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Evaluation Of The Chemical Stability Of Benznidazole-β-Cyclodextrin Complexes Through Forced Degradation Studies

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Abstract

Forced degradation studies are essential for pharmaceutical development, manufacturing, and packaging to determine active substance stability. Benznidazole treats acute Chagas disease. This study uses forced degradation studies to examine the chemical behaviour of Benznidazole (Bzn) and its complexes under various stress conditions to understand better how cyclodextrins (CDs) improve solubility. It will also determine if the molecule's chemical stability and solubility improve. The current study validated the chromatographic method for linearity, selectivity, precision, and filter saturation. To evaluate linearity, the correlation coefficient of the analytical curve was calculated from Bzn solutions at concentrations of 5, 20, 50, 80, 120, and 150 µg/mL in a 50:50 (v/v) acetonitrile: water mixture found that R^2 value 0.998. Linearity, precision, and filter saturation indicate that this Bzn quantification method is adequate. There were solubility values of 0.261 mg/mL for Bzn, 0.809 mg/mL for Bzn- βCD complexes, and 0.819 mg/mL for Bzn- CD-CP. Bzn degraded in alkaline, oxidising, and light-treated solutions. Bzn did not degrade in acidic conditions, aqueous solution at 90ºC, or a solid state under the light. The Bzn molecule's nitro group causes photodegradation. After 30 minutes of exposure, HPLC-DAD detected peaks at 200 nm in the photostability test Bzn sample. The study concluded that the complexes with cyclodextrin (βCD and βCD-CP) that increased drug solubility did not protect Bzn due to similar chemical behaviour under stress conditions as the un-complexed molecule.

Keywords: Benznidazole, forced degradation studies, solubility, HPLC-DAD validation

1. Introduction

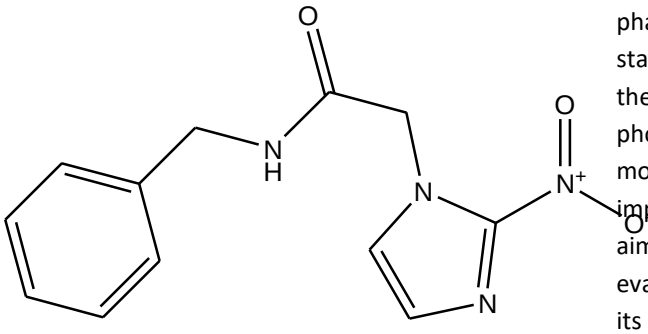
The success of pharmacological treatment is directly related to the triad of a drug's quality, safety, and efficacy. Quality is guaranteed by following the standards of good manufacturing practices and ensured by the quality control tests described in the main official compendiums [1-2]. The World Health Organization (WHO) defines a drug's stability as the ability of the pharmaceutical product to maintain its chemical, physical, microbiological and biopharmaceutical properties within specified limits throughout its shelf life [3]. Lack of stability, therefore, can cause changes in the physical and chemical properties of the drug that can be dangerous for patients due to the formation of toxic breakdown products or can lead to treatment failure due to loss of therapeutic effect [3].

Degradation products are a type of impurity arising from the decomposition of the drug or the excipients present in the formulation [4]. Several factors can favour the formation of these products, such as temperature, humidity, pH, oxygen, and light. Since drugs are exposed to all these factors, it is vital to develop studies on the degradation of drugs to determine not only their shelf life but also to detect potential degradation products and the conditions that are determinants for maintaining their stability [5-6]. Chromatographic techniques, especially HPLC, are used most in industrial and academic environments due to their high selectivity

and sensitivity [7]. HPLC can also be used to develop indicative stability methods [8].

Chagas disease is an infectious parasite disease caused by the protozoan *Trypanosoma cruzi* [9-10]. In India, benznidazole is available to treat the acute phase of the disease [11]. Very few methods indicating stability for benznidazole have been found in the literature using analytical techniques [12-15]. This fact demonstrates the need to develop an indicative method of stability using a technique widely used in analytical laboratories, such as liquid chromatography from discharge efficiency.

Despite its limitations, such as high toxicity and low solubility, Benznidazole (Bzn), chemically 2-Nitro-N-(phenylmethyl)-1H-imidazole-1-acetamide, is the drug of choice for the treatment of Chagas disease [16], whose molecule is shown in Figure 1, highlighting its functional groups. It has a molecular weight of 260.25 g/mol and is a crystalline powder, slightly yellowish, odourless, tasteless and stable in air. It is sparingly soluble in water, dimethyl sulfoxide, and hexane, as well as in ethanol, methanol, ethyl acetate, and dichloromethane. The melting range is 188ºC to 190ºC. The pH of the 26 mg /mL suspension is 5.5 [17].



(2-Nitro-N-(phenylmethyl)-1H-imidazole-1-acetamide).

Figure 1: Molecular structure of benznidazole.

On the other hand, cyclodextrins (CD) have been used to improve the solubility of poorly soluble drugs, increase stability, reduce the bitter taste of some substances, alleviate tissue irritation and enhance bioavailability. The bond with cyclodextrins can reduce the reactivity of the complexed molecule by protecting it inside, thus avoiding attacks from the external environment, consequently increasing its chemical stability [18]. In the work of Taio et al. (2024) [19], different types of Bzn-CD inclusion complexes were studied, concluding that the benznidazole-beta cyclodextrin (Bzn- βCD) and benznidazole- β cyclodextrin-copovidone (Bzn- βCD-CP) complexes were those that obtained the best solubility results, which went from 0.2 mg /mL, reaching 0.8 mg/mL and 0.9 mg/mL for the Bzn- βCD and Bzn- βCD-CP complexes, respectively.

Inclusion complexes using CD also increased the solubility and dissolution rate of Bzn, with the best results attributed to the complexes with randomly methylated βCD and the sulfobutyl ether βCD [19]. It is also essential to consider that the forced degradation study is a valuable tool for understanding a substance when there is little information available about the degradation products and for understanding possible degradation pathways of the drug during shelf life. This type of study also assists in pharmaceutical development, manufacturing process, and packaging material for the final product, aiming at the stability of the active substance. Forced degradation studies within stability research offer a methodology for identifying potential degradation products that may arise in accelerated stability-indicating methods for determining Benznidazole and its degradation products in active pharmaceutical ingredients or during long-term stability assessments. The stress test should include the effect of temperature, humidity, oxidation, photolysis, and other relevant tests based on the molecule under study. Thus, given the positive impact of CDs on the solubility of Bzn, this study aims to complement and deepen this knowledge, evaluating the chemical behaviour of the drug and its complexes under different stress conditions through forced degradation studies and verifying, then whether, in addition to the increase in solubility mentioned, there is also a gain in the chemical stability of the molecule.

2.0. Materials and Methods

2.1. Raw materials and reagents

Bzn (purity 99.33%) was commercially purchased from Toronto Research Chemicals Pvt Ltd. The reagents used in these studies were sodium hydroxide, hydrochloric acid, hydrogen peroxide analytical grade, βCD (Akhil Healthcare Private Limited, Kadak Bazar, Vadodara), povidone

(Kandivali West, Mumbai, Maharashtra, India), HPLC grade acetonitrile (Merck, India), ultra-purified water obtained by the Milli-Q Millipore® purification system.

2.2. Chromatographic System

An analytical method was developed on a Merck Hitachi LaChrom Elite ® high-performance liquid chromatograph (Germany) equipped with a L-2130 quaternary pump, L-2200 automatic injector, L-2300 column oven, L-2400 UV detector and other similar equipment with L-2455 diode array detector (DAD). The parameters established for the analyses were: Purospher RP-18 column, 250 x 4.6 mm, 5 µm particle, mobile phase composed of acetonitrile (ACN) and water in a ratio of 30:70 (v/v), flow rate of 0.5 mL /min at 25ºC, wavelength at 324 nm and injection volume of 20 µL.

Validation of the analytical method: The chromatographic method was validated for linearity, selectivity, precision and filter saturation [20]. Linearity was evaluated through the correlation coefficient of the analytical curve from Bzn solutions with concentrations of 5, 20, 50, 80, 120 and 150 µg /mL in acetonitrile: water 50:50 (v/v), prepared in duplicate from two stock solutions with concentrations of 1 mg /mL. Each point was injected in duplicate. Selectivity was evaluated with samples from forced degradation studies, maintaining the chromatographically pure Bzn peak in the presence of degradation products. Precision was assessed at intra-run precision (repeatability) and inter-run precision (intermediate precision). For repeatability, replicates of 3 determinations of a solution with a concentration of 100 µg /mL were prepared. For intermediate precision, replicates of the test concentration were tested on different days using different equipment. Samples were prepared at two concentrations (50 µg /mL and 100 µg /mL of Bzn), and each solution was filtered through a 0.45 µm Millex® PVDF syringe filter with three discard volumes: 0 mL, 3 mL and 5 mL.

Preparation of Bzn- βCD complexes: The binary Bzn- βCD and ternary Bzn- βCD-CP complexes were obtained using equimolar amounts of the drug about βCD, and for the ternary complex, the amount of CP was 1% of the total mass. Aqueous preparations of each component were obtained separately and, after being combined, were kept under stirring for 48 h and then frozen at -25ºC and dried by the lyophilisation process in a Thermo Scientific (USA) Super Modulyo Freeze Dryer lyophiliser, equipped with a Boc-Edwards (Italy) VLP-285 high vacuum pump.

The exact quantities of binary and ternary compounds were obtained by physical mixing (PM) for comparison purposes regarding the complexation process.

Solubility: The samples of Bzn, Bzn- βCD and Bzn-βCD-CP were added in excess to 10 mL of water and the suspensions obtained were subjected to shaking at 200 rpm for 72 hours at 37ºC in a Cole-parmer® Orbital Shakers model SH-200 Series in duplicate. Then, the samples were filtered through a 0.45 µm PVDF filter, diluted 10 times with acetonitrile: water in a 50:50 (v/v) ratio, and then analysed by HPLC.

2.3 Forced degradation studies

For the forced degradation studies, stock solutions of Bzn, Bzn- βCD and Bzn- βCD-CP, obtained by lyophilisation, were prepared in water at 100 µg /mL. The conditions used are described in Table 1. The binary and ternary complexes were subjected to the forced degradation study only under conditions in which Bzn was unstable.

The alkaline and acidic solutions were neutralised and diluted to a theoretical 50 µg /mL concentration. The solutions from the oxidation study were diluted twice, and the samples collected under neutral pH and photolysis conditions were not diluted. The samples and their respective blanks (with the same components of each condition under study without the drug) were analysed by HPLC in duplicate, filtering them through a 0.45 µm membrane and discarding the first 3 mL.

Table 1: Conditions used in the forced degradation studies for aqueous Bzn solution at room temperature

Study	Condition	Exposure time
Acid hydrolysis	HCl 0.1 M	50 hours
Alkaline hydrolysis	0.1M NaOH	50 hours
Neutral pH hydrolysis	Water at 90ºC	6 hours
Oxidative degradation	H_2O_2 3%	50 hours
Light	UV + visible (solution)	30 minutes
Light	UV + visible (solid state)	50 hours

3. Results

An analytical curve with a linear correlation coefficient (r) value of 0.9992 and a coefficient of determination (R^2) of 0.9984 (Figure 2) was obtained, thus presenting a good correlation within the concentration range of 5 to 150 µg /mL for the proposed chromatographic method.

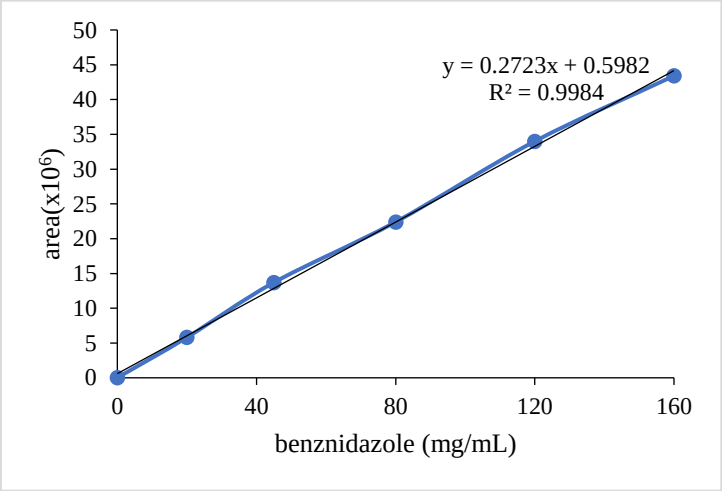


Figure 2: Analytical curve of Bzn using the HPLC method, acetonitrile: water mobile phase 50:50, flow rate 0.5 mL/min, at 25ºC, Purospher RP-18 column, 250 x 4.6 mm, 5 µm particle, reading at 324 nm with 20 µL injection.

The method's accuracy was proven by analysing Bzn samples at a concentration of 100 µg /mL (Table 2), where the coefficient of variation (CV) was less than 1%.

Table 2: Intra- and inter-run precision for Bzn solution

Day	equipment	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	average	coefficient of variation (%)
1	1	100.15	100.63	100.13	99.89	100.16	100.19	0.27
	2	99.84	100.23	99.84	99.2	99.34	99.69	0.42
	1	101.07	99.71	99.89	98.99	100.03	99.94	0.75
	2	100.25	100.36	101.33	100.14	100.47	100.51	0.47

Table 3: Filter saturation assessment for Bzn solutions. Disposal volumes in mL.

Theoretical concentration (µg/mL)	Unfiltered	0	3	5
50	100.07 + 0.03	99.89 + 0.00	100.00 + 0.01	100.00 + 0.02
100	100.45 + 0.04	100.10 + 0.06	100.27 + 0.03	100.30 + 0.06

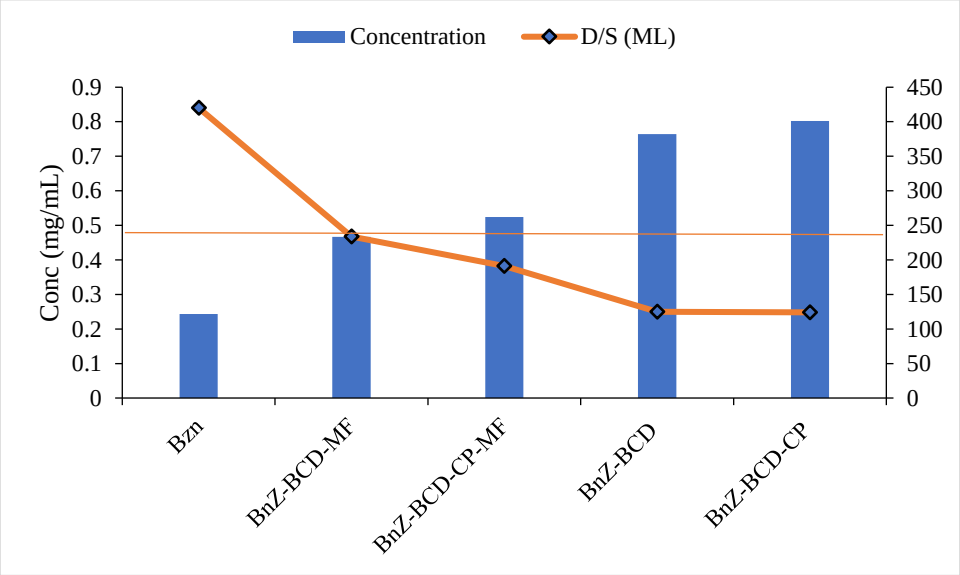


Figure 3: Solubility of Bzn and the binary Bzn- βCD and ternary Bzn- βCD-CP complexes obtained by lyophilisation and physical mixing in water. (adequate solubility for D/S<250 mL)

Table 4: Percentage of Bzn in the various conditions of the forced degradation study. Drug concentration data is converted to percentages, considering the initial value of each condition to be 100%. The initial concentration in µg /mL was: a) 47.4; b) 46.8; c) 97.0; d) 47.6; e) 68.2; f) 97.1. The highlighted columns represent the conditions where a drop in drug concentration is greater than 10%, showing strong evidence of susceptibility to degradation under these conditions.

	0.1 M-HCl	0.1 M-NaOH	Aqueous Media (90°C)	3% of Hydrogen Peroxide	Light (solution)	Light (solid)
Initial	100%	100%	100%	100%	100%	100%
1- minute	-	-	-	-	99.4%	-
5- minute	-	-	-	-	97.7%	-
30- minute	-	-	-	-	85.4%	-
2 hours	-	-	100.9%	-	-	-
3 hours	100.4%	97.7%	-	98.4%	-	-
4 hours	-	-	101.4%	-	-	-
6 hours	100.2%	97.3%	98.7%	96.9%	-	-

11 hours	100.4%	92.3%	-	-	-	-
30 hours	100.2%	80.2%	-	83.8%	-	-
50 hours	100.2%	79.3%	-	68.6%	-	98.6%

The photodegradation observed here can be attributed to the nitro group in the Bzn molecule, which is very susceptible to photochemical reactivity. It is known that visible and UV light can transform the nitro group into nitroso. Another functional group present in the molecule that can also undergo photolysis is the imidazole ring, which can be cleaved under the action of light, and such reactions appear to occur very quickly [19-21].

The Bzn sample from the photostability test (after 30 minutes of exposure) was analysed in HPLC-DAD, and it was possible to note the appearance of peaks at approximately 200 nm (Figure 5), which may be related to the degradation products of Bzn and are not detected at 324 nm in the method developed. Analyses at 200 nm are not viable due to the substantial interference of the mobile phase in this spectrum region [23].

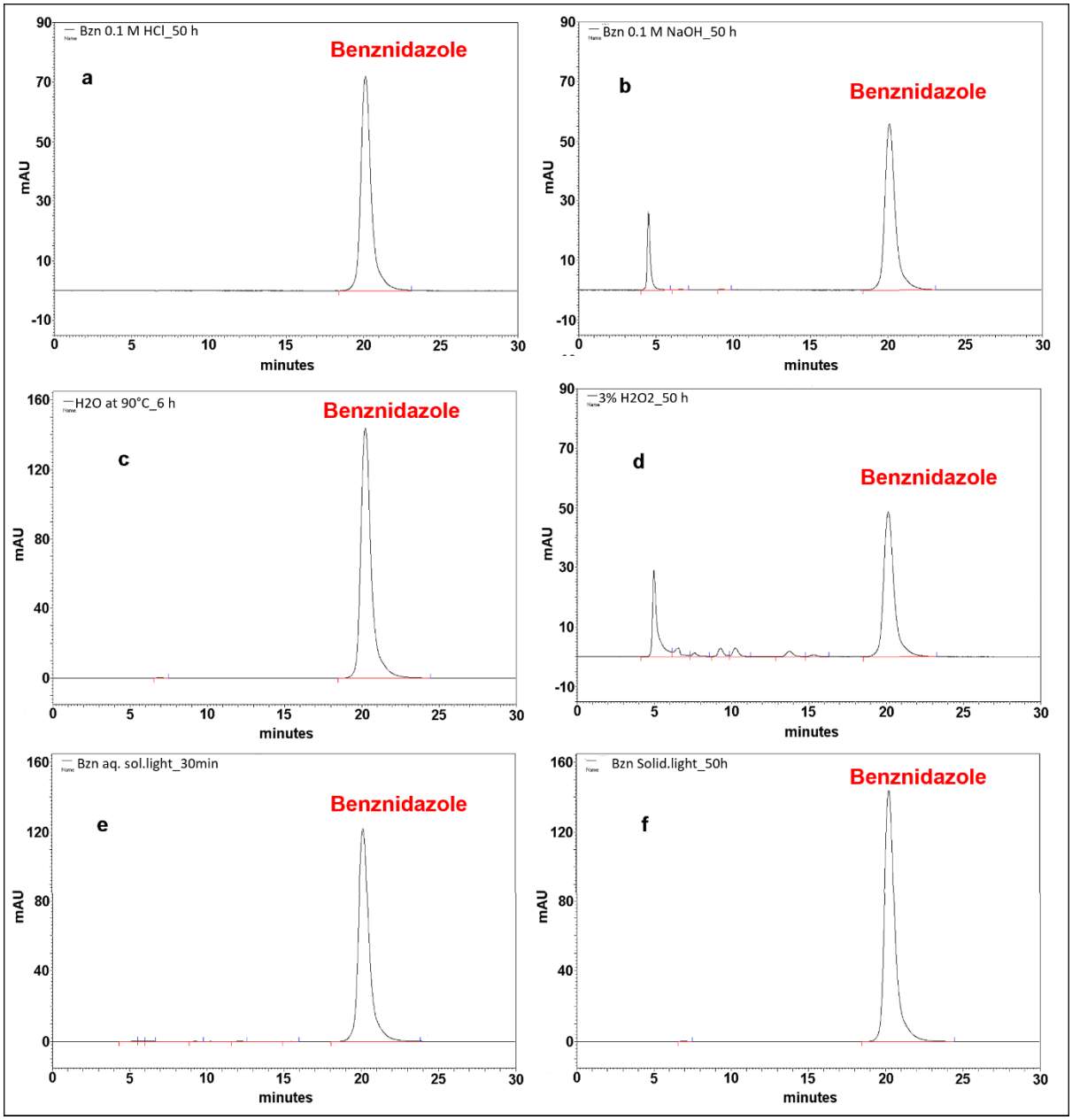


Figure 4: Chromatograms of Bzn (RT at 20 min) obtained by HPLC-UV at 324 nm resulting from exposure in 0.1 M HCl after 50 h (a), 0.1 M NaOH after 50 h (b), H_2O at 90°C after six h (c), 3% H_2O_2 after 50 h (d), the aqueous solution under light after 30 min (e), and Bzn in the solid state under light after 50 h (f). Initial theoretical concentration: 50 µg /mL for a, b, and d; 100 µg /mL for c, e, and f.

In this case, one hypothesis for the appearance of these bands may be the cleavage of a group of chromophores, probably the imidazole ring. Another hypothesis is that the bands of approximately 200 nm may represent part of the Bzn molecule, such as the benzyl group, in the absence of autochrome groups that cause a change in the absorptivity of the chromophore to longer wavelengths. These groups characteristically have unshared electrons, in this case, the NH group of the acetamide of Bzn [12].

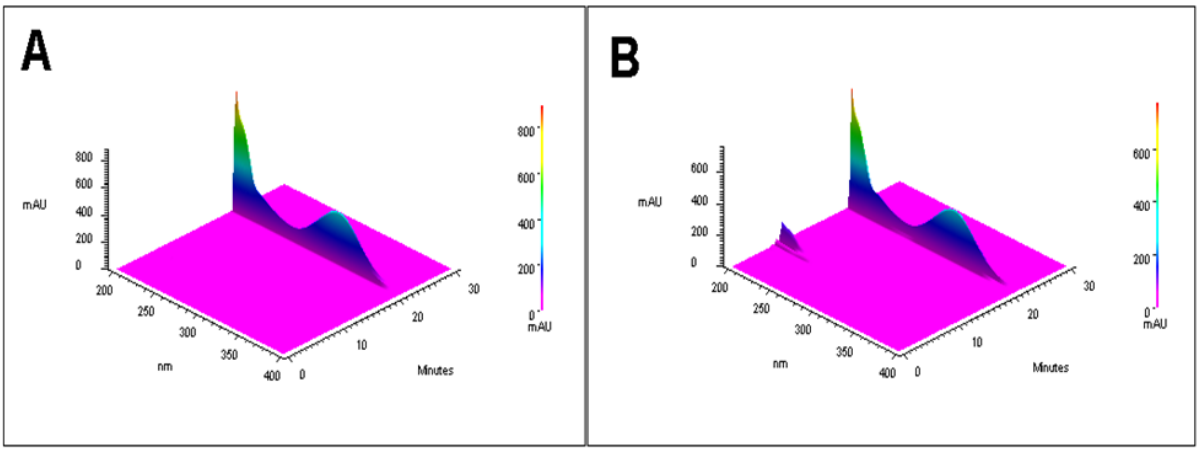


Figure 5: 3D chromatograms obtained by HPLC-DAD of the control Bzn solution (protected from light with aluminium foil) (A) and the Bzn solution exposed to light for 30 minutes (B) showed a decrease of approximately 15% in the drug content.

The Bzn- βCD and Bzn- βCD-CP complexes were tested under the same conditions as Bzn, which degrades in alkaline solution, peroxide, and light (Tables 5, 6, and 7).

Table 5: Percentage of Bzn to the exposure time in the photodegradation study in solution

Time (t)	Bzn	Bzn- βCD	Bzn- βCD-CP
0 min	100	100	100
30 min	87.5	90.7	88.3
60 min	77.5	78.3	76.3
120 min	60.5	57.3	56.2
150 min	51.5	49.6	47.7

Table 6: Percentage of Bzn about the exposure time in alkaline solution

Time (t)	Bzn	Bzn- βCD	Bzn- βCD-CP
0 hours	100	100	100
3 hours	99.1	98.2	96.7
6 hours	97.5	98	97.2

11 hours	94	95	93
30 hours	87.7	89	86.8
50 hours	82.7	83.7	80.6

Table 7: Percentage of Bzn about the exposure time in solution in the presence of peroxide

Time (t)	Bzn	Bzn- β CD	Bzn- β CD-CP
0 hours	100	100	100
2 hours	94.6	96.1	92.7
5 hours	95	96.3	97.3
10 hours	95.5	97.8	97.9
30 hours	76.7	79.2	83
50 hours	69.3	74.3	75.7

From the results presented in Tables 5, 6 and 7 and Figure 6, it can be seen that there was no gain in the chemical stability of Bzn under the conditions studied. The complexation of Bzn with β CD is energetically favourable; however, the enthalpy involved in the drug-CD bond is practically the same when the benzyl or imidazole group of the molecule is inserted into the cyclodextrin cavity, calculated through molecular modelling [24]. Thus, the imidazole and nitro groups susceptible to photolytic degradation, for example, will not be protected

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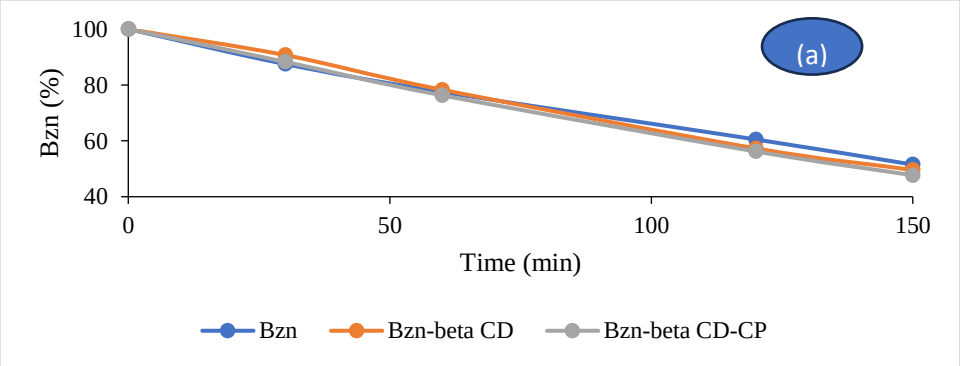

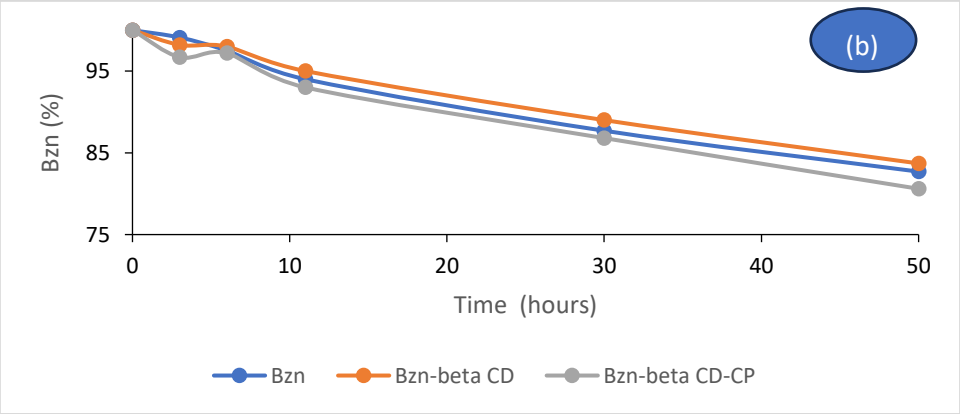

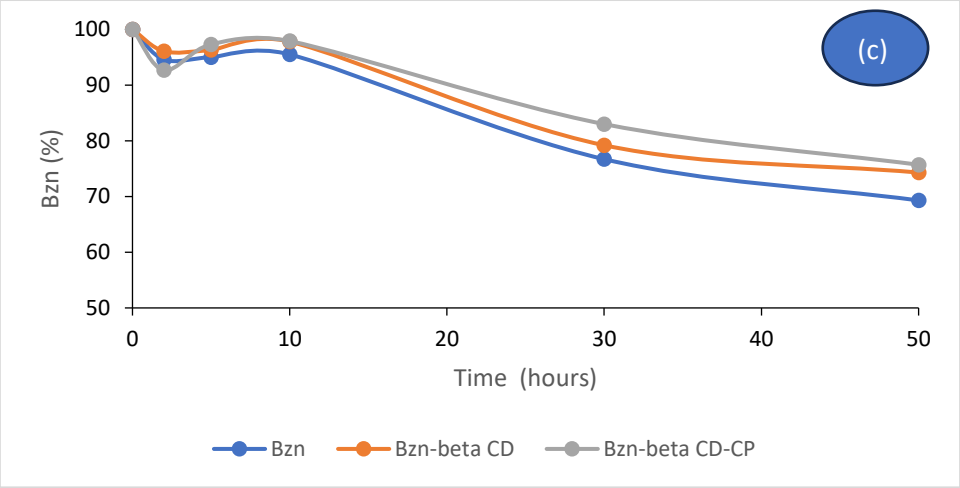

Figure 6: Graph of Bzn concentration in solution (converted to percentage considering the initial time as 100%) versus exposure time under UV + visible light (a), in the alkaline medium in 0.1 M NaOH solution (b) and the oxidative medium in 3% H_2O_2 solution (c).

4. Discussion

The saturation of the filter used to prepare the samples was evaluated. It was concluded that it is necessary to discard 3 mL of the solution containing Bzn (Table 3) to ensure the saturation of the compound in the filter.

Thus, given the results obtained for linearity, precision and filter saturation, the method proposed here for quantifying Bzn is adequate. According to the biopharmaceutical classification, a drug acquires adequate solubility when the dose/solubility ratio is less than or equal to 250 mL; in the case of Bzn, the dose corresponds to 100 mg. For a dose of 100 mg, 412 mL of water is required to solubilise the un-complexed Bzn, 131 mL for the binary complex and 125 mL for the ternary complex obtained by lyophilisation (Table 5). Thus, the drug was initially considered to have low solubility, but when complexed, it acquired adequate solubility for dissolving the dose in the gastrointestinal tract.

The solubility test was a comparison parameter of the extensive characterisation study already available in the Bzn-□CD complexes literature. The values found of 0.261 mg /mL for Bzn, 0.809 mg /mL for the Bzn-□CD complexes and 0.819 mg /mL for Bzn-□CD-CP are similar to the values of 0.2 mg /mL, 0.8 mg /mL and 0.9 mg /mL, respectively, for the values obtained by Espinosa et al. (2018) [21]. Bzn was subjected to several stress conditions where it was possible to detect its degradation in an alkaline solution (Figure 4b), oxidising environment (Figure 4d) and in the presence of light when in solution (Figure 4e). In acidic conditions, in aqueous solution at 90ºC and the solid state under the action of light, Bzn did not undergo degradation.

In alkaline solution, it is possible to observe in the chromatogram (Figure 4b) the emergence of another prominent peak resulting from degradation, in addition to the drug with a retention time of around 20 minutes. Degradation in this condition reached 20% (Table 4) and can be attributed to the hydrolysis of the acetamide functional group, which is susceptible to the nucleophilic attack of water [22].

In an oxidative environment, Bzn also degraded with the emergence of several degradation products (Figure 4d). The impurity with a retention time of 5 minutes, which appeared in both the Bzn chromatogram in alkaline and oxidative conditions, may be similar; however, it was impossible to compare the absorbance spectra since both peaks were not pure. Generally, the oxidative degradation of drugs occurs by autoxidation, which can be caused by forming radicals initiated by the presence of transition metals, peroxides or molecular oxygen (21-22).

The presence of peroxides in formulations is a relevant concern since they may be impurities present in usual excipients in the composition of solid pharmaceutical firms such as povidone, polyethene glycol, and hydroxypropyl cellulose,

among others. The concentrations of these peroxides may vary from batch to batch and manufacturer to manufacturer [19].

Likewise, the photodegradation study showed that the drug decomposes in solution when exposed to light since the Bzn peak disappeared from the chromatogram obtained after 50 hours of exposure (the time required for the photostability chamber to reach the conditions recommended by the ICH). Thus, the test was repeated for up to 30 minutes (Table 4 and Figure 4e); therefore, it was possible to observe a reduction in the area of the Bzn peak in the chromatogram. However, there was no appearance of any other significant peak detected by the applied method.

However, in the solid state, Bzn appears more stable under the conditions recommended by the ICH compared to the solution (Figure 4f and Table 4). After exposure, the Bzn powder presented a more intense yellow colour than the control sample; however, the colour change does not necessarily mean the degradation of the drug.

5. Conclusion

The study of Bzn forced degradation showed that the drug can break down in an alkaline solution, with peroxide, and when exposed to light. The complexes with β cyclodextrin (β-CD and β-CD-CP) that proved to increase the solubility of the drug were not able to protect Bzn since they did not present differences in chemical behaviour under the stress conditions of the study about the un-complexed molecule.

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