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Stability-Indicating Method for the Analysis of Amorolfine and its N-oxide Degradation Product

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This work aimed to validate an ionic pairing stability-indicating method for determining amorolfine (AMF) in topical formulations by HPLC-DAD. The tailing factor, capacity factor, and theoretical plates were optimized by employing the desirability function. AMF was quantified in a hydrophilic formulation produced in our laboratory and Loceryl® cream and lacquer. Reverse-phase HPLC method with detection at 218 nm showed selectivity (peak purity> 995), robustness (RSD <2.0%), linearity (35–325 µg.mL^{-1}), accuracy (≈100.0%), precision (RSD <2.0%) and limit of quantitation of 750 ng.mL^{-1}. Forced degradation of AMF in hydrogen peroxide resulted in a degradation product with pH-dependent kinetics. The N-oxide degradation product was purified and identified by mass spectrometry (MS) and hydrogen and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR).

Keywords: Amorolfine, N-oxide, HPLC, mass spectrometry, Loceryl

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Introduction

Amorolfine (AMF) is a tertiary amine derived from morpholine and available as a salt – hydrochloride. It is a synthetic drug which exhibits both fungistatic and fungicidal effects. It has a broad-spectrum antifungal activity, including dermatophytes, yeast, and dimorphic fungi. Lacquers and creams containing AMF are employed to treat onychomycosis and dermatomycoses (1,2).

The analytical development of methods for drugs and pharmaceutical formulations is a complex process. Usually, it involves choosing a separation technique, suitable detection, statistical tools to design and analyze data, and searching for potential interfering compounds. The use of statistical tools to optimize analytical methods has been broadening the understanding and quality of experimental findings. Multivariate analysis is essential for optimizing chromatographic methods since several outcomes are critical. The desirability function is extremely useful for multiple response optimization and transforms a response into a scale-free value ranging from zero to one (least to most "desirable") (3).

Forced degradation of drug substances and products is performed at more severe conditions than stability accelerated studies aiming to access the selectivity during the development of stability-indicating methods. Ideally, these methods should be capable of detecting the loss of the main compound and the increase in degradation products. The mechanism of drug reaction can be proposed from forced degradation studies, and it is essential to lead the development of the pharmaceutical formulation and packaging to ensure drug stability (4,5).

Tertiary amines tend to form N-oxides as oxidative degradation products. Hautauer et al. (2000) showed that the presence of peroxides increased the rate of raloxifene degradation to its N-oxide form. In solution, the oxidation of tertiary amines is a pH-dependent reaction, and when ionized, the oxidation rate is significantly reduced (6,7).

Experimental section

Reagents and Pharmaceutical Formulations

Amorolfine hydrochloride was purchased from Pharmasynthese® (Saint Pierre Les Elbeuf, France). All reagents were analytical or HPLC grade. Acetonitrile, methanol and ethanol were purchased from Merck® Co (Darmstadt, Germany). Purified water was obtained by a Millipore® Direct-Q 3UV (Molsheim, France).

Diluent 1 – ethanol : phosphoric acid 0.1% (v/v) (2:1, v/v).

Diluent 2 – ethanol : phosphoric acid 0.1% (v/v) (1:1, v/v).

Loceryl® cream (LOC) and lacquer (LOL) containing 0.25% and 5% of AMF, respectively, were commercially obtained. Our laboratory is studying the permeation of AMF employing *in-vitro* models that simulates human nails and, for this reason, a testing formulation was also analyzed (8). The E15 dispersion (DE15) contains 2.7%

(w/v) of AMF and is prepared as follows: add 0.75 g of polymer (Eudragit® E100) to 40 mL of ethanol 67% (v/v). Mix gently for 20 min and then add 1.25 g of acetylcysteine, 0.1 g of ascorbic acid, and 1.5 g of amorolfine hydrochloride. Keep mixing gently until the formulation is clear and add up to 50 mL with ethanol 67% (v/v).

Factorial Design and HPLC-DAD Method Optimization

Table 1 summarizes the factors and levels of the factorial experiment (2^4 with central point - two replicates for the corners and three for the center) used to optimize the HPLC-DAD stability-indicating method. The results were analyzed on the Design Expert software version 7.1.5 (Minneapolis, US). The tailing factor was the main issue in the analytical development and, therefore, given the highest importance. The targets and limits were established based on the results of the experimental design and the work of Dolan and Snyder (9). AMF at 250 µg.mL^{-1} was prepared in diluent 1, filtered through a 0.45 µm Polyvinylidene difluoride (PVDF) syringe filter and 15 µL injected.

Table 1. Factors and responses for the optimization of HPLC-DAD method to AMF determination.

Factor	Low Level	High Level		
Methanol (%)	64	70		
Phosphate Buffer (mM)	50	250		
pH	1.75	2.25		
Oven Temperature (°C)	25	45		
Response	**Target**	**Limits**	**Weights[a]**	**Importance**
Tailing Factor (*T*)	1.65	1.55 – 1.70	0.33	+++
Capacity Factor (*k*)	3.5	3.0 – 4.0	0.33	++
Theoretical Plates (*N*)	9,000	6 – 12k	0.10	+

[a] Weights less than 1 give less emphasis to the goal/target. Value equal to 0.1 gives almost same emphasis all over the range/limits.

Forced degradation conditions (acid, basic and oxidative medium) was used to test AMF chemical intrinsic stability and optimize the chromatographic method. A stock solution of AMF (SS AMF) at 250 µg.mL^{-1} was prepared in diluent 1 and the chemical agent added to get a final solution containing 0.2 M of Hydrocloric Acid (HCl) or Potassium Hydroxide (KOH), or 1% (v/v) of hydrogen peroxide (H_2O_2) (Table 2).

Validation of HPLC-DAD method

Chromatografic Conditions

The chromatographic analyses were performed in an Agilent® (Santa Clara, USA) HPLC series 1200 consisting of a quaternary pump, an autoinjector, an oven for column, and a diode array detector. Chemstation software (version B03.02) controlled the HPLC system, data acquisition and processing. An ACE® (Aberdeen, UK) phenyl column 150 × 4.6 mm, i.d. 3 µm, endcapped, pore size 100 Å, carbon load 9.5%, and surface area of 300 m^2.g^{-1} was used as reverse stationary phase. The flow rate was set to 0.9 mL.min^{-1}; injection volume of 15 µL; mobile phase methanol: phosphate buffer 250 mM pH 2.25 (62:38, v/v); oven temperature set at 45°C; detection at 218 nm (bandwidth 8 nm), run time 10 min. The mobile phase must be previously prepared making sure that the methanol is added to the phosphate buffer, since mixing the solvents by the quaternary pump mixer ends up clogging the system due to the phosphate precipitation.

Table 2. Solutions and conditions for forced degradation.

Condition	SS AMF (mL)	Chemical agent	pH[c]	Sampling (days)
Control[a]	1.36	0.34 mL of Diluent 1	3	0,1,2,3, and 4
Acid[a]	1.36	0.34 mL of HCl 1 M	1	1,2,3, and 4
Basic[a]	1.36	0.34 mL of KOH 1 M	12	1,2,3, and 4
Oxidative[b]	1.36	0.34 mL of H_2O_2 5%	4	1,2,3, and 4

[a] Kept in well closed vial at room temperature (20 – 25°C). [b] Kept in well closed amber vial at room temperature. [c] measured with Merck® pH (0-14) strips.

Precision

The method precision was demonstrated by repeatability (six determinations at 100%) and intermediate precision (two days). Three formulations were evaluated and the samples prepared as follows: 1) DE15 preparation – 85 µL of DE15 diluted in diluent 2 to obtain a final theoretical concentration of 250 µg.mL^{-1} of AMF; 2) Lacquer preparation – add 15 mL of phosphoric acid 0.1% into a 50-mL volumetric flask, add 50 µL of Loceryl® lacquer (mass of lacquer weighted by difference), and add ethanol to volume; 3) Cream preparation - transfer 500 mg Loceryl® cream, to a 30-mL beaker. Add 0.5 mL of phosphoric acid 0.1% and mix with a spatula. Add two more aliquots of 0.5 mL of phosphoric acid 0.1% and repeat the mixing process. Add 1 mL of diluent 1, mix, and transfer into a 25-mL volumetric flask. Wash the beaker with diluent 1 combining the portions in the volumetric flask. Add diluent 1 to volume. Filter through a quantitative filter paper. AMF standards were prepared in diluent 1 at final concentrations of 50 or 250 µg.mL^{-1}, for Loceryl and DE15 formulations, respectively. All samples and standards were filtered through 0.45 µm PVDF syringe filter membrane.

Selectivity

The method selectivity was assessed by visual evaluation of peak shape and chromatographic parameters (such as tailing factor and resolution) of AMF peaks (standard, formulations and degradation solutions). For the peak purity analyses, the spectra were recorded in the range 205-300 nm in a frequency of 5.12 Hz. Noise spectrum and background compensation were subtracted from peak spectra. The slope for integration was set to 10 and 20 for the AMF concentrations of 50 and 250 µg.mL^{-1}, respectively.

Robustness

The robustness was evaluated through a fractional factorial design (2^{4-1}), four factors and a central point in duplicate in a total of 18 runs for each sample. The factors (low and high levels) were: buffer concentration (phosphate at 240 and 260 mM), oven temperature (42 and 48°C), pH of the buffer (2.15 and 2.35), and concentration of methanol (60 and 64%).

Accuracy

The accuracy for the DE15 formulation was assessed by the standard addition method in which known quantities of the analyte were added to the placebo. For Loceryl® formulations known amounts of the standard were added to the test sample and the recovery calculated.

Limit of Quantification

The limit of quantification was established by analyzing AMF at concentrations below 1,000 ng.mL^{-1}. Data in the time range from 0.2 to 1.2 min was used for the six-sigma noise calculation. Signal-to-noise greater than 10 was employed as criteria to establish the limit of quantification.

AMF oxidative kinetics and pH effect

The oxidative kinetics was performed on samples containing final concentration of 250 µg.mL^{-1} of AMF and 3% hydrogen peroxide (w/v) in ethanol: phosphate buffer diluent (3:1, v/v) at different pH values (range 2.9 - 10.8). The degradative solutions were maintained at room temperature (25°C), protected from light, and analyzed during 36 hours by the HPLC-DAD validated method. Phosphate buffers at 140 mM and different pH were prepared using the Biological Buffer Calculator (John Wiley & Sons) app for Android platform. The pH of the buffer solutions was measured in a pHmeter and were in the range of ± 0.05 of the value predicted by the app.

Characterization of oxidative degradation product (NOx)

Preparation and purification of NOx

The AMF was degraded in oxidative medium at pH 10.8 to obtain the degradation product - NOx. AMF, 10.0 mg, was transferred to a 5 mL volumetric flask, solubilized in 2.5 mL of ethanol, 1.75 mL of phosphate buffer pH 10.8 and 0.75 mL of 30% hydrogen peroxide (w/v). It was stored at room temperature and protected from light for seven days. The purification was performed on a SPE (Solid-phase Extraction) Finisterre® C18/18, 500 mg/6 mL as follows: the degraded solution was added to the SPE cartridge and washed with 7 mL of purified water. Two more washes with 7 mL of water were performed to ensure removal of the phosphate buffer. Two portion of 10 mL of methanol were eluted and the fractions dried at 60°C in air circulating oven. A light yellowish solid (7.8 mg) was obtained, which was characterized by ^{1}H NMR and ^{13}C NMR , HPLC-DAD, LC-ESI/MS, and HRMS (High-Resolution Mass Spectrometry).

Characterization techniques

The NOx and AMF were analyzed by LC-ESI/MS in an Agilent (Santa Clara, US) Infinity 1260 (degasser, pump, auto injector, oven and UV detector) coupled with a single mass quadrupole detector model 6120B. The chromatographic conditions are described in Table 3.

Table 3. LC-ESI/MS conditions for the analyses of AMF and NOx.

Characteristic	Description
Column	Agilent Poroshell 120 EC-C18 (4.6x50 mm, 2.7 µm)
Mobile Phase[a]	ACN:H_2O:Formic acid (50:50:0.1)
Flow rate	0.35 mL.min^{-1}
Injection Volume	4 µL
Oven Temperature	40°C
Mass Detector	Capillar voltage +3.7 kV, drying gas flow 12 L.min^{-1}, nebulizer pressure 40 psig, drying gas temperature 280°C, fragmentor 150 V

[a] diluent for NOx and AMF solutions.

NOx and AMF were analyzed in a HRMS equipment for prediction of molecular formula. The samples were submitted to direct infusion in a LC-QTOF (Shimadzu (Kyoto, Japan) Nexera X2 and Bruker (Billerica, US) Impact II. The conditions were: source APCI (Atmospheric Pressure Chemical Ionization) in the positive mode, corona at 4 kV and temperature 450°C, nebulizer pressure 0.4 Bar, drying gas at 200°C and 4 L.min^{-1}, m/z range from 50 to 1000, and sodium formate as the calibrator.

^{1}H and ^{13}C NMR spectra of AMF and NOx were obtained on a Bruker (Billerica, US) Ascend™ 400 MHz spectrometer. Both compounds were dissolved in deuterated methanol with TMS (Tetramethylsilane) as internal reference. The results were acquired and processed by the software Bruker Top Spin 3.2 and

analyzed in software MestReNova (Santiago de Compostela, Spain) version 12.0.

AMF - ^{1}H NMR (400 MHz, Methanol-d4) δ 7.29 (d, J= 8.0 Hz, 2H), 7.16 (d, J= 8.0 Hz, 2H), 4.13 – 3.92 (m, 2H), 3.53 – 3.41 (m, 2H), 3.17 – 3.01 (m, 2H), 2.83 – 2.66 (m, 2H), 2.60 – 2.43 (m, 2H), 2.43 – 2.30 (m, 1H), 1.65 (q, J = 7.4 Hz, 2H), 1.26 (s, 6H), 1.21 (dd, J= 6.3, 3.4 Hz, 6H), 1.05 (d, J= 6.5 Hz, 3H), 0.65 (t, 3H). ^{13}C NMR (101 MHz, Methanol-d4) δ 148.67, 136.95, 129.98, 127.16, 70.29, 64.82, 58.42, 41.35, 38.54, 37.84, 31.30, 29.00, 18.65, 18.17, 9.49.

NOx - ^{1}H NMR (400 MHz, Methanol-d4) δ 7.28 (d, J = 7.3 Hz, 2H), 7.18 (d, J= 7.2 Hz, 2H), 4.33 – 4.15 (m, 2H), 3.29 – 3.25 (m, 1H), 3.17 – 3.10 (m, 1H), 3.02 – 2.95 (m, 2H), 2.94 – 2.87 (m, 1H), 2.77 – 2.70 (m, 1H), 2.61 – 2.52 (m, 2H), 2.52 – 2.46 (m, 1H), 1.69 – 1.59 (m, 2H), 1.26 (s, 6H), 1.14 (dd, 6H), 1.08 (d, 3H), 0.66 (t, 3H). ^{13}C NMR (101 MHz, Methanol-d4) δ 148.60, 137.95, 130.19, 127.12, 78.08, 69.92, 67.70, 43.31, 38.55, 37.87, 30.87, 29.04, 21.77, 18.23, 9.53.

Results and Discussion

Development and Optimization of HPLC-DAD Method

Release and permeation studies of formulations containing AMF usually employ liquid chromatography methods, but few chromatographic information is satisfactorily described (10–12). Gao et al. (2012) described a quantitative method for determination of topical formulation by HPLC employing C-18 column and acetonitrile, methanol and citrate buffer as the mobile phase. The AMF tailing factor (T) in this study was 2.95, a much higher value than the maximum recommended (T≤2.0) (13).

During the development of the method we have encountered the same issue - high tailing factor values that were hardly less than 3.0 (Figure 1). Some known strategies to decrease the value of the tailing factor were tested without success, such as: a) columns with different types of reverse phase loading (C-18, C-8, phenyl), column sizes (100, 150 and 250 mm) and diameters of particles (3 and 5 µm), in addition to Hydrophilic Interaction Liquid Chromatography (HILIC) and cyano normal phase columns; b) different organic solvents (acetonitrile, methanol and isopropanol); c) ionic pairing with pentane and heptane sodium sulfonate; d) triethylamine and dibutylamine; e) column temperature (25-65°C); f) aqueous phase pH (1.5-9.0); g) gradient with low and high elution time; h) drug concentration in order to investigate possible overload of analyte in the column; i) different types and concentrations of the buffer.

The tailing factor under none of the conditions tested was less than two. Although, increasing the concentration of phosphate buffer resulted in less asymmetric peaks (T ≈ 2.6 and 2.0 for 50 and 150 mM, respectively). A factorial experiment was designed to study the effect of relevant factors on different chromatographic parameters (Table 1). Phosphate buffer, in concentrations of 5 - 25 mM, is widely used for the pH control of mobile phases and mixtures with acetonitrile has presented better results for AMF peak width and system pressure than methanol. However, methanol kept phosphate buffer in solution at concentrations greater than 50 mM, preventing the tubing and column clogging. The response variables studied to optimize the method were tailing factor, capacity factor, and theoretical plates. The resolution was not included since there was no degradation product at this point of development, and the tailing factor was a problem that made it impossible to proceed with the analytical and stability studies.

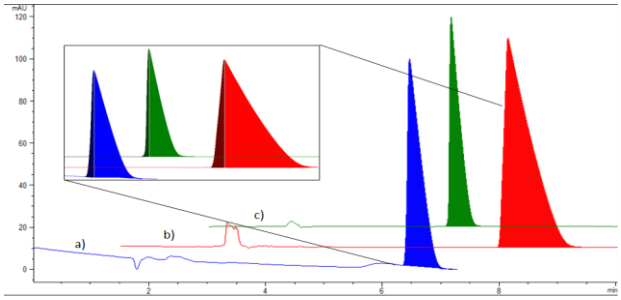

Figure 1. Overlapping chromatograms under the following conditions: a) mobile phase ACN: 0.5% phosphoric acid (60:40), flow rate 1.2 mL.min^{-1}, temperature 35°C, phenyl column; b) ACN: 0.2% triethylamine + 0.2% phosphoric acid pH 3.0 (50:50), 1.2 mL.min^{-1}, 35°C, column C18; c) ACN: 0.005 M hydrochloric acid pH 3.0 (50:50), 1.2 mL.min^{-1}, 35°C, column C18. Tailing factor values: a) 3.611; b) 3.676; c) 3.725.

All three response variables had statistically significant factors and the models fitted with excellent predictability (r^2> 0.988 for all models). Figure 2 shows the results of the factorial analysis.

The tailing factor had the buffer concentration as the most relevant factor; the higher the buffer concentration, the lower the tailing factor. Buffer concentration and pH contribute to 93.8% of the variation verified in the sum of squares and are responsible for the decrease observed in the tailing factor. The pKa of AMF is 6.6; at the pH values tested, it is protonated (14). The effect of pH on the tailing factor is indirect since the increase in pH increases the amount of phosphate in its anionic form that can pair ionically with the positively charged AMF.

The capacity factor had the percentage of methanol and temperature of the column as factors with more significant effects (54.13 and 26.15% of the variation on the sums of squares (SS), respectively). Capacity factor values lesser than 2.0 are not recommended and hardly generate methods capable of separating the analyte from the components of the formulation, impurities, and degradation products. The decrease in the percentage of methanol increases the capacity factor, with all experiments obtaining values of *k*>2.0. The lowest temperature tested (25°C) associated with the percentage of methanol in the lower level ended up increasing the capacity factor to values greater than 3.0.

The buffer concentration has the greatest influence (86.6% SS) in the number of theoretical plates. The

relation was positive, so increasing the buffer concentration caused an increase in the theoretical plates.

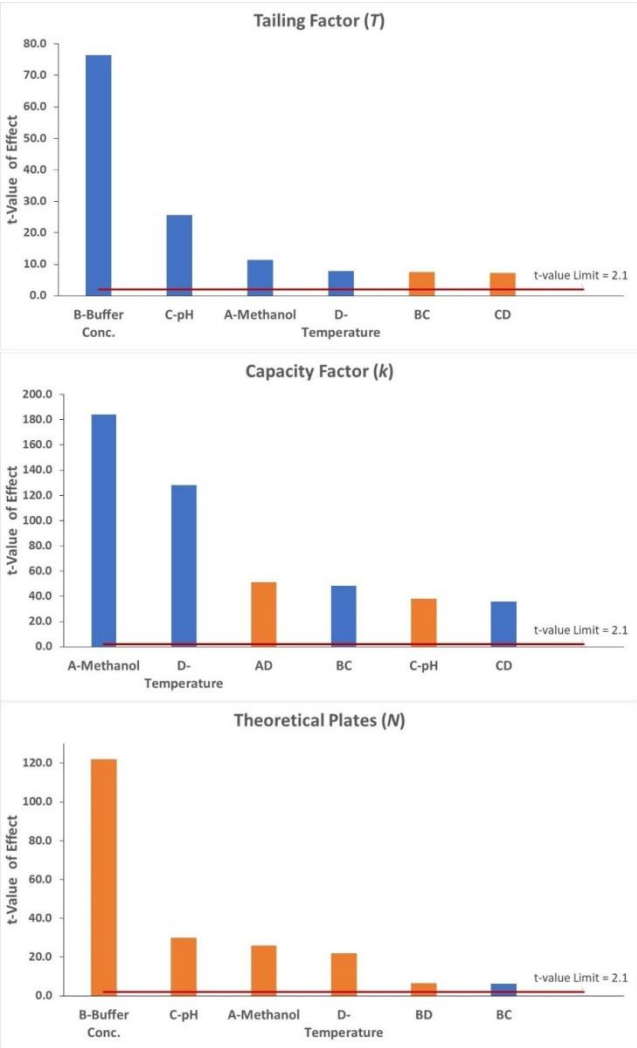

Figure 2. Pareto graphs of statistically significant factors and interactions for tailing and capacity factors, and theoretical plates. In orange, the positive effects (factor and response have a positive correlation) and in blue, the negative (factor and response are inversely correlated)

Briefly, the tailing factor and the theoretical plates are favored to a large extent by the increase of the buffer concentration and to a lesser degree by pH. In contrast, the capacity factor had better results in lower levels of methanol and column temperature. Indeed, there is influence of the four factors on the three evaluated responses, making very hard the decision to set up the method conditions. Several techniques are used to optimize multiple responses and Desirability Function is one of them. The method describes an objective function that reflects desirable values for each response ranging from zero to one (less to most desirable, respectively) (3). Figure 3 shows the result of the optimization of the chromatographic method using the Desirability Function. There is a big blue area where desirability function is zero and at least one response is out of the limits. Only a small area meets the specifications established for the responses (red-orange zone). Based on this, the condition selected was: 64% methanol, 250 mM phosphate buffer concentration, pH 2.25 and column temperature 30°C. The AMF was tested under these conditions and presented satisfactory values of tailing and capacity factor, as well as of theoretical plates (1.61, 3.49 and 10,135, respectively).

Samples of AMF were subjected to acid, basic, and oxidative conditions and then submitted to analysis on the optimized method. AMF was considered stable if the following criteria were met: 1) no secondary peaks other than those related to the diluent; 2) the peak of AMF kept the same retention time (± 5%) and was spectroscopically pure (peak purity > 995 and similarity curve does not cross the threshold curve); 3) the AMF percentage remained in the range of 98.0 - 102.0% (arbitrarily time zero was considered 100% and the areas of the peaks of the other times were related to time zero to obtain the percentages).

The control sample met the stability criteria and AMF was considered stable in diluent 1 for at least 4 days at room temperature in well closed vials. The acid and basic media did not change the chromatographic profile of the AMF and were also considered stable.

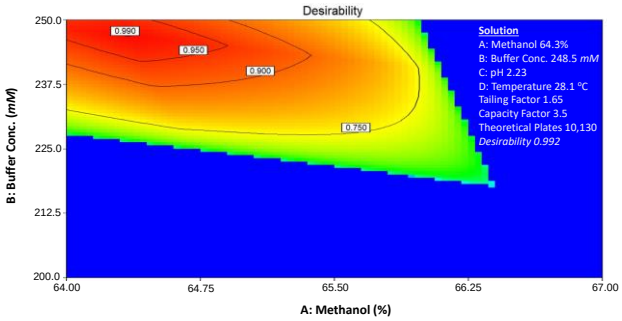

Figure 3. Desirability graph applied to development of chormatogrpahic method for separation of amorolfine and its degradation products.

The oxidative condition reduced the peak intensity of the AMF and revealed a degradation product (Figure 4.a). The AMF and degradation product peaks were not well resolved and a new adjustment in the method was necessary. Based on the previous results, the percentage of methanol was reduced in order to change the capacity factor of the AMF and the degradation product (NOx), improving the selectivity of the method. Methanol at 62% was able to separate the peaks, however there was an increase in system pressure (greater than 275 bar) and in the peak width (with reduction of the theoretical plates to undesired lower limit of 5,000). In order to minimize the effects of decreasing methanol, the column temperature was increased to 45°C obtaining a reduction in pressure (≈ 200 bar) and improvement on theoretical plates (≈ 6000) with a satisfactory resolution between the peaks (Rs> 2.0) (Figure 4.b). Thus, the validation procedure was performed using: 62% methanol, 250 mM buffer concentration, pH 2.25 and 45°C for oven temperature.

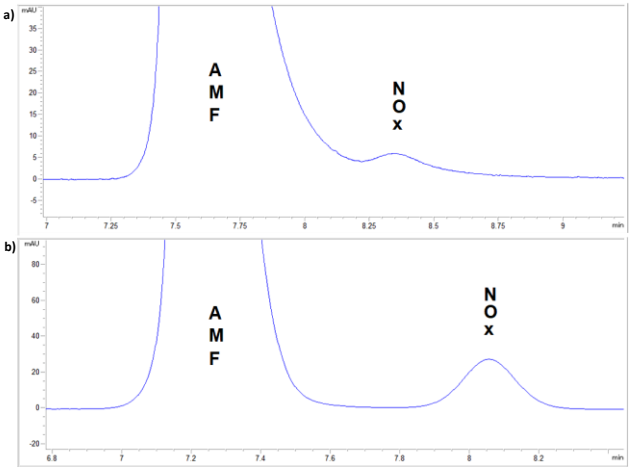

Figure 4. Chromatograms at 218 nm from AMF oxidative degradation solution. a) mobile phase - methanol: 250 mM phosphate buffer, pH 2.25 (64:36) and temperature 30°C; b) methanol: 250 mM phosphate buffer, pH 2.25 (62:38) and temperature 45°C.

Validation of HPLC-DAD method

The determination of AMF in Loceryl® formulations proved to be challenging. Using an aliquot (50 µL) of LOL resulted in high variations intraday, because part of the pipetted volume remained in the tip. The issue was overturned by weighting the volume of lacquer (relative standard deviation (RSD) lower than 2.0%) (Table 4).

The challenges of extracting the AMF from the LOC were even greater. Initially the cream was extracted and diluted to a 5-mL volumetric flask with different diluents, but the recovery of AMF was not satisfactory. The recovery problem has been solved adding small amounts of solvent, mixing, and repeating this process, and also increasing the final volume of diluent to 25 mL.

The DE15 formulation is hydrophilic and easier to prepare, and, for this reason, its central point was 250 µg.mL^{-1}. The use of higher concentration of AMF also allowed greater sensitivity in the analyses of forced degradation samples.

The selectivity was tested for the formulations and for forced degradation samples in comparison to the AMF standard. There was no visual alteration in the shape of the AMF peak nor in the chromatographic parameters (tailing factor, theoretical plates, and capacity factor) when comparing the peak of the standard and test samples. LOC showed an intense peak with a value of k <1 identified as the formulation preservative (2-phenoxyethanol). A split peak in the void volume could be observed for DE15 solution and corresponds to ascorbic acid and acetylcysteine. AMF was spectroscopically pure (impurity not detected in the similarity/threshold curve analysis and peak purity index greater than 995) in the samples under test and the resolution between the peak of the drug and the oxidative degradation product remained higher than 2.0.

The robustness assay had RSD values lower than <2.0% for the three formulations, demonstrating that the method is robust. The accuracy was satisfactory with a narrow 95% confidence interval (CI) including 100.0% (RSD < 2.0%) for all formulations.

The linear regression lines were obtained plotting the peak area at 218 nm versus the concentration of AMF. The coefficients of correlation (r) close to 1.00 demonstrated that virtually all the variation on the peak areas can be explained by the concentration of the drug. The random residuals showed that the first-order model is adequate and values of standardized residues lesser than |2.0| indicate the absence of outliers. The two calibration curves showed linearity and were equivalent (slopes and intercepts are not statistically different for α=0.05) since both are in the linearity range of the detector (peak height between 80 - 160 and 400 - 800 mAu for the curves with the center points at 50 and 250 µg.mL^{-1}, respectively).

Repeated injections of AMF at 0.75 µg.mL^{-1} showed signal-to-noise ratios greater than 10 and average of 14. The limit of quantification of 0.75 µg.mL^{-1} was considered high and it is mainly due to the high noise observed at 218 nm (0.2 mAu) which was associated with large concentrations of phosphate buffer and methanol in the mobile phase.

Table 4. Summary of analytical parameters results for the determination of AMF in different pharmaceutical formulations.

Analytical Parameter		Loceryl® Lacquer	Loceryl® Cream	DE15 dispersion
Linearity[a]	Central Point (range)	50 µg.mL^{-1} (35 – 65)		250 µg.mL^{-1} (175 – 325)
	Equation (r)	y = 26.24x -85.59 (0.998)		y = 27.48x -166.99 (0.999)
	Residuals	Random, standardized residuals between -2.0 and +2.0		
Precision	Repeatability[b]	1.09 and 1.33%	1.34 and 1.64%	1.65 and 1.05%
	Intermediate[c]	1.63%	1.58%	1.32%
Accuracy	Mean[d]	100.5%	100.7%	100.1%
	CI 95%	99.4 – 101.5%	100.0 – 101.4%	98.9 – 101.2%
Robustness	RSD[e]	0.39%	0.69%	0.33%
Quantification Limit[f]		0.75 µg.mL^{-1}		

[a] *n*=15; [b] two days; [c] RSD of *n*=12; [d] *n*=9; [e] *n*=9; [f] *n*=5.

AMF oxidative kinetics and pH effect

Figure 5 presents the results of the percentage of AMF as a function of time for the different pH under conditions of forced oxidative degradation (H_2O_2 3%). The experiment demonstrates that the stability of the AMF in the oxidative medium depends on the pH. At pH 2.9, there was no reduction in the AMF content, and the analytical error can explain the observed variations.

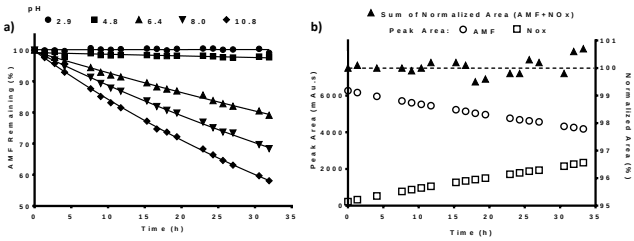

Figure 5. Kinetic reaction of AMF. a) Oxidative (H_2O_2 3%) degradation kinetics of AMF at different pH; b) Representative mass balance of AMF degradation at pH 8.0.

The results were modeled, and the first-order kinetic model obtained better adjustments. However, we suggest that the reaction must be classified as pseudo-first order because the hydrogen peroxide is in excess and despite being consumed in the reaction, its concentration remains practically unchanged.

Table 5 shows the calculated reaction constants and $t_{90\%}$ values. A significant increase in degradation is noted when the pH is changed from 4.8 to 6.4. The pKa value of AMF ≈ 6.6 can explain this and the assumption that tertiary amines oxidize easily when not ionized (14,15). As the concentration of unionized AMF increases, the higher reaction constant (k) and at pH 10.8, only 6.3 h is required for the drug content to be reduced by 10%.

Table 5. Reaction constant values (*k*) and t90% for degradation of AMF in oxidative medium at different pH.

pH	**4.8**	**6.4**	**8.0**	**10.8**
***k* (h^{-1})**	$7.39.10^{-6}$	$1.17.10^{-4}$	$1.93.10^{-4}$	$2.81.10^{-4}$
$t_{90\%}$ (h)	237.7	15.0	9.1	6.3
% Unionized[a]	1.6	38.7	96.2	>99.9

[a] Estimative considering the pKa of the tertiary amine group of the AMF equal to 6.6.

Characterization of NOx

AMF and NOx UV spectra are practically identical from 205 to 300 nm with maximum absorption at 218 nm. The UV library search for NOx peak apex spectrum resulted in a similarity greater than 999.0 compared to the AMF standard spectrum (Figure 6.a). It indicates that the chemical change in NOx did not occur around the AMF chromophore group (aromatic ring and its direct substituents). The purified NOx peak had satisfactory spectroscopy purity by HPLC-DAD, percentage area close to 100% and therefore was considered pure enough for NMR and MS analyses.

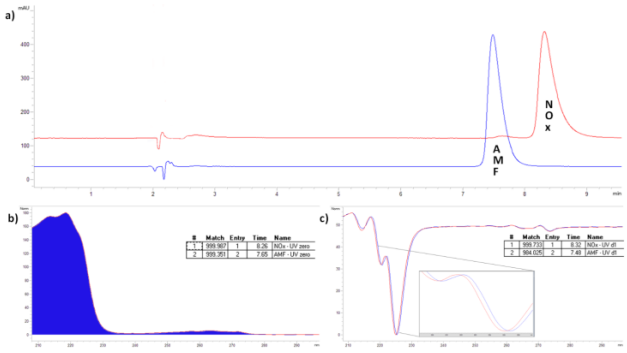

Figure 6. a) overlapped UV spectra of NOx and AMF (max. at 218 nm); b) d1-UV spectra of NOx and AMF (min. at 226 and 225 nm, respectively).

The LC-ESI/MS results showed the base peak at m/z 318.3 for AMF corresponding to its molecular ion $[M-H]^{+,}$ with the isotopic abundance very close to the theoretical one. NOx had a base peak at m/z 334.2. An increase of 15.9 in the m/z ratio is due to the addition of oxygen to the AMF molecule. Also, there is a signal at m/z 667.6, which can be attributed to the NOx dimer $[2M-H]^+$ (Figure 7.b). The N-oxides have a strong tendency towards dimer formation in ESI and APCI techniques (16,17). According to IUPAC (2013), a dimeric ion in mass spectrometry is: "Ion formed by ionization of a dimer or by the association of an ion such as $[M]^{+\bullet}$, $[M+H]^+$, or $[M–H]^-$ with its neutral counterpart M." (18). Ibrahim et al. (2012) compared the formation of N-oxide dimers in mass analysis and state that dimerization is greater in the ESI technique than in the APCI (19).

The mass spectrometry results for NOx were detailed by HRMS which had a base peak at m/z 334.2741 and a prediction of the elemental formula for the molecular ion $[M-H]^+$ as $C_{21}H_{36}NO_2$ (-1.3 ppm error). The NOx formula differs from AMF by only one oxygen. The analysis also showed peak at m/z 667.5397 which might be associated with NOx dimerization in the APCI source. These experimental results strongly suggest that the degraded AMF is an N-oxide.

For the NMR analysis, it is important to emphasize that AMF is in the form of salt, hydrochloride, which makes nitrogen more electronegative, similarly to the N-oxide group. The signal count and the number of hydrogens in the ^1H NMR spectra were identical for AMF and NOx molecules confirming that both have 35 hydrogens. The assignment of the chemical shifts was complex, especially for the methylene groups in H8 and H11 whose experience different environments for its hydrogens due to the chiral carbon in C9 (diastereotopic methylenes) (Figure 8.a). Thus, instead of 2 signals, 4 signals are expected for these two methylene groups. This stereocenter has the same effect for H13 and H19. So, the region from 2.3 to 3.5 ppm presented a great number of signals and overlaps that makes it hard to interpret. The

chemical shifts of the other groups of the AMF and NOx molecules remained practically unchanged (Figure 9).
Assignments for ^{13}C NMR spectra were much simpler (Figure 10). C11 had a signal at 64.82 ppm for AMF and 78.08 ppm for NOx. This variation of more than 10 ppm is considered significant and indicates that the carbon atom is more deshielded in the NOx molecule. The carbons of the morpholine ring, C13 and C19, also experienced similar effect indicating that there was a change in the electronegativity of the nitrogen that binds to C13, C19 and C11 atoms. These results are complementary to the mass spectrometry results and confirm the oxidation of the AMF to its n-oxide form (Figure 7.b).

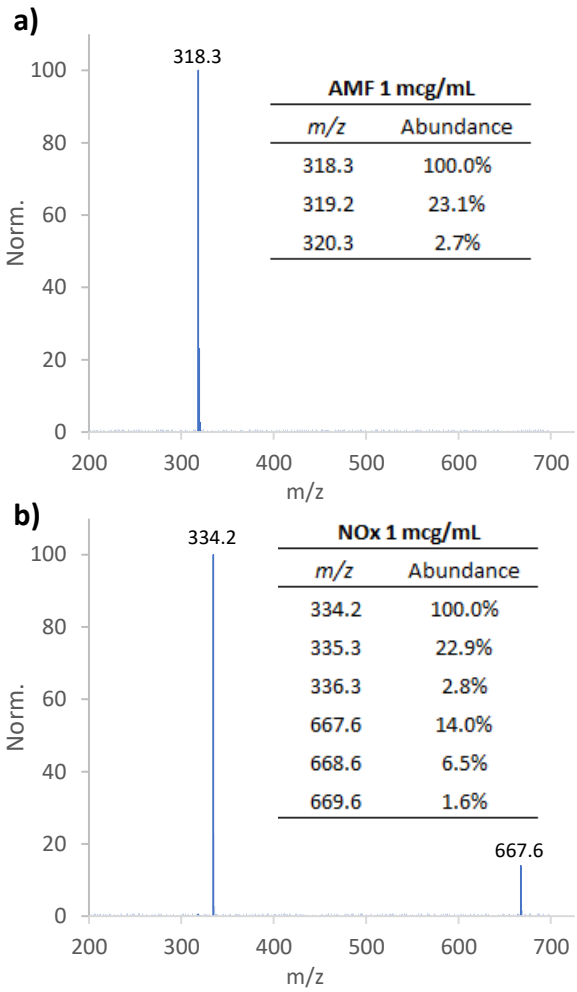

Figure 7. a) MS spectra of AMF; b) MS spectra of NOx.

NOx and AMF discrimination by derivative UV spectroscopy

The UV spectra of AMF and NOx were practically identical and could not be unambiguously discriminated by the similarity index (Figure 5.b). The DAD detector and the Agilent Chemstation software can generate math *derivatives* (dA/dλ) of the UV spectra collected and these functions contain more *maxima* and *minima* that can be extremely useful for identifying and tracking peaks (20). The first devivative UV (d1-UV) spectra of AMF and NOx were calculated from their average peak apex spectra and recorded on the UV Library. Smoothing is very important to minimize the influence of noise. Savitzky-Golay method was set to 7 points since greater number of points (for example, 15) makes the bands broad. The spectra were splined by 5 new data points to become more pleasing.

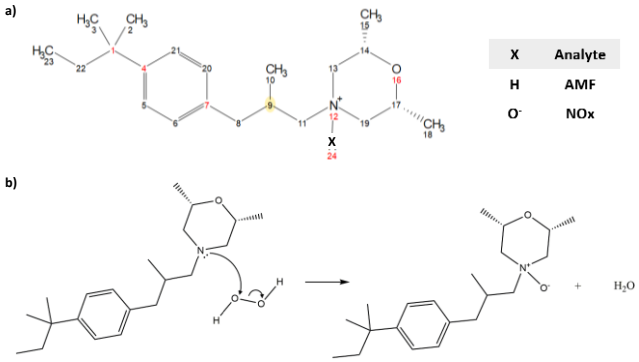

Figure 8. Chemical structures. a) Numbered AMF and NOx for NMR interpretation; b) reaction scheme of AMF oxidation to NOx.

The d1-UV was able to better discriminate NOx and AMF (Similarity < 985.0) from a small change in the shape of the spectra (*maxima* of 225 and 226 nm for AMF and NOx, respectively) (Figure 5.c). These results were reproducible and confirm that d1-UV is a potent tool for the identification of compounds of the same class having very similar UV spectra.

Mass Balance

Mass balance is a measure of a drug loss and the increase of its degradation products. It suggests the correctness of a stability-indicating method showing that all degradation products were detected. The use of area-percent normalization for mass balance assumes same response factor for the drug and its degradants which is not always right, especially for UV detection (6,21).
The oxidation of AMF to NOx occurred in the nitrogen atom that is not part of the chromophore group and, therefore, a similar response factor is expected at 218 nm for these compounds. The sum of the normalized areas was very close to 100% and the small variation verified can be justified by the analytical error (Figure 5.b). This indicates that NOx is the only degradation product under the conditions tested.

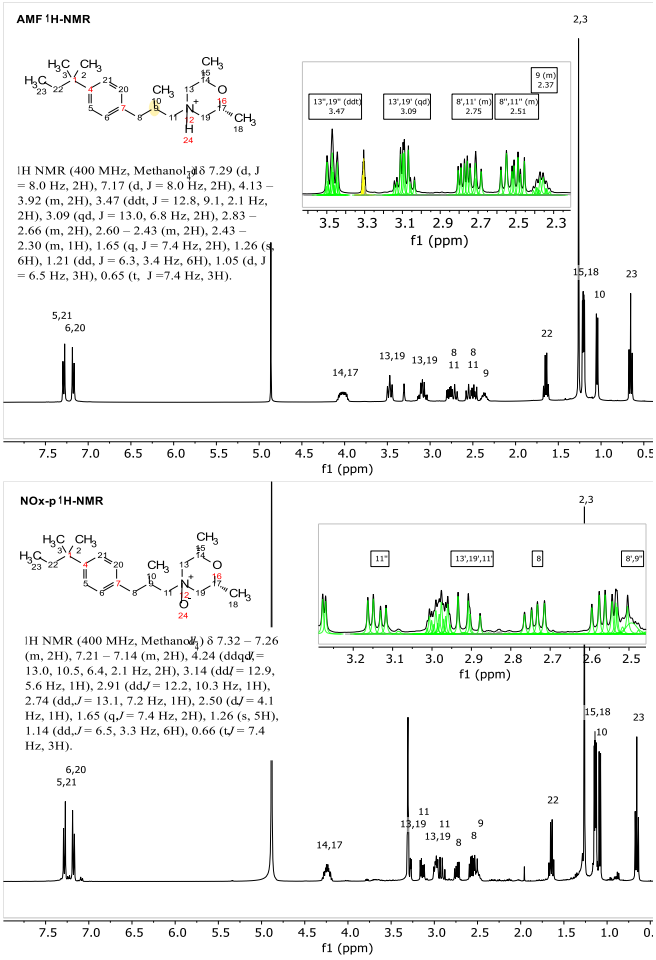

Figure 9. Chemical shifts and assignments of AMF and NOx ^{1}H-NMR.

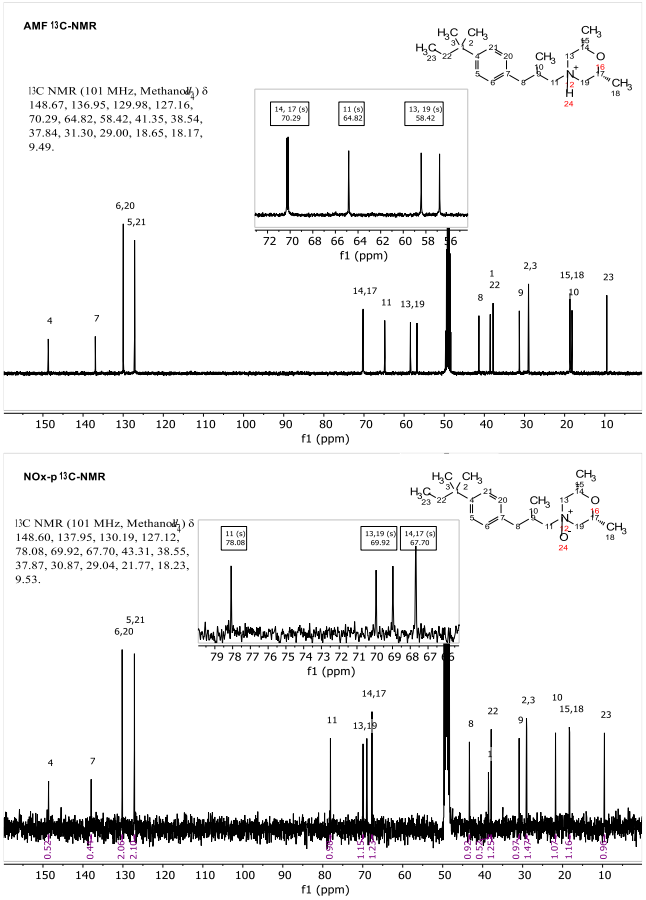

Figure 10. Chemical shifts and assignments of AMF and NOx ^{13}C-NMR.

Conclusions

An ionic pairing HPLC-DAD stability-indicating method was developed and validated showing specificity, linearity, robustness, precision and accuracy for the quantification of amorolfine in the DE15, and Loceryl® lacquer and cream formulations. Standard AMF solutions are stable for at least 4 days at room temperature. AMF is stable under acid and basic conditions and degrades in oxidative medium, but it is pH dependent. The greater the amount of non-ionized drug (pH > pKa), the greater the value of the reaction constant (k) and faster the degradation. LC-MS and ^{1}H and ^{13}C NMR confirmed the N-Oxide as an oxidative degradation product of AMF. UV-derivative is a powerful technique for identification of compounds that have similar UV spectra.

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Conflict of interest

None to declare.

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