

Stability-indicating HPLC method for determination of amiodarone hydrochloride and its impurities in tablets: a detailed forced degradation study

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Amiodarone hydrochloride is one of the most important drugs used to treat arrhythmias. The USP monograph for amiodarone hydrochloride describes an HPLC method for the quantification of seven impurities, however, this method shows problems that result in unresolved peaks. Moreover, there is no monograph for tablets in this compendium. Thus, a stability indicating HPLC method was developed for the determination of amiodarone, its known impurities and degradation products in tablets. A detailed forced degradation study was performed submitting amiodarone API, tablets and placebo to different stress conditions: acid and alkaline hydrolysis, oxidation, metal ions, heat, humidity, and light. Amiodarone hydrochloride API was susceptible to degradation in all stress conditions. The tablets also showed degradation in all environments, except in acidic condition. The analytes separation and quantification were achieved on an Agilent Zorbax Eclipse XDB-C18 column (100 x 3.0 mm, 3.5 µm). The mobile phase was composed of 50 mM acetate buffer pH 5.5 (A) and a mixture of methanol-acetonitrile (3:4, v/v) (B) in gradient elution. The method was validated in the range of 350-650 µg/mL for assay and 10-24 µg/mL for impurities determination. Therefore, this method can be used both for stability studies and routine quality control analyses.

Keywords: Amiodarone hydrochloride. Impurities. Forced degradation. Stability indicating methods. High-performance liquid chromatography.

INTRODUCTION

Cardiovascular diseases are the number one cause of death in the world. It is estimated that 17.7 million people died in 2015 from cardiac diseases, representing 31% of all global deaths (WHO, 2018). Among cardiovascular diseases, arrhythmia can be defined as an abnormal heart rhythm. This means that the electrical impulses of the heart can happen faster, slower or erratically. Arrhythmias can range from asymptomatic and transitory clinical findings to life-threatening abnormalities (American Heart Association, 2018).

Amiodarone hydrochloride (Figure 1) is one of the drugs used to treat arrhythmias, although none of its pharmacological effects is clearly related to arrhythmia-suppressing properties (Mason, 1993). Oral amiodarone is indicated to treat patients with recurrent ventricular tachycardia, fibrillation resistant to other drugs and to

maintain sinus rhythm in patients with atrial fibrillation (Connolly, 1999). Despite its potential for serious adverse effects and uncertainties about its mechanism of action, amiodarone is one of the first-line drugs for the treatment of common cardiovascular diseases (Roden, 2010).

Amiodarone hydrochloride is used worldwide and its monograph is described in a large number of pharmacopeias, including the United States Pharmacopoeia (USP, 2017) and the British Pharmacopoeia (BP, 2018). The monographs for amiodarone hydrochloride raw material presented in those pharmacopeias describe an isocratic reversed-phase HPLC method for the quantification of seven impurities (A-G) (Figure 1). The same analytical conditions are presented for amiodarone tablets monographs in BP and USP Pharmacopoeial Forum. Additionally, these compendia report other methods for the quantitative amiodarone assay (BP, 2018; USP, 2018).

Karmakar *et al.* (2014) observed problems with the USP method for amiodarone related substances that resulted in unresolved peaks due to impurities A, D and E. The authors optimized the USP method using quality

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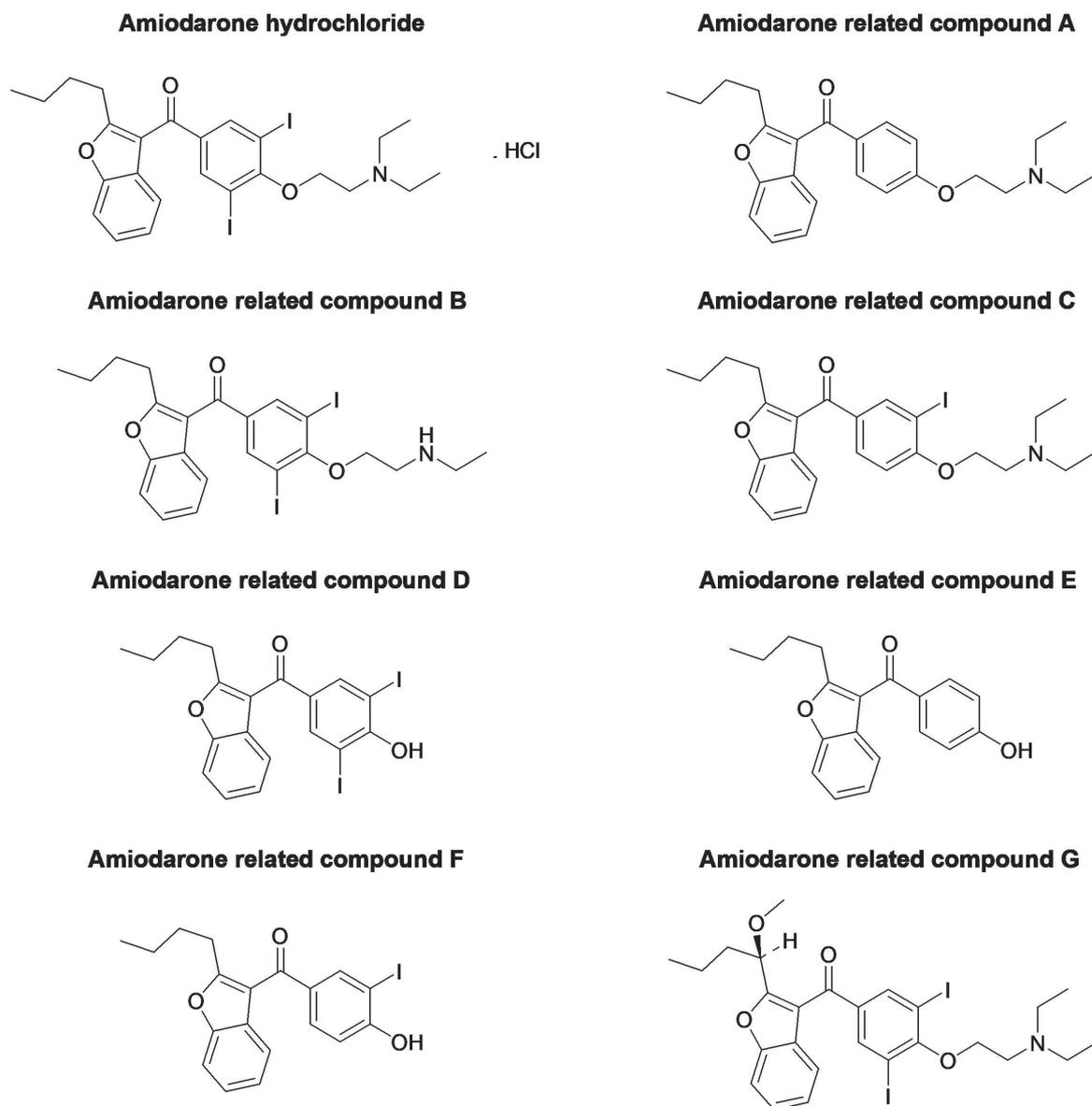


FIGURE 1 - Chemical structures of amiodarone hydrochloride and its impurities (A-G).

by design approach and altered the column brand, column temperature and pH of the mobile phase. However, they did not perform a forced degradation study to verify the separation of the related compounds in the presence of degradation products.

It is known that forced degradation tests are the main tool to predict drug degradation, to identify possible degradation products of an active pharmaceutical ingredient (API) or drug product and to develop stability indicating methods. Therefore, it is not possible to guarantee that a method is stability indicating only because it is capable of separating some known impurities (Baertschi, Alsante, Reed, 2011; Bakshi, Singh, 2002; Silva *et al.*, 2009).

Khan *et al.* (2005) developed an isocratic HPLC method for the estimation of purity and quantitative

determination of amiodarone API in the presence of five of its known impurities. In order to demonstrate the specificity of the method, samples of amiodarone were subjected to various stress conditions such as acid, base, hydrogen peroxide, heat, humidity, and light. Amiodarone presented significant degradation (8%) only under acidic hydrolysis.

Christopherson, Yoder, and Miller (2004) developed a gradient HPLC method for the determination of amiodarone and two of its known impurities in amiodarone injection. The authors described a degradation study submitting amiodarone to acid, base, peroxide, heat and light conditions. Amiodarone was stable under all conditions, except for photolytic treatment (13% degradation).

Despite being an important and widely used drug, there are only a few studies reporting the stability of

amiodarone under stress conditions. Furthermore, the stability indicating methods described do not prove to be able to resolve the peaks due to the seven known amiodarone impurities and the degradation products.

In this context, the aim of this study was to perform a detailed and comprehensive study to evaluate the stability of amiodarone in API and tablets under acidic, alkaline, oxidative, heat, humidity and light conditions and in the presence of metal ions, according to current legislation (Anvisa, 2015). Furthermore, a simple and selective gradient HPLC stability indicating method for quantification of amiodarone and its seven related compounds in the presence of its degradation products was developed and validated.

MATERIAL AND METHODS

Reagents and standards

Amiodarone hydrochloride reference standard was obtained from Fiocruz (Rio de Janeiro, Brazil). Impurities D and E were purchased from U.S. Pharmacopeial Convention (Rockville, USA) and impurities A, B, C, F, and G were obtained from PGS (San Francisco, EUA). Tablets containing 200 mg of amiodarone hydrochloride and placebo were provided by Hipolabor Farmacêutica (Sabará, Brazil). Amiodarone hydrochloride API was purchased from Zhejiang Sanmen (Sanmen, China). The reagents used for the analysis were: acetonitrile (ACN), methanol (MeOH) and dimethyl sulfoxide (DMSO) from Merck (Darmstadt, Germany), hydrogen peroxide from Soltech (Rio de Janeiro, Brazil) and acetic acid, hydrochloric acid, iron (III) sulfate, ammonium hydroxide and sodium hydroxide from Neon (São Paulo, Brazil). Purified water was obtained from an Elga Purelab Option-Q system.

Preparation of mobile phase and diluent

The diluent was prepared by mixing 500 mL of ACN and 500 mL of purified water. Mobile phase A was prepared by mixing 3 mL of acetic acid and 1000 mL of purified water. In the optimized phase, pH was adjusted to 5.5 with ammonium hydroxide. Mobile phase B was composed of 300 mL of MeOH and 400 mL of ACN. After preparation, both mobile phases were filtered through a 0.22 µm filter.

Instrumentation and analytical conditions

Chromatography was carried out using a Thermo Ultimate 3000 HPLC system equipped with a quaternary

gradient pump, an autosampler, a column oven, and a photodiode array detector. Software Chromeleon 7 was used for data acquisition and analysis. Separation was performed on an Agilent Zorbax Eclipse XDB-C18 column (100 x 3.0 mm, 3.5 µm). After optimization, the column was maintained at 50 °C. A gradient elution was used (Table I), with a flow rate of 0.7 mL/min. The injection volume was 2 µL and UV detection was performed at 240 nm.

TABLE I - Mobile phase gradient used

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	35	65
25.0	15	85
25.1	35	65
35.0	35	65

Mobile phase A: 50 mM acetate buffer pH 5.5; Mobile phase B: mixture of methanol-acetonitrile (3:4, v/v).

Preparation of impurities mix standard for method development

Separate standard stock solutions of amiodarone hydrochloride and of each impurity (A-G) at a concentration of 500 µg/mL were prepared by dissolving an accurately weighed amount of 5 mg in 10 mL of methanol. Then, aliquots of 1 mL of each stock solution were taken and transferred to a 10 mL volumetric flask. The volume was completed with diluent. The final concentration of each analyte was 50 µg/mL.

Preparation of standard and sample solutions for the assay testing

The assay test was performed at 500 µg/mL of amiodarone hydrochloride. The standard stock solution was prepared by weighing 50 mg of amiodarone hydrochloride reference standard and transferring to a 50 mL volumetric flask. The volume was completed with methanol. Then, an aliquot of 5 mL of this solution was taken and transferred to a 10 mL volumetric flask. The volume was completed with diluent. For the sample preparation, 20 tablets were accurately weighed and ground to fine powder. An amount of powder equivalent to 50 mg of amiodarone hydrochloride was dissolved in 50 mL of methanol. An aliquot of 5 mL of this solution was taken and transferred to a 10 mL volumetric flask. The volume was completed with diluent.

Preparation of standard and sample solutions for impurities determination

The sample solution was prepared at a concentration of 10 mg/mL for amiodarone hydrochloride, dissolving an amount of powder equivalent to 100 mg of amiodarone hydrochloride in 10 mL of methanol. The standard solution was prepared at a concentration of 20 µg/mL of amiodarone hydrochloride, corresponding to the specified limit for impurities (0.2%). An aliquot of 4 mL of the assay standard solution (500 µg/mL) was taken and transferred to a 100 mL volumetric flask. The volume was completed with diluent. All impurities were calculated in comparison to the amiodarone peak.

Forced degradation

A comprehensive forced degradation study was performed submitting amiodarone hydrochloride API, tablets and placebo to different stress conditions: acidic, alkaline, oxidative, metal ions, heat, humidity, and light. In dry heat condition, the API, placebo and tablets powder were submitted to high temperature (105 °C in an oven) for seven days. The influence of humidity on stability was tested by exposing the samples powders in a 420/CLD stability cabinet (Nova Ética) to 40 °C and 75 ± 5% relative humidity for five days. Light stress studies were performed subjecting the samples powder to the UV-A radiation (200 Wh/m²) and visible radiation (1.2 million lux-hours) during five days. To prepare the samples submitted to hydrolytic degradation, oxidation (with hydrogen peroxide) and degradation by metal ions, approximately 50 mg of amiodarone hydrochloride were accurately weighed and transferred to 50 mL volumetric flasks, followed by the addition of 5 mL of cosolvent (DMSO was used for alkaline hydrolysis and MeOH for the other conditions). Then, 10 mL of degradation solutions were added, and the samples were exposed to the following conditions: acid hydrolysis (5.0 M HCl solution at 25 °C for 5 days), alkaline hydrolysis (1.0 M NaOH solution at 60 °C for 15 hours), oxidation (30% H₂O₂ solution at 25 °C for 3 days) and metal ions (0.1 M Fe₂(SO₄)₃ solution at 25 °C for 2 days). After this period, resulting solutions were neutralized, if applicable, and diluted to 50 mL with methanol. Then, aliquots of 5 mL of each solution were taken and transferred to 10 mL volumetric flasks. The volume was completed with diluent. These solutions were filtered and injected in the chromatographic system. Solutions of placebo and diluent employed for preparing the samples in each stress condition were injected in order to evaluate the presence of possible interference peaks.

The control samples were also prepared without adding the degradation solutions to quantify the percentages of amiodarone degradation.

Method validation

The stability-indicating method was validated for assay and related substances following the recommendations described in the Brazilian legislation RDC N° 166/2017 (Anvisa, 2017) and in ICH Guidance for Industry Q2 (R1) Validation of Analytical Procedures: Text and Methodology (ICH, 2005). Some acceptance criteria applied were defined based on the Guidelines for Standard Method Performance Requirements (AOAC, 2016). The following parameters were evaluated: selectivity, limit of detection (LOD) and limit of quantification (LOQ), linearity, precision, accuracy, robustness, and stability of solutions.

Selectivity

Selectivity of the developed method was proved by stress degradation studies in which the drug was intentionally degraded under different stress conditions (Anvisa, 2015). The presence of interferents in the retention time of amiodarone and its known impurities was evaluated. The acceptance criteria for adequate separation was the resolution value greater than 1.5. Moreover, the peak purity was analyzed using a photodiode array detector.

LOD and LOQ

Limits of detection and quantitation were estimated at a signal to noise ratio of 3:1 and 10:1 respectively, according to ICH (2005). For this approach, decreasing analyte concentrations were analyzed.

Linearity

Linearity was evaluated in two ranges, one for impurities and the other for assay. For impurities determination, linearity was evaluated at six concentration levels, corresponding from 0.10% to 0.24% of the working concentration (10 mg/mL). The linearity of the assay was determined in the range from 70% to 130% of the working concentration (500 µg/mL) using five different concentration levels. In both cases, solutions were prepared in triplicate. The obtained data were statistically analyzed to prove that they met the assumptions for a linear regression. The Grubbs test was used in order to evaluate the presence of outliers. Shapiro-Wilk and Cochran tests were performed to assess the normality and homoscedasticity of residuals, respectively (Cochran,

1941; Shapiro, Wilk, 1965; Souza, Junqueira, 2005). Also, the regression significance and adjustment to the linear model were evaluated.

Precision

For the impurities method, the intra-day precision was evaluated by carrying out six preparations of placebo spiked with amiodarone hydrochloride at 0.20% of nominal sample concentration. The inter-day precision was performed by repeating the same procedure on another day by another analyst. The precision of the assay method was assessed in terms of intra-day and inter-day precision by the quantification of amiodarone in six independent replicates prepared at 100% of the working concentration. Results were reported in terms of relative standard deviation (RSD).

Accuracy

To assess accuracy, placebo was spiked with known amounts of amiodarone hydrochloride in three levels: 0.10%, 0.20% and 0.24% of the working concentration for impurities (10 mg/mL) and 70%, 100% and 130% of the working concentration for the assay (500 µg/mL). Each solution was prepared in triplicate and the recovery percentage of amiodarone was calculated.

Robustness

The robustness of the method was evaluated analyzing samples prepared in the precision test. Samples were assayed under nominal conditions and by the variation of the following analytical parameters: column temperature (48 and 52 °C), flow rate (0.6 and 0.8 mL/min), buffer pH (5.3 and 5.7) and column batch. Moreover, the retention factor, tailing factor, and theoretical plate number were evaluated for five injections of the standard solution in each condition to set the system suitability parameters.

Stability of solutions

The standards and samples solutions used in the precision test were stored in the autosampler in amber glass vials at 10 °C. Solutions were analyzed by the HPLC instrument at different time intervals (0 h, 24 h, 48 h, and 72 h).

RESULTS AND DISCUSSION

Method optimization

In order to optimize the stability indicating method, the impurity mixture standard was employed. Different columns were used and the best results were obtained

with Agilent Zorbax Eclipse XDB-C18 (100 x 3.0 mm, 3.5 µm). The column temperature was tested in the range of 25 °C to 50 °C. The peak shape was improved increasing temperature. Thus, the temperature was set at 50 °C. It is known that changes in the pH of the mobile phase are one of the best strategies to enhance the resolution between acidic or basic substances. Amiodarone hydrochloride is a weak base with a pKa value of 6.56. The pKa values for the impurities were predicted using MarvinSketch software (ChemAxon, Budapest, Hungary) and the following values were obtained: 8.58 (impurity A), 9.40 (impurity B), 8.52 (impurity C), 5.61 (impurity D), 7.80 (impurity E), 6.70 (impurity F) and 8.47 (impurity G). Amiodarone and its impurities have similar chemical structures and properties. Because of that, it is hard to predict the behavior of these analytes, especially considering pKa only. Thus, during the method development, the pH of the buffer was tested in the range of 3.0 to 6.0. The resolution between the critical peak pair (impurities E and A) was higher with pH 5.5, therefore, this condition was set. Several gradients were tested and the one with the best results is shown in Table I. The chosen wavelength was 240 nm, once amiodarone and its impurities showed a good absorption at this condition. The chromatogram obtained for the standard mixture, in the optimized condition, is shown in Figure 2.

Forced degradation

Table II shows a summary of the results obtained in the forced degradation study for both API and tablets. In acidic hydrolysis, 5.0 M HCl was used for 120 hours. Amiodarone API showed around 10% degradation, while amiodarone tablets were stable. The only degradation product formed with amiodarone API was impurity B. In alkaline hydrolysis, amiodarone API and tablets were exposed to 1.0 M NaOH for 15 hours at 60 °C in an oven. Both API and tablets showed degradation over 20%. The chromatogram obtained for amiodarone tablets is shown in Figure 3. Impurity D was formed, with several unknown impurities. Two kinds of oxidations mechanisms were tested. With 30% H₂O₂ for 96 hours, both API and tablets showed degradation over 20%. The chromatogram obtained for tablets is shown in Figure 4. There was a formation of one unknown impurity, with a relative retention time (RRT) of 0.47, and impurities B, C and D. With 0.1 M Fe₂(SO₄)₃ for 48 hours, only amiodarone API showed degradation over 10%. Impurities B and D were formed in this condition. When exposed to dry heat (105 °C for 168 hours), amiodarone tablets and API showed degradation around 10%. Impurities B, C, D, E, and F were formed for amiodarone tablets, as well as an

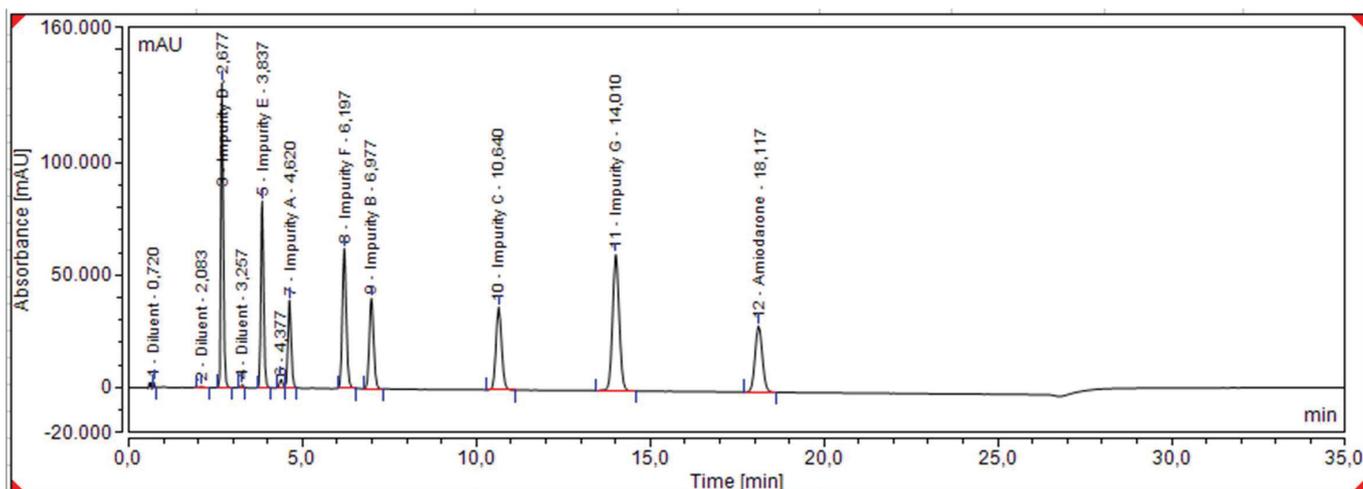


FIGURE 2 - Representative chromatogram of the impurity mixture standard.

unknown impurity with RRT of 0.47. The chromatogram obtained is shown in Figure 5. After exposure to humid heat (40 °C and 75% R.H. for 120 hours), only amiodarone API showed degradation over 10%. Two unknown impurities were formed, with RRT of 0.47 and 0.59. Also, impurities B and D were formed, for both API and tablets. Amiodarone API was more susceptible to photolysis than tablets when exposed to UV light for 120 hours. After the exposure, there was a formation of impurities B, C and D and an unknown impurity with RRT of 0.47. The extent of degradation under all conditions tested was lower for tablets than for API. The main degradation products formed in all conditions were impurities B, C and D and an unknown impurity with RRT of 0.47. The optimized method was able to resolve the peaks due to amiodarone, its known impurities, and degradation products.

A literature survey revealed that only two forced degradation studies for amiodarone hydrochloride are to be found (Christopherson, Yoder, Miller, 2004; Khan *et al.*,

TABLE II - Results obtained in the forced degradation study for amiodarone API and tablets

Stress conditions	Degradation (%)	
	Amiodarone API	Amiodarone tablets
5.0 M HCl – 120 h	9.51	1.97
1.0 M NaOH – 60 °C – 15 h	30.82	20.35
30% H ₂ O ₂ – 96 h	34.93	21.61
0.1 M Fe ₂ (SO ₄) ₃ – 48 h	30.82	7.89
105 °C – 168 h	11.99	8.38
40 °C, 75% R.H. – 120 h	15.04	6.92
UV light – 120 h	16.44	7.11

2005). However, both used mild stress conditions that resulted in a small extent of amiodarone degradation. In this study, the stress conditions employed resulted

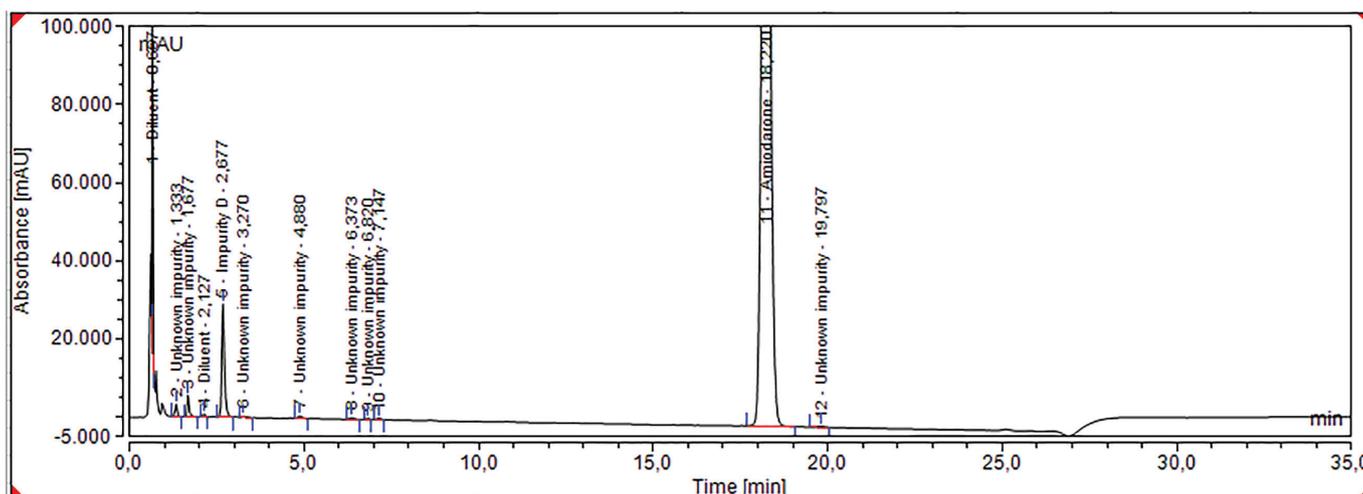


FIGURE 3 - Representative chromatogram of amiodarone hydrochloride tablets after degradation by alkaline hydrolysis.

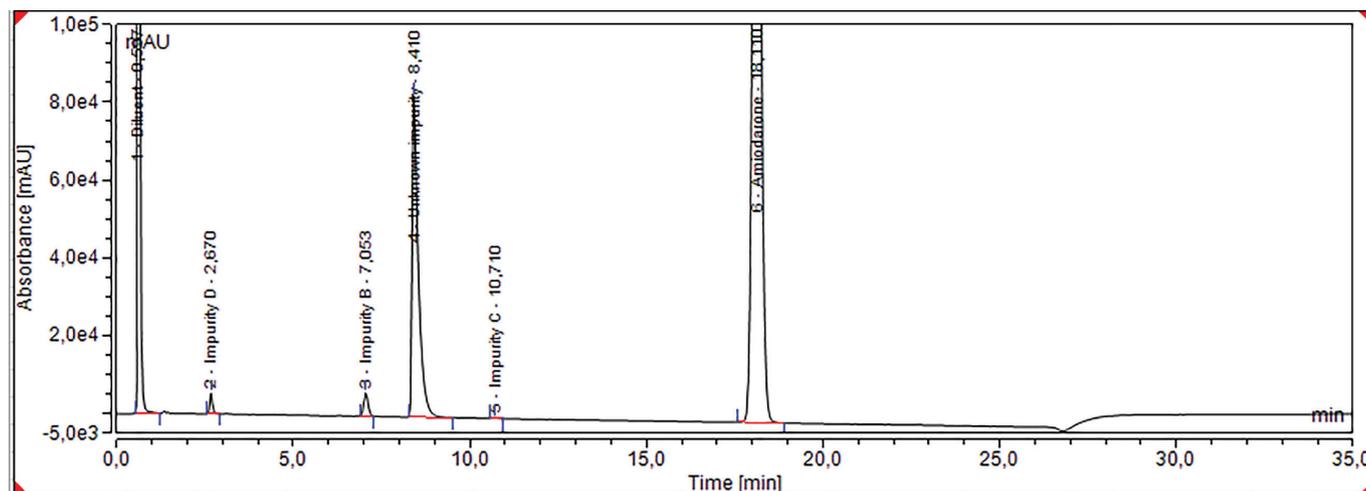


FIGURE 4 - Representative chromatogram of amiodarone hydrochloride tablets after degradation by oxidation.

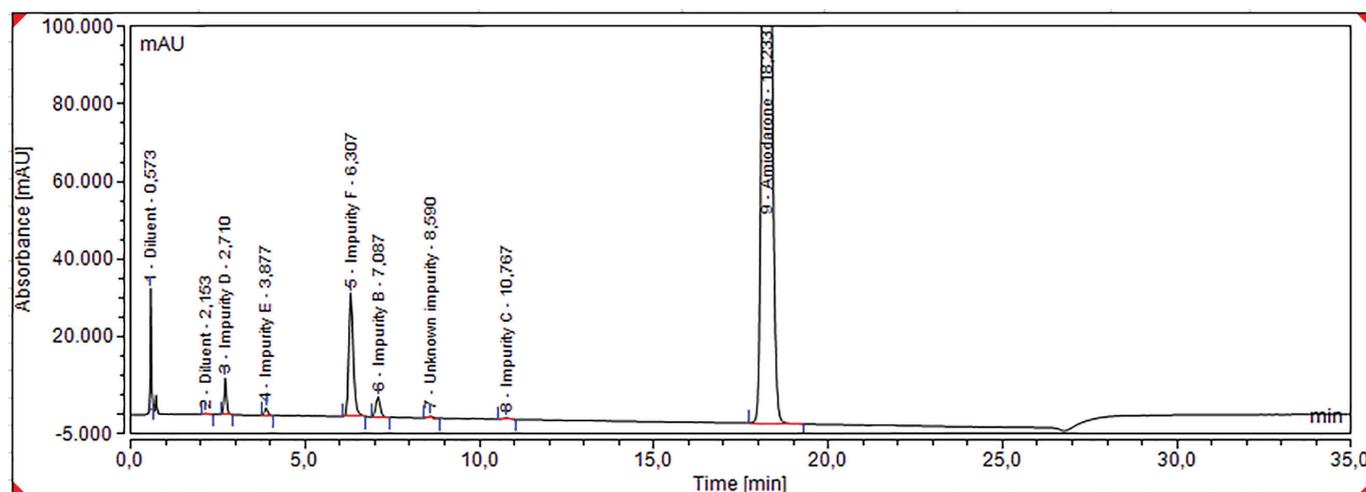


FIGURE 5 - Representative chromatogram of amiodarone hydrochloride tablets after degradation by dry heat exposure.

in amiodarone degradation under all conditions and in adequate extension (about 10 to 30%). It is hard to compare the degradations obtained, once the pharmaceutical forms used are different and API from different manufacturers can lead to distinct degradation profiles.

Method validation

Selectivity

The selectivity of the method was investigated by injecting the stress samples. There were no peaks eluting in the same retention time than amiodarone, its related substances or degradation products peaks. All peaks were well separated and the resolution was in all cases not less than 1.5. Moreover, a photodiode array detector was used to scan the amiodarone and all known impurities peaks in the range from 200 to 400 nm. Spectra indicated that no co-elution was present, once the purity match was above 950, according to Chromeleon 7 software.

LOD and LOQ

Employing the signal-to-noise ratio method, the detection and quantitation limits were 8 $\mu\text{g/mL}$ and 9 $\mu\text{g/mL}$, respectively.

Linearity

Statistical tests were used to verify if the obtained data met the premises for the use of the ordinary least squares method. The results obtained for the tests of normality and homoscedasticity of residuals showed that the residuals are in agreement with all the least squares method assumptions. Moreover, the residuals presented random distribution, the regression was statistically significant and lack of adjustment to the linear model was not observed. The equations of the calibration curves obtained were $y = 147.54x + 44.734$ for impurities method and $y = 151.12x - 877.23$ for assay method. For both of them, the correlation coefficient was higher than 0.990, as required by the validation guideline (Anvisa, 2017).

Precision

For the impurities method, the RSD obtained was 5.2%, for both intra-day and inter-day precision. Concerning the assay method, the RSD obtained in the intra-day and inter-day precision were 1.0% and 1.2%, respectively (Table III). The developed method was found to be precise as the RSD values were less than 5.3% for impurities and 2.0% for the assay (AOAC, 2016).

TABLE III - Amiodarone hydrochloride precision (*data are means \pm SD)

	Theoretical concentration (%)	Concentration obtained (%) [*]	RSD (%)
Impurities			
Day 1 (n = 6)	0.20	0.20 \pm 0.01	5.2
Day 2 (n = 6)	0.20	0.19 \pm 0.01	5.2
Inter-day (n = 12)	0.20	0.19 \pm 0.01	5.2
Assay			
Day 1 (n = 6)	100.00	102.53 \pm 1.02	1.0
Day 2 (n = 6)	100.00	101.28 \pm 0.99	1.0
Inter-day (n = 12)	100.00	101.90 \pm 1.17	1.2

SD: standard deviation

Accuracy

Accuracy was assessed using placebo solutions spiked with known amounts of amiodarone hydrochloride in three levels. The recovery percentages were between 90.0 and 107.0% for impurities and 98.0 and 102.0% for assay. Moreover, the RSD values were below 5.3% and 2.0%, respectively (Table IV) (AOAC, 2016).

Robustness

In the robustness study, each chromatographic factor selected was changed one by one to estimate the effect of the change on the results. The method was not robust only for changes in the pH of the buffer solution, and therefore, this parameter must be properly controlled.

Stability of solutions

Solutions were considered stable for 72 hours since no evidence of degradation of the analyte was observed until this time.

CONCLUSIONS

A comprehensive stability study was carried out, subjecting samples of amiodarone hydrochloride API and tablets to degradation by acid and alkaline

TABLE IV - Amiodarone hydrochloride recovery (data are means \pm SD, n = 3)

Theoretical concentration (%)	Recovery (%) (mean \pm SD)	RSD (%)
Impurities		
0.10	98.85 \pm 0.28	0.29
0.20	97.66 \pm 1.26	1.29
0.24	96.79 \pm 1.39	1.43
Assay		
70	99.72 \pm 0.72	0.72
100	99.16 \pm 0.68	0.69
130	98.57 \pm 0.88	0.89

SD: standard deviation

hydrolysis, oxidation, metal ions, heat, humidity, and light. Amiodarone hydrochloride API was susceptible to degradation in all stress conditions. The tablets also showed degradation in all environments, except in acidic condition. Moreover, in all cases, the extent of degradation for the tablets was lower than for the API. In this sense, the use of proper adjuvants and packaging materials should be considered during drug product development. The developed stability-indicating method was completely validated showing to be selective, linear, precise, accurate and robust and can be used both for stability studies and routine quality control analyses.

ACKNOWLEDGMENTS

The authors thank Pedro Henrique Gouvêa, Clara Coelho and Raquel Arribada for the assistance in the preparation of the manuscript. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Received for publication on 09th April 2018
Accepted for publication on 22nd August 2018