Stability-Indicating LC Assay for Butenafine Hydrochloride in Creams Using an Experimental Design for Robustness Evaluation and Photodegradation Kinetics Study

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Abstract

A stability-indicating liquid chromatography method for the determination of the antifungal agent butenafine hydrochloride (BTF) in a cream was developed and validated using the Plackett-Burman experimental design for robustness evaluation. Also, the drug photodegradation kinetics was determined. The analytical column was operated with acetonitrile, methanol and a solution of triethylamine 0.3% adjusted to pH 4.0 (6:3:1) at a flow rate of 1 mL/min and detection at 283 nm. BTF extraction from the cream was done with *n*-butyl alcohol and methanol in ultrasonic bath. The performed degradation conditions were: acid and basic media with HCl 1M and NaOH 1M, respectively, oxidation with H_2O_2 10%, and the exposure to UV-C light. No interference in the BTF elution was verified. Linearity was assessed ($r^2 = 0.9999$) and ANOVA showed non-significative linearity deviation ($p > 0.05$). Adequate results were obtained for repeatability, intra-day precision, and accuracy. Critical factors were selected to examine the method robustness with the two-level Plackett-Burman experimental design and no significant factors were detected ($p > 0.05$). The BTF photodegradation kinetics was determined for the standard and for the cream, both in methanolic solution, under UV light at 254 nm. The degradation process can be described by first-order kinetics in both cases.

Introduction

Butenafine hydrochloride (BTF) is a benzylamine derivative antifungal agent. It has a structure that resembles the allylamines; however, a butylbenzyl group replaces the allylamine group (Figure 1) (1). This antifungal agent has activity against dermatophytes such as *Thrichophyton mentagrophytes, Microsporum canis* and *Thrichophyton rubrum* which cause tinea infections (2,3). The drug inhibits the fungal enzyme squalene epoxidase, blocking the biosynthesis of ergosterol, which is an essential component of fungal cell membranes (1–3). Also, the benzylamine antifungal agent demonstrates inherent anti-inflammatory properties in vivo, as demonstrated by reduced cutaneous erythema response after UVB irradiation (4). BTF was introduced in the Brazilian market in 2007.

There is no study describing suitable analytical conditions of a method that is stability indicative for both BTF raw material and commercial cream, objectifying to quantify the drug in presence of its degradation products (DP) and to elucidate inherit stability characteristics of the standard and the active substance in the semisolid formulation. Due to these reasons, a LC method was developed and validated by specificity, enclosing placebo and stress conditions as acid, basic, oxidation and UV light, linearity, accuracy, repeatability, intermediate precision, robustness, limit of quantification (LOQ), and limit of detection (LOD). A Plackett-Burman two-level experimental design approach was applied for the robustness test and it is justified as it is capable of examining potential sources of variability by screening a large number of factors in relatively small number of experiments. The design allows examining N-1 factors in N experiments, which will lead to a decrease in time and costs to execute the robustness test. (5,6). Therefore, the reliability of the analysis with respect to deliberate variations in method parameters can be evaluated, as recommended by ICH Guideline (7).

According to ICH (8), stress testing of the drug substance can help identify the likely degradation products, which can cooperate to establish the degradation pathways and the intrinsic stability of the molecule. In agreement with this guide, the light testing should be an integral part of the stress testing. Consequently, a study of the BTF photodegradation kinetics was performed in order to provide evidence on how the quality of the drug varies with the time under the influence of light. To realize this study, the developed stability-indicating method was applied.

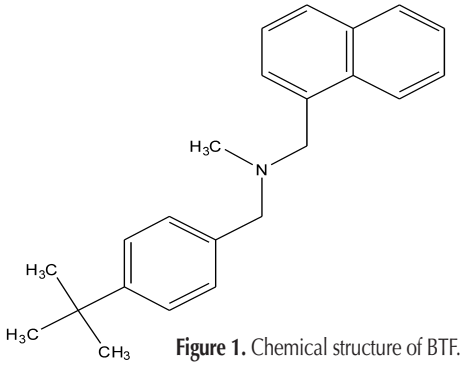

Figure 1. Chemical structure of BTF.

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The objective of the present study was to develop and validate a stability-indicating LC method in compliance with the ICH Guideline (7) and the USP (9) for the determination of BTF in creams using the Plackett-Burman experimental design for robustness evaluation as well as to determine the photodegradation kinetics of the drug in methanolic solution, as there is no published study with this information.

Materials and Methods

Materials

The reference standard was kindly supplied by Brainfarma (Rio de Janeiro, Brazil) and the commercial cream Tefin was obtained in the local market. Creams are packed in metallic tubes and claimed to contain 1% (w/w) of BTF and the following inactive ingredients: benzyl alcohol, ethyl alcohol, cetyl alcohol, sodium benzoate, emulsifying wax, edetate disodium, polyethylene glycol 40 stearate, liquid petrolatum, polysorbate 60, propylene glycol, simethicone, white petrolatum, sodium hydroxide and water. All reagents were analytical or HPLC grade. Acetonitrile and methanol were purchased from Tedia (Fairfield, USA), the phosphoric acid from Merck (Darmstadt, Germany) and the *n*-butyl alcohol from Synth (Diadema, Brazil). Purified water was obtained by a Millipore® Direct-Q 3UV with pump (Molsheim, France).

Chromatographic system

The HPLC system (Agilent 1200 series, Santa Clara, CA) consisted of a G1311A quaternary pump, G1322A vacuum degasser, G1316A thermostat column compartment, G1329A standard auto sampler and G1315B diode array detector set at 283 nm. The analytical column, a Shim-pack CLC – C8 (M) (250 mm × 4.6 mm i.d., 5 µm particle size) (Shimadzu Corporation, Tokyo, Japan), and the guard column, a Shim-pack CLC G-C8 (4) (4.0 mm i.d., 10 mm long) (Shimadzu), were operated in ambient temperature (25°C). The final selected mobile phase was acetonitrile, methanol, and a solution of triethylamine 0.3% adjusted to pH 4.0 with phosphoric acid 10% (6:3:1; v/v/v) in isocratic mode at a flow rate of 1 mL/min and the sample injection volume was 20 µL. The triethylamine solution was prepared freshly or kept in refrigerator (5°C ± 3°C) for a maximum time of 24 h.

Sample preparation for LC analysis

The stock solution of BTF reference standard was prepared in methanol. The working standard solution (20 µg/mL) was obtained by the dilution of the stock solution in a mixture of acetonitrile, methanol and water (6:3:1, v/v/v). The use of water instead of a solution of triethylamine and phosphoric acid in the diluent mixture was verified and no modification in the chromatographic profile or in the measured areas was observed.

For the commercial cream, a quantity equivalent of 10 mg BTF was transferred to a 50 mL volumetric flask, and then it was added 15 mL of co-solvent *n*-butyl alcohol (that was selected after the testing of several solvents), followed by 5 min in ultrasonic bath. After that, 15 mL of methanol were added to the volumetric flask, followed by more 5 min in ultrasonic bath. Methanol was added until the concentration of 200 µg/mL of BTF. This solution was filtered through cellulose paper filter and one aliquot of the filtrated fluid was diluted with a mixture of acetonitrile, methanol and water (6:3:1, v/v/v) until the final concentration of 20 µg/mL. Also, here the use of water instead of a solution of triethylamine and phosphoric acid in the diluent mixture was verified and no modification in the chromatographic profile or in the measured areas was observed. Both sample and standard solutions were then filtered through a 0.45 µm membrane filter (Millipore) prior to the injection.

Validation procedure

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose (7). The method was validated for linearity, detection limit, quantification limit, precision (repeatability and intermediate precision), accuracy, specificity, robustness, and system suitability.

To test linearity, standard plots were constructed with six concentrations in the range of 10–40 µg/mL of the drug prepared in triplicates. The linearity was evaluated by linear regression analysis that was calculated by the least square regression and by ANOVA for compliance of the linear model.

The quantification and detection limits were obtained based on signal-to-noise approach. The background noise was obtained after injection of the blank, observed over a distance equal to 20 times the width at half-height of the peak in a chromatogram obtained by the injection of 20 µg/mL of the reference standard (10,11). The signal-to-noise ratio applied was 10:1 for the LOQ and 3:1 for the LOD. The results were verified experimentally.

The repeatability was verified from six independent sample preparations in the same day, obtained as described in "Sample preparation for LC analysis." The intermediate precision was tested by assaying freshly prepared sample solutions at the same concentration in two other days. Precision was reported as %RSD.

The accuracy was determined by the recovery of known amounts of BTF reference standard added to the samples in the beginning of the preparative process. The added levels were 20%, 40%, and 60% of the nominal drug concentration. The results were expressed as the percentage of BTF reference standard recovered from the sample.

Two types of specificity experiments were performed. In the first one, it was assessed by comparing the chromatograms obtained from the pharmaceutical preparation and the standard solution with those obtained from excipients which take part in the commercial cream and verifying the absence of interferences. In the second type, forced degradation protocols were performed in order to provide suitable analytical conditions for stability study of BTF. The accelerated degradation conditions applied were: light, acid, basic and oxidant media. Samples were analyzed against a freshly prepared control sample (with no degradation treatment) and under light protection. The peak purity was determined using the tools of the Agilent Chemstation software. Excipient solutions were submitted to the same degradation conditions in order to demonstrate no interference. Specific details of the experiments conditions are described in the following sections.

Effect of UV light

One mL of a solution containing 0.1 mg/mL of BTF reference

standard in methanol was placed in a closed 1 cm quartz cell. The cells were exposed to a UV chamber (100 × 18 × 17cm) with internal mirrors and UV fluorescent lamp CSR F30W T8 emitting radiation at 254 nm for 15, 30, 60, 120, and 180 min. Before the final study a preliminary evaluation was conducted for 17.5 h. The same procedure was realized for sample solution from the commercial cream, after the BTF extraction as described in Sample preparation for LC analysis. Protected samples, wrapped in aluminum foil (in order to protect from light) were submitted simultaneously to identical conditions and used as control. After the degradation treatment, the samples were diluted to 20 µg/mL with a mixture of acetonitrile–methanol–water (6:3:1, v/v/v) and immediately analyzed.

Effect of oxidation

BTF reference standard was dissolved in methanol (1 mg/mL) and 5 mL of this solution were transferred to a volumetric flask, where hydrogen peroxide solution (30%) was added until the final concentration of 10% and the volume was completed with methanol. After 20 h the solution was diluted until the final concentration of 20 µg/mL, filtered, and analyzed. This degradation period was defined based on previous studies. Similar procedure was realized for the commercial cream, when 25 mL of the initial solution 200 µg/mL of BTF, obtained as described in "Sample preparation for LC analysis," were transferred to a volumetric flask and submitted to degradation. A control solution containing the excipients was prepared under the same circumstances of the commercial cream solution.

Effect of acid and alkaline hydrolysis

Five milliliters of the BTF reference standard solution were transferred to a volumetric flask and HCl 2.5 M (acid degradation) or NaOH 2.5 M (alkaline degradation) was added until the final concentration of 1M in both cases. After 5 h (basic degradation) and 1 and 6 days (acid degradation), one aliquot of the solution was neutralized HCl 1M (alkaline degradation) or with NaOH 1M (acid degradation) and diluted with acetonitrile, methanol and water (6:3:1, v/v/v) until the final concentration of 20 µg/mL for LC analysis. Similar procedure was realized with the commercial cream, when 25 mL of the initial solution 200 µg/mL of BTF (obtained as described in "Sample preparation for LC analysis") were transferred to a volumetric flask and submitted to the degradation. A control solution containing the excipients was prepared under the same circumstances of the commercial cream.

During the stability assays the peak purity tool was applied to confirm the absence of other substances co-eluting in the same retention time.

The robustness of the analytical method was investigated with the two level factors summarized in Table I, where the –1 level is the low parameter level and the +1 is the high parameter level. The six factors selected were examined in a Plackett-Burman design (8 experiments) in different arrangements (5,6).

The effect (Ex) of each factor and the estimate experimental error $(SE)_e$ were calculated according to Heyden et al. (2001) (6). The statistical interpretation provides a numerical limit value that makes it possible to define what is significant and what is not. This limit value to identify statistically significant effects is usually derived from the t-test statistical method, in accordance with the equation:

$$t = \frac{|E_x|}{(SE)_e}$$

An effect is considered significant at a given a level if t calculated > t critical.

The BTF standard and the sample were analyzed under identical experimental conditions and for this reason no additional experiments were necessary. Another approach was also applied to analyze the results: the estimation of the error from the distribution of effects, done according to the Dong algorithm, which is a suitable tool to identify significant effects in small designs. The Dong algorithm allows the determination of the so-called margin of error (ME) and the simultaneous margin of error (SME), both critical effects, calculated according to Heyden et al (2001). One effect that exceeds the ME, but is below the SME is called possibly significant and an effect that is above the SME is considered significant (6).

The system suitability was verified through the evaluation of the obtained parameters for the standard elution, such as theoretical plates, peak asymmetry and retention factor, verified in different days of the method validation. The resolution was calculated according to USP (9) using the chromatogram obtained from the worst case of accelerated degradation conditions, which is the chromatogram that presented the nearest degradation product peak (DP) to the BTF peak. The injection precision was calculated according to USP (9) and also from the difference of duplicate injections of five sample solutions, as described by Ermer and Ploss (12).

BTF photodegradation kinetics

The study was carried out with quartz cells containing 0.5 mL of BTF standard in methanolic solution 400 µg/mL exposed to UVC radiation (254 nm). The experiment conditions for the drug irradiation were the same described for the specificity analysis under UV light as described in "Validation procedure" at preestablished times (40, 60, 80, 100, 120, and 140 min), the total volume contained in the quartz cell was transferred to a volumetric flask (n = 3) and diluted with acetonitrile, methanol, and water (6:3:1, v/v/v) to achieve the final theoretical concentration of 20 µg/mL. These solutions were protected from light and analyzed by LC, employing the validated method.

Table I. Factors and Levels Applied to the Robustness Test by LC Method

Factor	Units	Level (–1)	Level (+1)	Nominal
pH of the TEA* solution	–	3.9	4.1	4
Conc. of the organic phase	%	88†	92†	90
Column temperature	°C	20	30	25
Wavelength of the detector	nm	281/282	284/285	283
Column manufacturer	–	Shimadzu	Merck	Shimadzu
TEA concentration	%	0.27	0.33	0.3

* TEA = triethylamine

† The modification on the organic phase composition was done proportionally to the nominal composition (30% methanol and 60% acetonitrile).

The photodegradation kinetics of BTF in the presence of the cream excipients was evaluated through the same experimental conditions described for the BTF standard. The cream solution, prepared as described in "Sample preparation for LC analysis," was diluted in methanol. Aliquots of 1 mL of this solution (100 µg/mL) were transferred to quartz cells and exposed to UVC light (254 nm). Also, a solution containing the excipient ingredients was submitted to this stress condition to evaluate a possible interference of its degradation in the BTF elution. At pre-established times (30, 60, 90, 120, 150, 180, 210, and 240 min), the total volume contained in the quartz cell was transferred to a volumetric flask (n = 3) and diluted with acetonitrile, methanol and water (6:3:1, v/v/v) to achieve the final theoretical concentration of 20 µg/mL. As described for the standard, these solutions were protected from light and analyzed by LC, employing the validated stability-indicating method.

In order to evaluate a possible contribution of thermally induced degradation, protected samples prepared from the standard and the commercial cream were wrapped in aluminum foil and exposed to the same conditions described above. They were kept in the chamber until the withdrawn of the last samples and analyzed. In addition, the temperature inside the chamber was controlled during the experiment in both cases.

The BTF photodegradation kinetic rate was determined by plotting the drug concentration (zero-order process), the log (first-order process) and the reciprocal (second-order process) concentration versus time. The determination coefficients (R^2) were obtained and the best observed fit indicated the reaction order. The kinetic parameters constant (k) and $t_{0.5}$ were calculated.

Results and Discussion

Selection and optimization of the chromatographic conditions

The effect of the composition of the column and mobile phase on the retention time of BTF and on its chromatographic parameters was initially investigated. At the first stage, C18 column chemistry (15 cm) and a solution 0.3% triethylamine in water were used with acetonitrile as the organic solvent. Modifications in the organic phase percentage resulted in inadequate long retention time or inadequate peak symmetry.

Table II. BTF Precision Study by LC Method

	Label claim (%)	RSD (%)
Intra-day precision (n = 6)		
Day 1	101.13*	0.76
Day 2	99.09	1.62
Day 3	98.26	0.88
Inter-day precision (n = 17)		
	99.4	1.64

* One sample was identified as an outlier, according to Dixon's Q Test (α = 0,05) (13) and excluded.

Subsequently C8 analytical column (25 cm) was also evaluated. Adequate analytical parameters were obtained. Due to the high quantity of acetonitrile required, methanol was tested, as a part of the organic phase, to reduce the analysis costs. The composition of the mobile phase varied from: 45% to 80% (acetonitrile), 10% to 45% (methanol), 10% (triethylamine solution 0.3%), and both 3 and 4 pH values were evaluated (as BTF is a basic compound with pKa value 7.87 ± 0.5, calculated with the ACD/ChemSketch software). A composition of acetonitrile, methanol and triethylamine solution (6:3:1, v/v/v) with pH value of 4.0 was finally chosen. The addition of triethylamine was done to block sample retention by ionized silanols, which could cause tailing of basic compounds.

Validation of LC method

Before the beginning of the validation procedure, a stability test was performed with two solutions: one of them containing reference standard and the other containing the commercial cream, both in a mixture of acetonitrile, methanol and water (6:3:1, v/v/v) and prepared according to the sample preparation for LC analysis section. After 20 h of analysis there was no significant alteration in the measured areas (the variation was smaller than 2%).

Linearity, LOD, and LOQ

Over the concentration range of 10–40 µg/mL, the slope and the intercept obtained from the three standard curves analyzed together were 22.415 and 6.524, respectively, and the determination coefficient was 0.9999. The analysis of variance revealed that the obtained results correspond to a linear regression ($p < 0.05$) with adequate fitting ($p > 0.05$). Regarding the intercept, it was significantly different from the theoretical zero value ($p < 0.05$), although it was less than 2% of the area obtained for 20 µg/mL of BTF reference standard (100% of analyte level), therefore, there is no interference on the validation (10). LOQ and LOD were 0.0542 and 0.0166 µg/mL, respectively.

Precision

Repeatability and intermediate precision results are expressed as relative standard deviations (%RSD). The %RSD values presented in Table II were low for both the repeatability and the intermediate precision (%RSD maximum 1.64%), demonstrating the good precision of the developed method for the semisolid dosage form.

Accuracy

The data for accuracy were expressed in terms of percentage recoveries of BTF from the real samples. These results are summarized in Table III. The mean recovery data were within the range of 100.48% to 103.21% and the mean %RSD was 0.86%, satisfying the acceptance criteria for the study.

Table III. BTF Accuracy Study by LC Method

Added levels (%) (n = 9)	Added conc. (µg/mL)	Mean recovery (%) ± RSD (%)
20	4	103.21 ± 0.90
40	8	101.28 ± 1.32
60	12	100.48 ± 0.38

Specificity

Placebo injections were performed to demonstrate the absence of interference of the excipients components with the BTF elution. The results demonstrate that there was no interference of other materials from the cream formulation, as shown in Figure 2A.

BTF solutions were submitted to different stress conditions to induce degradation. Under UV radiation (254 nm), total degradation was observed after 17.5 h for the standard and for the commercial cream solutions. After 30 min the BTF content in both analytes exhibited some decrease and additional peaks were detected quicker than in other stress conditions (Figure 2B). The results obtained in this preliminary stability study indicate that BTF is very susceptible to the photodegradation. However, UV spectra's from BTF peak and from other two peaks of DP were overlaid (Figure 3). Two of them presented similarity to the BTF peak, suggesting that these DP molecules have few differences from the BTF molecule or that the structures just present the same absorptive properties. These findings indicate that the developed LC method is capable of providing separation among similar molecules and/or molecules with similar absorption profiles from the analyte.

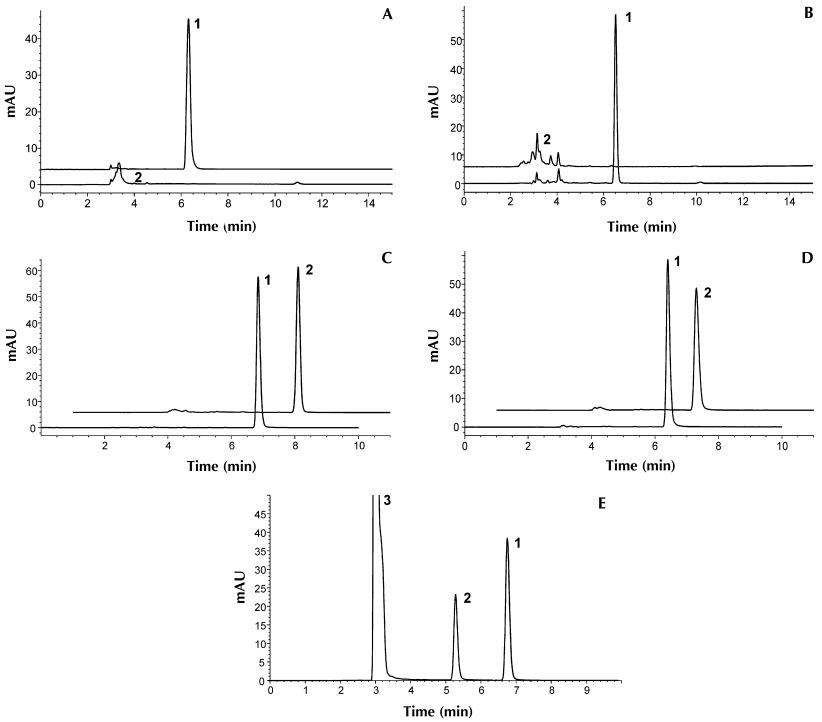

Figure 2. A: Representative chromatograms obtained for 1, BTF reference standard 20 µg/mL and for the 2, placebo. B: Photo degradation of BTF 20 µg/mL from the commercial cream in methanolic solution exposed to 254 nm UV light. 1, 30 min of exposure and 2, 17.5 h of exposure. C: Chromatograms obtained from 1, reference standard 20 µg/mL and from the 2, commercial cream solution 20 µg/mL of BTF, both in 1 M hydrochloric acid for 6 days. D: Chromatograms from the 1, BTF reference standard 20 µg/mL and from the 2, commercial cream solution 20 µg/mL of BTF, both in 1 M sodium hydroxide for 5 h. E: 1, BTF and 2, it's DP in a chromatogram from the commercial cream solution 20 µg/mL of BTF in hydrogen peroxide 10% for 19 h. 3, H_2O_2 stabilizing. Chromatographic conditions: see Chromatographic system.

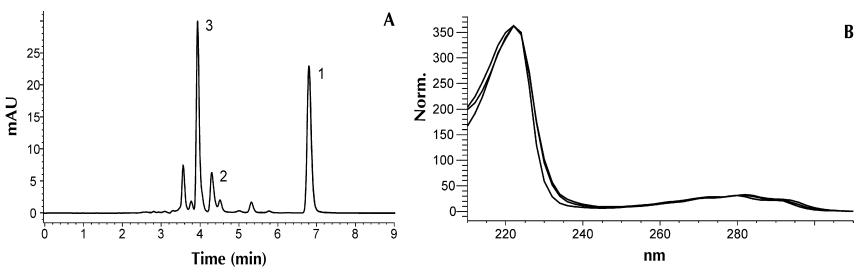

Figure 3. A: Chromatogram from the BTF reference standard exposed to UV light for 2 h. 1) BTF, 2) DP, 2) majority DP. B: The overlapping of the UV spectra from peaks indicated in the chromatogram obtained by DAD resources. A: indicating similarity between the molecules 1, 2, and 3 or same absorptive properties. Chromatographic conditions: see "Chromatographic system."

When acid degradation was performed, no degradation peaks were detected and the concentration of butenafine hydrochloride remained constant during the exposure time to the degradation condition, according to "Validation procedure" (Figure 2C). Under basic conditions the standard remained stable, although the drug from the commercial cream solution did not (Figure 2D). The possible reason can be due to the action of one or more excipient ingredients present in the solution that facilitate the degradation reaction.

The oxidation by H_2O_2 caused the degradation of standard and commercial cream and just one DP peak could be observed. However, the extent of oxidation was not the same for the sample solutions. After 20 h it remained 96.23% of BTF on the standard solution and only 62.42% of the drug lasted on the commercial cream solution (Figure 2E). Also, this higher degradation in sample solution can be due to the action of one or more excipient ingredients that favor the product formation.

Considering the evaluated factors, the highest susceptibility of the drug was detected when its methanolic solution was exposed to UVC light, for both standard solution and commercial cream solution.

Robustness

The Plackett-Burman design selected factors, the applied combination of factor levels (+1 or –1) to each experiment and the obtained responses are summarized in Table IV for both wavelength variations of 1 nm and 2 nm. The responses are the percentages of BTF in the commercial cream (relative to its label claimed concentration) obtained in relation to the standard solution in each experiment.

After the calculation of the effects for each parameter, the statistical interpretation, through the t-test, allowed to define what is significant and what is not. No significant

factors were detected for all the experiments in the tested wavelengths (Table IV), as t-calculated values were minor than the t-critical value ($\alpha = 0.05$).

Another way to evaluate the results is creating a half normal-plot, to rank the effects according to the increasing absolute effect size. The effects are plotted against values called rankits, which are a scale defined by portioning the right half of the normal distribution in equal parts and by taking the median of each slice. The rankits often used to analyze 8 experiments Plackett-Burman design were obtained from Heyden et al. (2001) (6). The effect of each factor was calculated and the results are summarized with the respective rankit in Table V (just for the wavelength variation of two nm). These effect values were compared with the ME (0.44) and the SME (0.89). None of the results exceeded the ME or SME values, so the effects of each parameter were not significant, confirming the results obtained from the t-test, for this wavelength variation.

Also, the performance parameters were analyzed. In all the performed experiments the theoretical plates were higher than 5000 and the tailing factor was smaller than 1.4, in agreement with the literature (14,15). The retention factor observed in all analysis was higher than 1 (16).

Table IV. Plackett-Burman Design Factors and Obtained Response to Each Experiment

Experiment	pH	Column	Dummy	Wave-length	% Organic Phase	Temp.	% TEA*	Response (± 1 nm)	Response (± 2 nm)
1	+1	+1	+1	–1	+1	–1	–1	99.41	99.46
2	–1	+1	+1	+1	–1	+1	–1	99.67	99.91
3	–1	–1	+1	+1	+1	–1	+1	99.15	99.12
4	+1	–1	–1	+1	+1	+1	–1	99.11	99.08
5	–1	+1	–1	–1	+1	+1	+1	99.57	99.5
6	+1	–1	+1	–1	–1	+1	+1	99.22	99.24
7	+1	+1	–1	+1	–1	–1	+1	99.35	99.35
8	–1	–1	–1	–1	–1	–1	–1	99.45	99.47
$t_{calculated}$ (± 1 nm)	–1.333	1.902	–0.053	–0.658	–0.800	0.373	–0.622		
$t_{calculated}$ (± 2 nm)	–1.166	1.756	0.442	–0.281	–1.086	0.442	–0.952		
$t_{critical}$	2.365								

* TEA = triethylamine

System suitability

The analysis of the BTF standard evaluated at each day presented the approximate results: 16600 theoretical plates, 0.97 peak asymmetry and 2.09 for the retention factor. The resolution of BTF and DP was obtained through the analysis of the oxidative degradation chromatogram that has the nearest DP from the BTF peak and the calculated value was 12.4. For injection repeatability, calculated according to two different ways (as described in "Validation procedure"), a RSD of 0.2% was determined for the standard and sample solution. The obtained results establish that the LC system and procedure are capable of providing data of acceptance quality (14,15).

BTF photodegradation kinetics

Through the evaluation of the determination coefficients obtained by plotting the drug concentration (zero-order process), the log (first-order process), and the reciprocal (second-order process) concentration versus time, the degradation of BTF in methanolic solutions could be better described as first order kinetic for both the standard solution and the cream solution, under the applied experimental conditions. The obtained determination coefficient values for the standard and the cream solutions, respectively, were: for zero order process 0.9789 and 0.9271; for first order process 0.9891 and 0.9755; for second order process 0.9809 and 0.8853. Therefore, the degradation speed is proportional to one component (17), the BTF itself. The obtained degradation rate constants (k) and $t_{0.5}$ were: 0.284 h^{-1} and 2.44 h for the standard solution; 0.422 h^{-1} and 1.64 h for the drug in the cream solution. The rate constants were quite different probably because of the action of one or more excipient ingredients that influence the drug degradation in the cream solution, as they are in the sample solution because of its preparation procedure. The plots containing the logarithm of the remaining BTF in the standard and in the commercial cream versus time are in Figure 4.

The temperature inside the chamber was always below 34°C. There was no consider-

Table V. Effects from the Seven-factor Plackett-Burman Design for The Wavelengths of 281 nm and 285 nm

Factor (definition)	Effect	Rankit
Detection Wavelength (A)	0.0525	0.09
Temperature (B)	0.0825	0.27
Dummy (C)	0.0825	0.46
% TEA* (D)	0.1775	0.66
% Organic phase (E)	0.2025	0.90
pH of TEA* solution (F)	0.2175	1.21
Column (G)	0.3275	1.71

*TEA = triethylamine

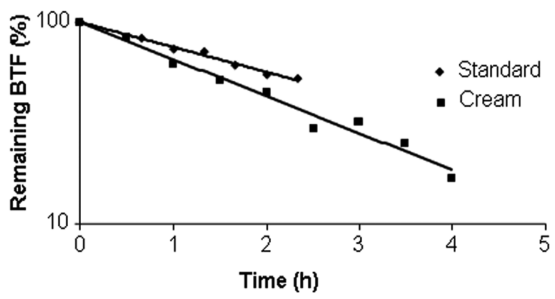

Figure 4. First-order reaction plots (logarithmic scale) of remaining BTF from the standard and from the cream versus time, after phtodegradation with UV-C light.

able variation in the BTF concentration in the solutions containing BTF standard and commercial cream, wrapped in aluminum foil and submitted to the photodegradation conditions, as thermal controls. The peaks generated by the solution containing the excipient ingredients and exposed to the same UV-C radiation had no interference in the BTF migration time.

Conclusion

The reverse phase LC method proposed was found to be simple, fast, accurate, precise, linear, robust, and specific and it is a powerful tool to investigate chemical stability of BTF. The robustness of the method was verified with small variations on pH, concentration of triethylamine solution, concentration of organic phase, detector wavelength, column manufacturer and analysis temperature, using the Plackett-Burman experimental design to examine potential sources of variability. All the parameters meet the criteria of the ICH guidelines for method validation. Its chromatographic retention time of 6.8 min allows the analysis of a large number of samples in an adequate period of time. The method could therefore be recommended for routine quality control analysis of raw material and of commercial cream, as well as for protocols of BTF stability study.

The method applicability to stability studies was proved through the evaluation of the main factors that affect the drug content in solution and through the BTF degradation kinetics in methanolic solution exposed to UVC light. Both methanolic solutions of BTF, reference standard and commercial cream, showed stability in relation to the applied acid condition and instability when submitted to basic, oxidation and light conditions, among these factors the highest susceptibility was detected at photodegradation conditions. The degradation kinetics was defined as first order for the drug in both standard and cream methanolic solutions, establishing that its speed is dependent on the drug concentration. The developed method and the stability study are essential to future studies of isolation and identification of the degradation products, objectifying the analysis of biological and toxicological aspects, aiming the maintenance of efficacy and safety of the pharmaceutical product.

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