction

Nitazoxanide (NTZ) is a thiazolide antiparasitic drug agent, that was first described in 1984 as a human cestocidal drug which was effective in a single dose against *Taenia saginata* and *Hymenolepis nana* [1, 2]. NTZ is active against a broad range of organisms including protozoa, helminths, viruses and bacteria. *In vivo*, NTZ is rapidly converted to its also active metabolite tizoxanide through a deacetylation [1,2]. The activity of NTZ and main active metabolite is believed to be due to its interference with the reaction of electron transfers dependent on the pyruvate:ferredoxin oxidoreductase enzyme, essential for the anaerobic energy metabolism [1-4].

Chemically, NTZ (Figure 1) can be denominated 2-acetolyloxy-*N*-(5-nitro-2-thiazolyl) benzamide with molecular formula $C_{12}H_9N_3O_5S$. It is characterized as a bright yellow crystalline powder, practically insoluble in water and poorly soluble in ethanol. NTZ is commercially available as coated tablets (500 mg) and oral suspension powder (20 mg/mL) [5].

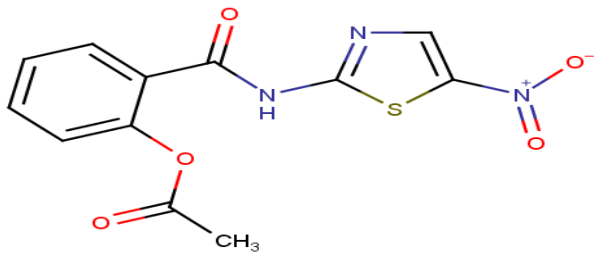

Figure 1. Chemical structure of NTZ.

The chemical stability of NTZ is reported in some studies. Malesuik et al. (2012) studied the behavior of the drug in solution and solid state under stress conditions, where the major product formed was identified as the result of NTZ deacetylation [6]. Photodegradation of NTZ was studied by Malesuik et al (2009) by exposing the drug to UV-C light at 254 nm. Under this condition, the drug showed zero-order degradation kinetics, and the suggested reactions for drug degradation is through cleavage of the amide group, forming aminonitrothiazol and acetilsalisilic acid, and reductive nitro compounds [7]. Ali et al (2012) also reported NTZ degradation when diluted in 1.0 M HCl and 0.1 M NaOH [8]. Considering the influence of pH on drug degradation and the already reported instability of NTZ in HCL and NaOH aqueous solutions, the present work aimed to evaluate the degradation kinetics of NTZ over a wide pH range, identifying the degradation products generated through LC-MS/MS.

Experimental

Chemicals and apparatus

Nitazoxanide was donated by Althaia S/A Pharmaceutical Industry (Atibaia, São Paulo, Brasil) with declared purity of 99.50%. Acetonitrile was obtained from Merck (Darmstadt, Germany). Orthophosphoric acid and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Sodium hydroxide and potassium phosphate monobasic from Synth (São Paulo, Brazil). Purified water was obtained using a Milli-Q Plus® system (Millipore, Bedford, USA).

Quantitative analyzes were performed by high performance liquid chromatography (HPLC), using a method developed and validated by Malesuik et al. (2008), according to the

following conditions: Column XBridge™ C-18 (250 x 4.6 mm, i.d., 5 μm particle size) (Waters, Wexford, Ireland); mobile phase o-phosphoric acid 0.1% (v/v) acetonitrile (45:55, v/v); pH of aqueous phase adjusted to 3.0 to obtain a better resolution between NTZ and degradation product; UV–vis detector set to 240; injection volume of 20 μL [9].
The analyzes were performed on a Prominence® Liquid Chromatograph Shimadzu, equipped with a degasser DGU-20A5, an LC-20AC pump, a SIL-20AC autosampler, an SPD-M20A PDA detector and a CTO-20 AC column oven (Shimadzu, Kyoto, Japan). LC Solution V. 1.24 SP1 system software was used to control the equipment and to calculate data and responses from the LC system.

Sample preparation

A stock solution of NTZ 400 μg/mL was firstly prepared. For it, 40 mg of NTZ was weighted and transferred to 100 mL volumetric flask, 70 mL of acetonitrile was added and the sample was taken to ultrasonic bath for 10 minutes, followed by adding the same solvent to make up to volume. Starting from the stock solution, samples were diluted to 100 μg/mL using 1 M HCl (pH 0.01) and 0.1 M HCl (pH 1.0) and in 25 mM phosphate buffer at pH 4.0; 6.0; 8.0; 10.0; 12.0. All samples were stored at 40 °C, and aliquots were withdrawn in time intervals, until a decay of approximately 50% of its initial content. The samples were diluted 1:1 in mobile phase and immediately analyzed by HPLC.

Kinetic calculations

The degraded samples were quantitatively analyzed by HPLC and the degradation rate kinetics were determined by plotting concentration of drug remaining vs time (zero-order process); ln concentration of drug remaining vs time (first-order process) and the inverse of the concentration vs time (second-order process). The regression coefficients (r) were obtained, and the best fit observed indicates the reaction order. According to the reaction order, the degradation rate constant (k), half-life time ($T_{1/2}$) and time where 90% of original concentration of the drug was left unchanged ($T_{90\%}$) were calculated.
These kinetic models can be represented by the following equations [10, 11]:

Zero-order reaction:

$$C = C_0 - kt \quad t_{1/2} = C_0 - 2k \quad t_{90\%} = 0.1C_0/k$$

First-order reaction

$$lnC = lnC_0 - kt \quad t_{1/2} = \ln 2\,/k \quad t_{90\%} = 0.106/k$$

Second-order reaction

$$1/C = 1/C_0 - kt \quad t_{1/2} = 1/kC_0 \quad t_{90\%} = 1/9kC_0$$

Where C is the final concentration, C_0 is the initial concentration, k is the reaction rate constant and t represents the reaction time.

LC-MS/MS analyzes

The LC-MS/MS analyses were performed on Waters Acquity® Ultra Performance Liquid Chromatographic system (Waters Corp., Milford, USA) coupled to mass spectrometer Micromass Q-Tof *micro* (Waters Corp., Milford, USA), equipped with an ESI interface operating in positive ion mode using a capillary voltage of 3.0 kV. The other optimum values of the ESI-Q-TOF parameters were source temperature 100 °C; Desolvation temperature 300 °C; Desolvation gas (N_2) flow 600 L/Hour. The detection was made considering a mass range of 100 – 800 *m/z*. The mobile phase consisted of water containing 0.1% formic acid and acetonitrile with 0,1 % formic acid (45:55 v/v) at a flow rate of 0.2 mL/min. A Zorbax Eclipse Plus C-18 column (2.5 x 50 mm, i.d., 1.8 μm particle size) (Agilent Technologies, CA, USA) was used.

Results and discussion

Kinetic degradation

Kinetics reactions for NTZ were studied in order to recognize the drug behavior along decomposition time, and to determine the reaction order and related parameters. The pH-rate degradation profile was determined in the pH range 0.01 - 10.0 at 40 °C. According to Yoshioka and Stella (2002) the effect of pH on degradation rate can be explained by the catalytic effects that hydronium or hydroxide ions can have on various chemical reactions [12]. The reactions dependent on the activity of hydronium and hydroxide ions, performed at constant pH, generally follow pseudo-first order kinetics. In a reaction in which hydronium ion, hydroxide ion, and water catalysis are observed can be described by:

$$k_{obs} = k_{H^+}a_{H^+} + H_2O + k_{OH^-}a_{OH^-}$$

Where k_{obs} is the sum of the degradation constants specific for each degradation pathway (acid or alkaline), and a_{H^+} and a_{OH^-} correspond to the activity of the hydronium and hydroxide ions, respectively.
For the determination of the reaction order, the degradation kinetic was calculated for all conditions through the decrease in drug concentration by time. The concentration, ln, and reciprocal concentration plots of remaining drug versus time were evaluated, and according to the correlation coefficient obtained, it can be concluded that the degradation of NTZ under the studied conditions follows first-order reaction kinetics. The obtained correlation coefficients presented in Table 1.The influence of pH on the reaction rate constant is shown in Table 2 and Figure 2. In the study we can observe a greater stability of the drug in solutions with slightly acidic pH, being more stable between pH 1.0 and 4.0. The conditions leading to the highest drug degradation were pH 0.01 and 10.0 which showed a constant reaction rate of 0.5882 x 10^{-2} and 0.7418 x 10^{-2} min^{-1}, respectively. The stability of NTZ at pH 12 at 40 °C was also evaluated. In this condition, total drug degradation occurred after 20 minutes of storage.

Table 1. Correlation coefficient values for reaction order obtained in determining the stability of NTZ at different pH values at 40 °C.

pH	Correlation coefficient (r)		
	Zero-order	First-order	Second-order
0.01	0.9856	0.9980	0.9714
1.0	0.9980	0.9984	0.9973
4.0	0.9983	0.9986	0.9982
6.0	0.9988	0.9998	0.9936
8.0	0.9955	0.9986	0.9903
10	0.9831	0.9962	0.9508

Table 2. Degradation rate constant (k), $t_{90\%}$ and half-life ($t_{1/2}$) for NTZ at different pH values and a temperature of 40 °C

pH	reaction rate constant (k) (min^{-1})	$t_{90\%}$ (min)	$t_{1/2}$ (min)
0.01	0.5882×10^{-2}	18.02	117.84
1.0	0.0885×10^{-2}	119.77	783.22
4.0	0.0689×10^{-2}	153.85	1006.02
6.0	0.2634×10^{-2}	40.24	263.15
8.0	0.3401×10^{-2}	31.17	203.81
10	0.7418×10^{-2}	14.29	93.44

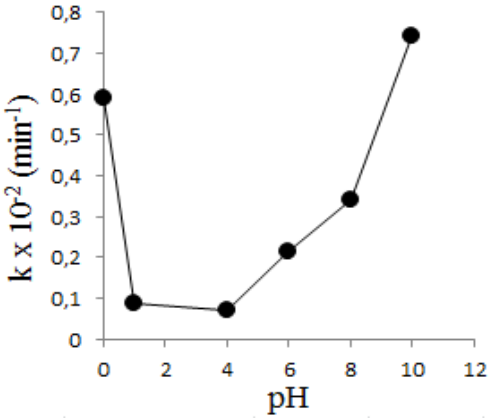

Figure 2. pH-rate profile for the degradation of NTZ at 40 °C.

Degradation products

Through chromatographic analyzes it was possible to observe the formation of a major degradation product, named DP-1. This degradation product was formed in the pH range of 0.01 - 10.0, and started to be detected in all these conditions, after 20 minutes of storage. It was also possible to visualize a second degradation product named DP-2, which started to be detected after 40 minutes in a pH 10.0 solution.

Figure 3 shows representative chromatograms of the analyzes carried out for the samples solubilized at pH 6, where it is possible to observe the increase in the concentration of DP-1 over time. This same behavior was observed in the pH range of 0.01 to 8.0. Figure 4 shows the representative chromatograms of the analyzes performed for the samples solubilized at pH 10.0, where it is possible to observe the formation of the second degradation product after 40 minutes of storage, indicated as DP-2. For samples solubilized at pH 12.0, an extensive degradation of the drug was observed, and it is not possible to calculate its kinetic parameters. Also in this condition, the formation of the degradation product DP-1 was not observed, only DP-2 was generated.

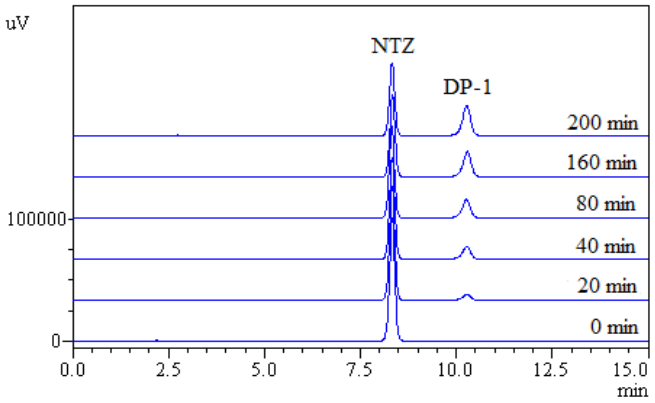

Figure 3. Chromatograms showing decomposition of NTZ in pH 6 solution at 40 °C, at different times of degradation.

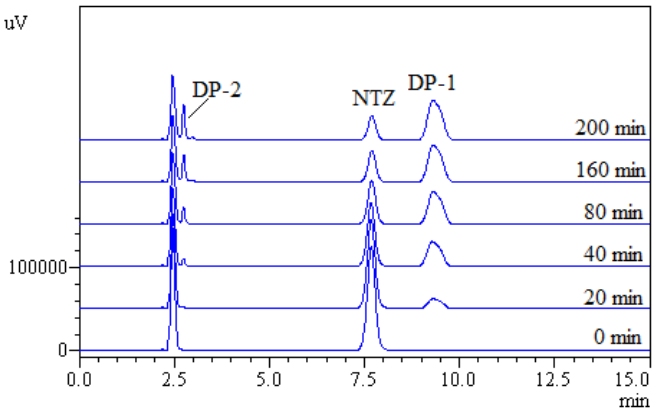

Figure 4. Chromatograms showing decomposition of NTZ in pH 10 solution at 40 °C, at different times of degradation.

In the pH range from 1.0 to 8.0, the formation of the degradation product DP-1 occurred directly proportional to the decay of the NTZ concentration, at all times analyzed, indicating only the formation of this degradation product. The same profile was not observed at pH of 0.01 and 10.0, due to the formation of a second degradation product (DP-2), visualized in the chromatographic analysis, and also the possible formation of other undetected products.

The Relative Mass Balance Deficit (RMBD) was calculated to demonstrate the correlation between the degradative losses of NTZ with the increase measured in the degradation products. The concentration of degradation products was calculated through the relative peak area, assuming both to have the same response factor in HPLC analysis.

The RMBD was calculated according to the equation [13,14]:

$$RMBD = 100\% \times \frac{(M_{ntz,0} - M_{ntz,X}) - (M_{dp,X} - M_{dp,0})}{M_{ntz,0} - M_{ntz,X}}$$

Where, $M_{ntz,0}$ represents the concentration of NTZ in the initial time, $M_{ntz,X}$ the concentration of NTZ after a determined time of degradation. $M_{dp,0}$ represents the concentration of the degradation products at the initial time and $M_{dp,X}$ the concentration of the degradation products after a determined time of degradation. For all pH tested, RMBD was calculated when the drug showed a degradation of approximately 10 % of its initial content. The calculated RMBD values are shown in Table 3.

Table 3. Values of the relative deficit of the mass balance obtained in the evaluation of the stability of NTZ at different pHs at 40 °C. considering a decay of approximately 10 % of its initial content.

pH	NTZ (%)	DPs (%)	RMBD
0.01	84.02	15.49	3.05 %
1.0	90.43	9.56	0.10%
4.0	89.55	10.33	1.10 %
6.0	91.97	8.11	0.02 %
8.0	89.94	9.80	2.49 %
10.0	84.07	15.54	2.42 %

In the case of a perfect mass balance, the RMBD will have a zero value. The same will be positive when the measured increase in degradation products is less than the loss of parent, and negative when the measured increase in degradation products exceeds the loss of parent [19,20]. In our study, considering a degradation of approximately 10% of its initial content, the low values of RMBD presented demonstrate that the degradation products are generated proportionally to the decay of the NTZ concentration. When calculated considering the final experiment time (degradation of approximately 50%), the RMBD presented higher values, possibly due to the formation of secondary degradation products.

Identification of degradation products

For the identification of the formed degradation products, mainly the major product, named as DP-1, the samples were analyzed by LC-MS/MS. A chromatogram representing the analysis is shown in Figure 5. In figure 5(A) a total ion chromatogram is shown, where two peaks are observed, in the retention times of 2.30 and 2.98 min. The peak at 2.30 refers to NTZ ($[M + H]^+$ 308), and the peak at 2.98 refers to degradation product DP-1 ($[M + H]^+$ 266). Figures 5(B) and 5(C) show the extracted ion chromatograms from masses 308 and 266, respectively.

To aid identification, additional fragmentation experiments (MS/MS) of these ions were performed fragmentation by a collision-induced dissociation (CID) process was performed with collision energy of 15 eV, and the spectra are shown in Figure 6.

The chemical structures, as well as the structure of the most probable of fragments formed are shown in Figures 7 and 8. Figure 7 shows the characteristic fragmentation of the NTZ molecule, with deacetylation giving rise to the *m/z* 266 fragment followed by the cleavage of the amide, resulting in *m/z* 121 fragment. The *m/z* 163 fragment is generated by cleavage of the amide, without deacetylation.

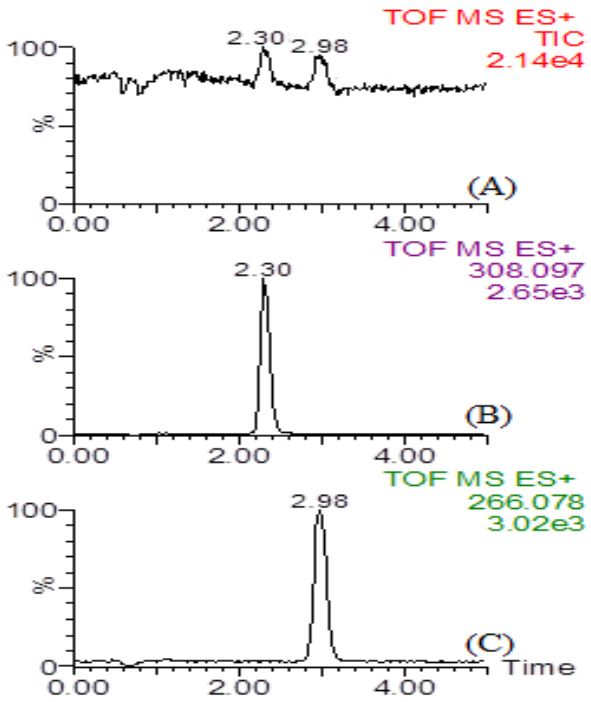

Figure 5. Representative chromatogram of the LC-MS/MS analysis of the degraded NTZ sample at pH 10.0 at 40 °C. 5(A) total ion chromatography. 5(B) and 5(C) extracted ion chromatogram *m/z* 308 and *m/z* 266, respectively.

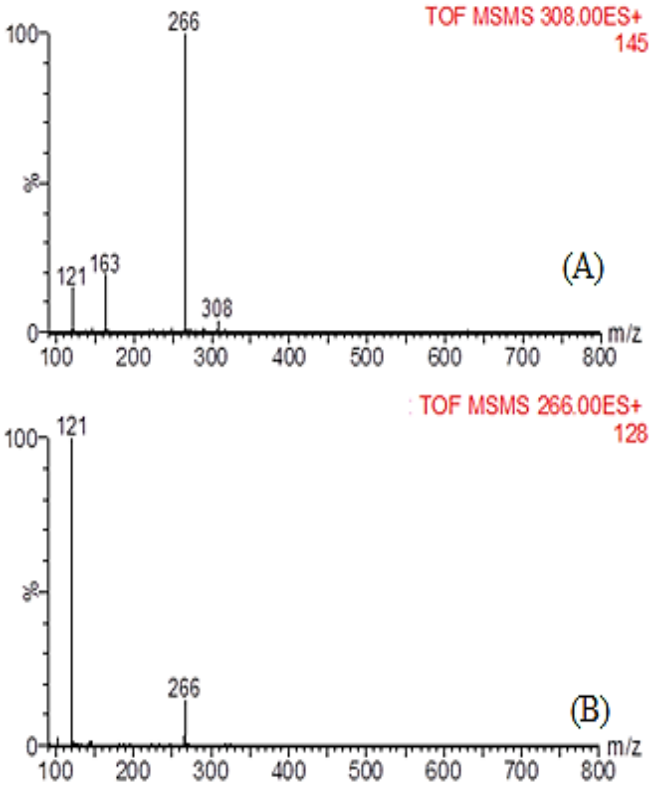

Figure 6. MS/MS spectra of $[M+H]^+$ ions from NTZ (A); and degradation product (DP-1) (B). CID at 15 eV.

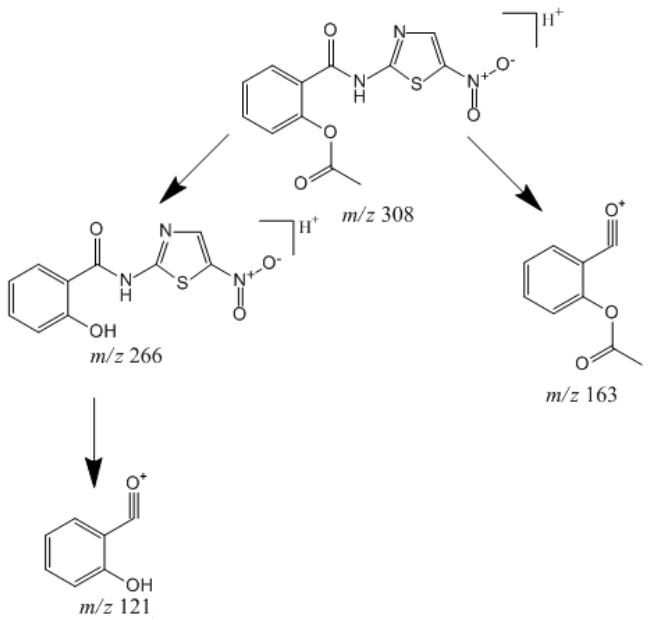

Figure 7. Chemical structure and proposed fragmentation route for the protonated NTZ molecule, identified from the MS/MS analysis.

The degradation product DP-1 shown in figure 8, has the same structure as tizoxanide, the main active metabolite of NTZ. In vivo, this reaction occurs rapidly through the action of plasma stearases [2,3]. In work carried out by Ali and collaborators (2012), the NTZ was subjected to different conditions of forced degradation, and this degradation product is also reported. In this work, it was formed after degradation in 0.1 M NaOH at room temperature, and after degradation in 1.0 M HCl under reflux [8]. In our study, this reaction occurred more quickly in an alkaline medium, due to the nucleophilic attack on carbonyl.

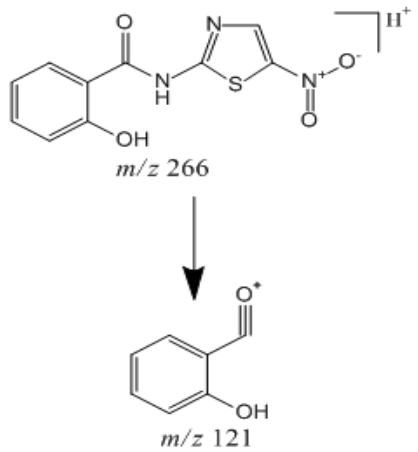

Figure 8. Chemical structure and proposed fragmentation route for the protonated DP-1 molecule, identified from the MS/MS analysis.

Conclusion

The determination of kinetic parameters of NTZ showed greater stability in acidic pH, between 1.0 and 4.0, and more unstable at pH close to neutrality and slightly alkaline. The calculated RMBD showed low values indicating the detection of all formed degradation products, and the analysis by LC-MS/MS allowed the identification of the main formed degradation product. The results obtained contribute to a better understanding about intrinsic stability characteristics of nitazoxanide.

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