

Stability study of azithromycin in ophthalmic preparations

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A stability study of azithromycin in ophthalmic preparations was developed by submission to different types of light, temperature and pH, using the biodiffusion assay (cylinder 3 x 3) for the quantifications. *Bacillus subtilis*, ATCC 9372, was used as test organism. The used concentration range was of 50 to 200 µg/mL. The study demonstrated that the drug suffered degradation when submitted to the ultraviolet light, germicide light, solar luminosity, acid solution, basic solution and hydrogen peroxide solution. The results were analyzed by the analysis of variance (ANOVA).

Uniterms: Azithromycin/stability study. Bioassay. Photodegradation. Ophthalmic preparations.

O estudo de estabilidade de azitromicina em preparações oftálmicas foi realizado após exposição a diferentes tipos de luz, temperatura e pH, utilizando o método de difusão em ágar (cilindros 3 x 3) para as quantificações. A faixa de concentração foi de 50 a 200 µg/mL. O estudo demonstrou que o fármaco sofreu degradação quando submetido às luzes ultravioleta, germicida e solar, e a soluções ácida, alcalina e de peróxido de hidrogênio. Os resultados foram analisados através da análise da variância (ANOVA).

Unitermos: Azitromicina/estudo de estabilidade. Bioensaio. Fotodegradação. Preparações oftálmicas.

INTRODUCTION

Stability is defined as the capacity of a drug substance or drug product to remain within established specifications to maintain its identity, strength, quality, and purity throughout the retest or expiration dating periods (Brasil, 2005; Nudelman, 1975; Yoshida, Sato, Moroi, 1993). Due to their possible composition, pharmaceuticals are especially sensitive to environmental factors. Strict storage conditions are necessary for the maintenance of integrity and product activity (Waterman, Adami, 2005).

Stability test of an active substance or finished product provides evidence on the drug quality and its variation with the time, influenced by a variety of environmental factors such as temperature, humidity and light (Tonnesen, 2001). The Brazilian stability studies guidelines were published at the document RE nº 1, on July 1st, 2005, which determined the specifications for this specific analysis (Brasil, 2005).

The azithromycin (Figure 1), is 1-oxa-6-azacyclo-

pentadecan-15-one, 13-[(2,6-dideoxy-3-C-methyl-3-O-methyl- α -L-ribo-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,0,12,14-heptamethyl-11-[3,4,6-trideoxy-3-(dimethylamino)- β -D-xylo-hexopyranosyl)oxy], dihydrate, is an azalide, a subclass of macrolide antibiotics. The macrocyclic lactone expansion and the presence of nitrogen atom in the lactone ring are responsible for the best pharmacological and microbiological azithromycin characteristics. Azithromycin has similar antimicrobial spectrum as erythromycin, but is more effective against certain gram-negative bacteria, particularly *Haemophilus influenzae*.

Azithromycin prevents bacteria from growing by interfering with their protein synthesis. Azithromycin binds to the 50 S subunit of the bacterial ribosome, and thus inhibits translation of mRNA. Nucleic acid synthesis is not affected (Korolkovas, 2000; United States Pharmacopeia, 2005; Zanini *et al.*, 1998).

Azithromycin is commercialized by Brazilian pharmacies as ophthalmic solution, with concentration of 1.667 mg/mL in physiological buffered vehicle (Zanini *et al.*, 1998).

For many aqueous drug solutions, stress conditions such as pH, temperature, light and oxidizing atmosphere

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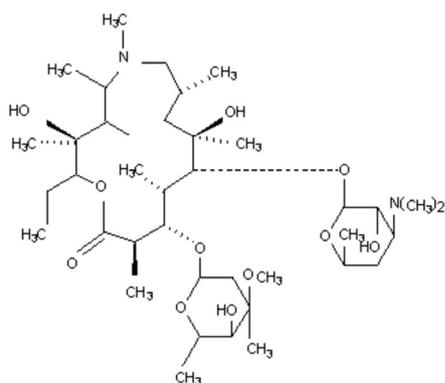


FIGURE 1 - Chemical structure of the azithromycin ($C_{36}H_{72}N_2O_{12} \cdot H_2O$; M_r 785.02; CAS 83905-01-5).

can sometimes affect the maximum stability of the drug (Kommanaboyina, Rhodes, 1999). For this reason, the aim of the study was to evaluate the stability of azithromycin in ophthalmic solutions face to several stress conditions, such as temperature, light and acid, alkaline and hydrogen peroxide solutions.

MATERIAL AND METHODS

Material

Azithromycin reference substance was gently supplied by the Ariston Laboratory (São Paulo, Brazil). Azithromycin raw material and ophthalmic solutions were gently supplied by the Bandeirantes Pharmacy (Araraquara, Brazil). Azithromycin ophthalmic solutions were claimed to contain 1.667 mg/mL (as anhydrous base) of the drug. All used chemicals were of analytical grade.

Preparation of azithromycin reference substance

The standard and sample stock solutions were prepared by dissolution in water (100 mg in a 50 mL volumetric flask) and then diluted with potassium phosphate buffer solution pH 8.0 and sonicated by 10 min (2000 μ g/mL). Standard solutions were freshly prepared in the concentrations of 50.0, 100.0 and 200.0 μ g/mL by transferring aliquots of 250.0, 500.0 and 1000 μ L, respectively, of the stock solution to 10 mL volumetric flasks and diluting to volume with potassium phosphate buffer solution pH 8.0.

Potassium phosphate buffer solution

Amounts of 16.73 g of dipotassium hydrogen phosphate and 0.52 g of potassium dihydrogen phosphate were dissolved in 1000 mL of distilled water (pH = 8.0).

Preparation of the azithromycin solutions

A commercial ophthalmic solution composed by azithromycin 25.0 mg in 15.0 mL of physiological solution with concentration of 1.667 mg/mL was used to prepare the sample solutions: 3.0 mL, 6.0 mL, and 12.0 mL of azithromycin commercial ophthalmic solution (10 samples) were transferred to a 100 mL volumetric flask and diluted to volume with potassium phosphate buffer solution pH 8.0, to give final concentrations of 50.0, 100.0 and 200.0 μ g/mL, respectively.

Organism and inoculum

The quantitative measurement of the azithromycin was carried out through diffusion in a test-agar using *Bacillus subtilis* ATCC 9372 as indicator microorganism. The cultures of *B. subtilis* ATCC 9372 were stored on Grove-Randall number 1 agar (Merck, Brazil) at freezer and peeled to another Grove Randall number 1 agar (24 h before assay) that was kept at 37°C. The bacteria were suspended in brain heart infusion broth (Merck, Brazil) using a glass homogenizer. Diluted culture suspensions of 25% \pm 2% turbidity were obtained at 580 nm (Farmacopéia Brasileira, 1988), using a suitable spectrophotometer (Merck SQ118, Germany) and a 10 mm diameter test tube as an absorption cell against brain heart infusion broth as blank.

Biodiffusion assay

The agar was composed of 2 separate layers, and only the upper layer was inoculated. The Grove-Randall number 11 agar (20 mL) was poured into microbiological petri dish (100 mm x 20 mm) for the base layer. After solidification, the inoculum (5.0 mL) was distributed to the surface of each base layer. Six stainless steel cylinders of uniform size (8 mm x 6 mm x 10 mm) were placed on the surface of inoculated agar and an exactly measured volume (200 μ L) of the corresponding dilution was deposited into each cylinder. Three alternated cylinders were filled with 200 μ L of reference substance and sample solutions. In this way, each dish contained three cylinders with three different azithromycin reference substance dilution and three cylinders with the corresponding sample concentrations. The bioassay plates were aerobically incubated at 35 \pm 2 $^{\circ}$ C for 18 h. The diameter (in mm) of growth inhibition zones was carefully measured using a caliper (Mitutoyo, Japan) with a precision of at least 0.1 mm. Six plates were used in each assay as shown in Figure 2.

The percentage activity of azithromycin in ophthalmic solutions was calculated by Hewitt equations

(Hewitt, 1977). The assay was statistically calculated by linear parallel model and by means of regression analysis, being then verified using analysis of variance (ANOVA).

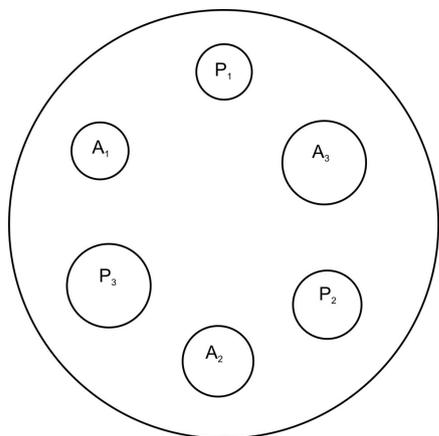


FIGURE 2 - Delineation 3 x 3, demonstrating the standard solutions (P) and sample solutions (A) disposed in Petri dishes, where P₁ (50.0 µg/mL); P₂ (100.0 µg/mL); P₃ (200.0 µg/mL) and A₁ (50.0 µg/mL); A₂ (100.0 µg/mL); A₃ (200.0 µg/mL).

Azithromycin degradation tests

Sample solutions (50.0, 100.0 and 200.0 µg/mL) prepared for the biodiffusion assay were stored in glass bottles closed and submitted the following expositions: temperature (50 and 100°C per 20 and 60 days); ultraviolet light 284 nm (10 cm from the source for 1, 2, 7, 14, 21 and 28 days); solar light (for 2, 5, 7 and 10 days); ultraviolet germicide light 254 nm (10 cm from the source for 2, 6, 12, 24, 36, 48 and 72 hours). The lamps used in the test were UV Lamp SM808 (Zhejiang, China) and UV Germicidal Lamp (Zhejiang, China).

Portions of 10 mL of the ophthalmic solutions (1.667 µg/mL) were transferred to three flasks in order to develop the chemical degradation study with 0.1 mol/L hydrochloric acid, 0.1 mol/L sodium hydroxide and 0.3 % (v/v) hydrogen peroxide solutions:

Acid degradation

5.0 ml of 0.1 mol/L hydrochloric acid solution was added to the ophthalmic solution (1.667 µg/mL). The solution was warmed in a bath at 70 °C, ± 2 °C, during 6 hours, cooled and neutralized with 5.0 mL of 0.1 mol/L sodium hydroxide solution. Portions of 1.5, 3.0 and 6.0 mL were transferred to 25 mL volumetric flasks and diluted to volume with purified water to give final concentrations of 50.0, 100.0 and 200.0 µg/mL, respectively.

Alkaline degradation

5.0 ml of 0.1 mol/L sodium hydroxide solution was added to the ophthalmic solution (1.667 µg/mL). The solution was warmed in a bath at 70 °C, ± 2 °C, during 6 hours, cooled and neutralized with 5.0 mL of 0.1 mol/L hydrochloric acid solution. Portions of 1.5, 3.0 and 6.0 mL were transferred to 25 mL volumetric flasks and diluted to volume with purified water to give final concentrations of 50.0, 100.0 and 200.0 µg/mL, respectively.

Degradation with hydrogen peroxide

5.0 mL of 0.3 % (v/v) hydrogen peroxide solution was added to the ophthalmic solution (1.667 µg/mL). The solution was warmed in a bath at 70 °C ± 2 °C, during 6 hours, cooled and added 5.0 mL of purified water. Portions of 1.5, 3.0 and 6.0 mL were transferred to 25 mL volumetric flasks and diluted to volume with purified water to give final concentrations of 50.0, 100.0 and 200.0 µg/mL, respectively.

After the sample expositions, the assays for drug quantification were developed by biodiffusion assay (Salgado, Roncari, 2005) and the potency calculated by Hewitt equations (Hewitt, 1977). The assay was statistically calculated by linear parallel model and regression analysis using analysis of variance (ANOVA) (AOAC, 1990).

RESULTS AND DISCUSSION

Pharmaceutical products quality is of vital importance for patient safety. The presence of impurities may influence the efficacy and safety of pharmaceuticals. Impurities and potential degradation products can cause changing of chemical, pharmacological and toxicological properties of drugs, having significant impact on product quality and safety (Tonnesen, 2001). Stability studies of pharmaceutical preparations become necessary, to assure product purity, safety, potency and effectiveness until use (Nudelman, 1975).

Stress studies are said to be useful to determine if accidental exposures to conditions other than those proposed have been deleterious to product; in order to evaluate which specific test parameters would be the best indicators of product stability; and to reveal the degradation patterns. Stress conditions are also mentioned in literature on establishment of stability-indicating assays. The total information was tabulated for different types of activities, such as hydrolysis in acid, alkaline and neutral conditions, oxidation and photolysis (ICH, 1996).

According to ICH (1996), the most used stress

conditions for decomposition study in acid medium were based on hydrochloric acid at strength of 0.1 mol/L. The temperature range varied from 40 °C up to 110 °C, and drugs were kept under temperature conditions for periods ranging from few minutes up to 2 months.

The stress conditions used for the hydrolysis of drugs under alkaline conditions run in parallel to those used for acid conditions. Sodium hydroxide is most used, at strength of 0.1 mol/L. Just like acidic degradation, a lot of variation is observed respecting time and exposure temperature of drugs to alkali.

The hydrogen peroxide seems to be much more popular for the purpose than any other oxidizing agents. The used strength of hydrogen peroxide varies between 1% to 30%. In some drugs, extensive degradation is seen when exposed to 3% hydrogen peroxide, for very short time periods, at room temperature. These conditions are not expected to show any change, even in the presence of high dose of oxidizing agents. The drug may be declared to be “practically stable” if no products are formed on subjecting the drug to this condition.

Light exposure can induce chemical degradation in susceptible molecules and the most obvious result of drug photodecomposition is the product loss of potency (Tonnesen, 2001). Mostly, drugs are exposed to short/

long wavelength UV light, or fluorescent light of varying illumination. Efforts are usually made to maintain the temperature around room temperature. The period of exposure ranges from a few hours to several months, depending on the light source intensity. Like oxidation, based on whether a drug is photolytic or not, varied types of decomposition behaviors are seen. Photolability studies are done on drugs in either solid or solution forms (ICH, 1996).

The sample solutions presented loss of the drug potency after the stress expositions, according to Table I. The ultraviolet light (284 nm) showed considerable potency loss (18.86%) after 28 days when compared with other exposition types, such as temperature, ultraviolet germicide light (254 nm) and solar light. However, the chemical degradation by acid, alkali and hydrogen peroxide presented almost total potency loss of the drug (11.5%), indicating drug susceptibility for direct degradation. No interference from the sample excipients and vehicles could be observed under experimental conditions.

The biodiffusion assay can reveal subtle changes not demonstrated by chemical methods, such as chemical degradation, and allows evaluation of azithromycin potency (Andrews, 1999; Breier *et al.*, 2002; Turcinov, Pepeljnjak, 1998). Figure 3 represents the drug potency decline after different types of expositions: temperature

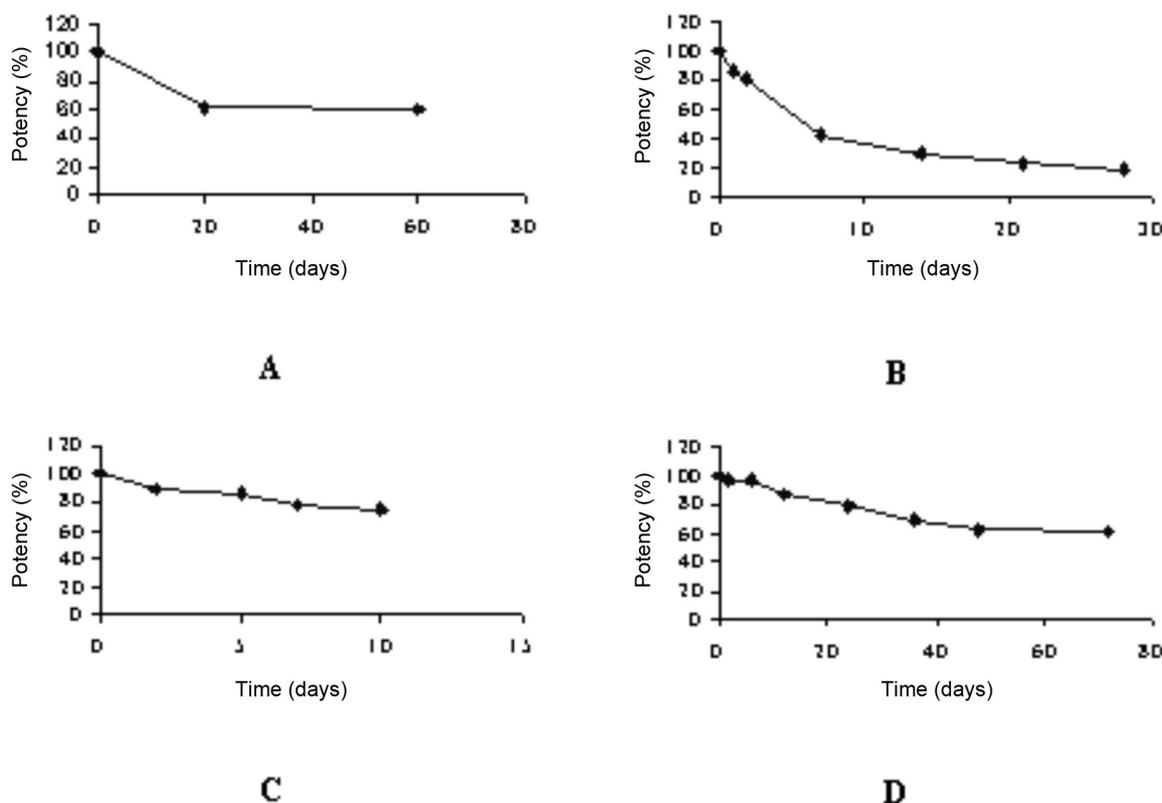


FIGURE 3 - Azithromycin potency decline after different types of expositions: temperature 50 °C (A); ultraviolet light (B); solar light (C); ultraviolet germicide light (D).

50°C (A); ultraviolet light (B); solar light (C); ultraviolet germicide light (D).

According to the Figure 3 the loss of potency was considerable when the samples were exposed to temperature (A) and ultraviolet light (B), showing the product susceptibility to light and thermal degradation.

The inhibition zones of azithromycin without degradation are represented in Figure 4, and the Figures 5, 6, 7 and 8 showed the zone diameters for the azithromycin under chemical degradation (acid, alkaline and hydrogen peroxide solutions), UV light (284 nm), UV germicide light (254 nm) and solar light, respectively.

There were clear reduction of zone diameters, specially for the azithromycin under chemical degradation, when compared with the inhibition zone at zero time (product not degraded) (Figures 4 and 5). The Figures 6, 7 and 8 showed the photodegradation of azithromycin. According to figures, the UV light (284 nm) exposition was more deleterious than UV germicide light (254 nm) and solar light expositions. Biological products such as antibiotics are particularly sensitive to stress conditions.

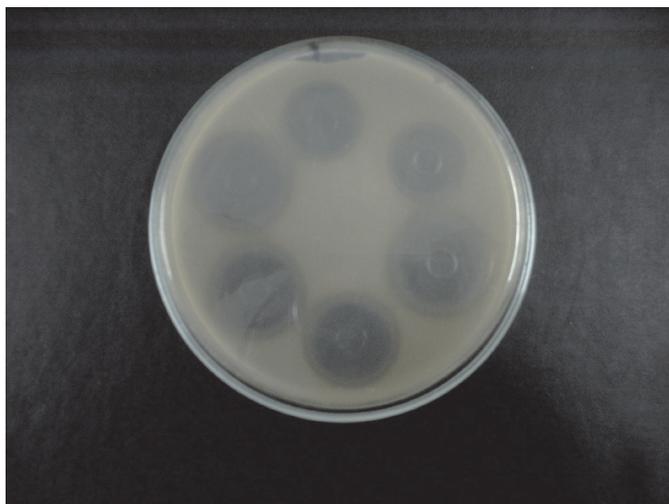


FIGURE 4 - Zone diameters for the azithromycin at zero time.

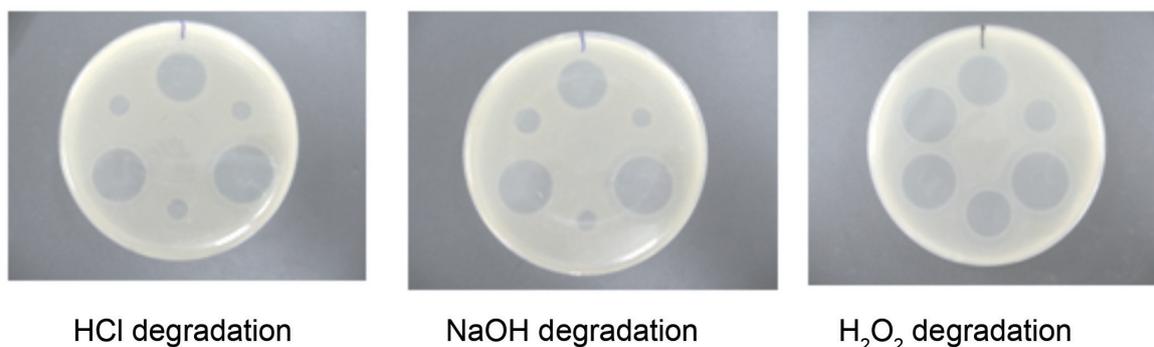


FIGURE 5 - Zone diameters for the azithromycin under different chemical conditions of degradation studies (acid, alkaline and hydrogen peroxide solutions).

Their main routes of decomposition can also vary, depending on the used chemical agents.

Segregation and quantification of antibiotic components by chemical methods such as HPLC, although precise, may not provide a true indication of their biological activity, and attempts to correlate antibiotic bioassay results with those from chemical methods have been usually disappointing. So, these results demonstrate that bioassays continue to play an essential role in the manufacture and quality control of antibiotic medicines.

The biodiffusion assay used for the azithromycin quantification was validated by Salgado and Roncari (2005). The statistical analysis demonstrated the applicability of the method: there were no deviations from parallelism and linearity with results obtained ($P < 0.05$). Table I shows the results obtained for azithromycin determinations after the stress expositions.

The stability study has as main goals to explore the nature, kinetic course and degradation of drugs, as well as determining for how much time products can conserve their original constitution (Marona, Zuanazzi, Schapoval, 1999; Marona, Schapoval, 2001). Quality control in the pharmaceutical industry is very important to assure effectiveness and confirm the quality of the medicines being commercialized for the population.

CONCLUSIONS

In this study, we concluded that it is necessary to be worried about the form, place and storage time of drugs, so that degradation reaction could be avoided: pH must be controlled, avoiding extreme values, as the chemical degradation presented almost total loss of drug potency; and light expositions showed considerable loss of drug potency as well. These procedures can avoid the drug degradation and the consequent product loss of effectiveness and security. These studies are important to establish the type and adequate places for storage of pharmaceutical

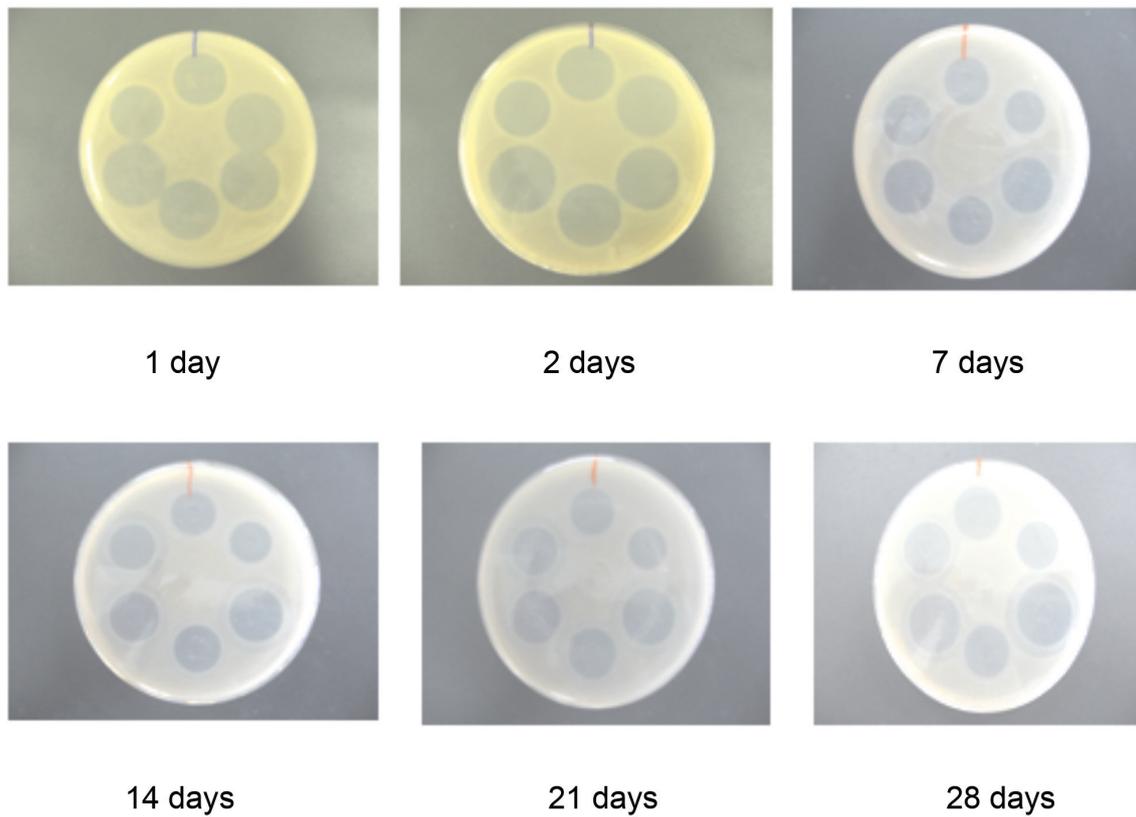


FIGURE 6 - Zone diameters for the azithromycin under UV light (284 nm) exposition.

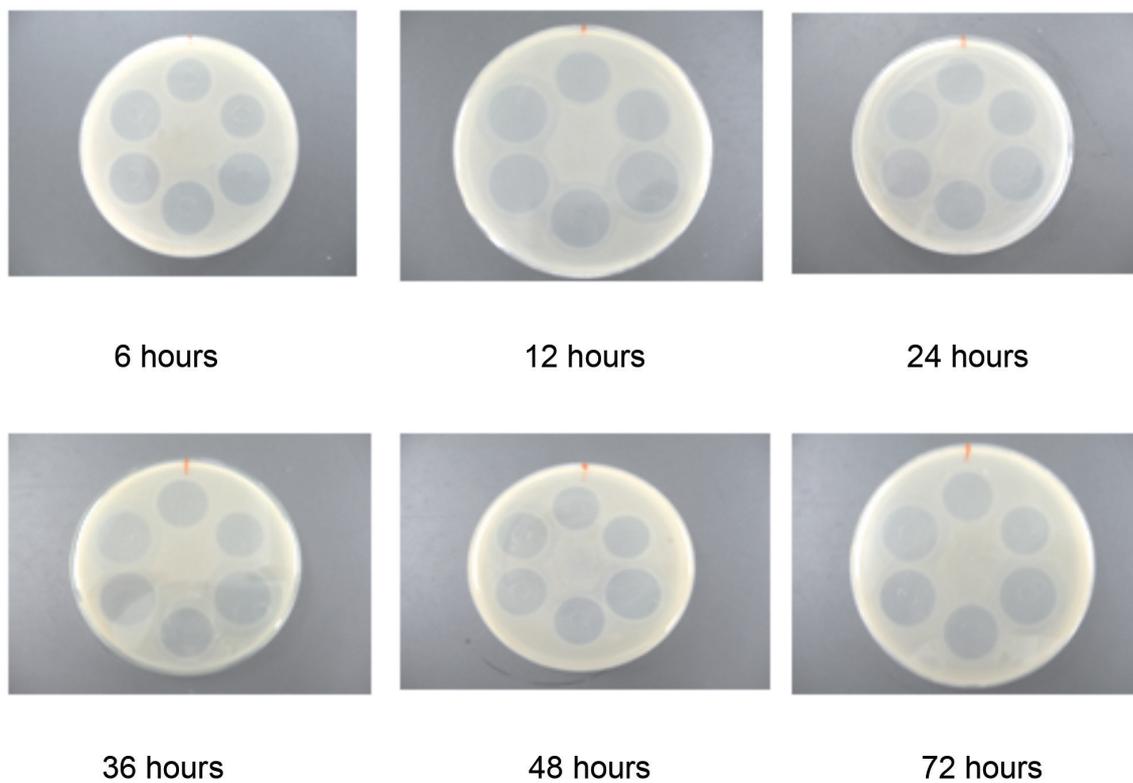


FIGURE 7 - Zone diameters for the azithromycin under UV germicide light (254 nm) exposition.

preparations, in order to prevent the degradation after exposures, assuring the drug integrity, and product quality and safety for the user.

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