



Development of stability-indicating LC method assisted by Design of Experiment for fenticonazole cream analysis in presence of degradation product

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ABSTRACT

Fenticonazole is an antifungal drug widely used in a cream formulation including as a generic medicine. Stability studies of fenticonazole in a cream formulation are very scarce. In this research, we intent to contribute to generic medicines quality control and provide reliable data seeking for insertion of fenticonazole monograph in official compendia. Therefore, in this work it was studied the behavior of fenticonazole under several conditions and developed a stability-indicating LC method to separate the degradation products and quantify the drug in presence of them. It was used the Design of Experiments (DoE) as tool to achieve robust and easy transferable method. Fenticonazole stability was evaluated under aqueous, alkaline (0.1 M NaOH), acidic (0.1 M HCl) and oxidative (3% v/v, H₂O₂) at ambient temperature and heating at 90°C, over 6 hours. The drug shows to be unstable under all stressed test conditions. It was completely degraded under acid medium with arising of degradation products. The robust and stability indicating LC method was validated. It is able to reveal the fenticonazole instability and to separate its degradation product with accuracy and precision (RSD < 2%) and without any placebo interferences.

Keywords: Liquid Chromatography. Design of Experiment. Degradation Product. Fenticonazole. Drug Stability. Quality Control.

INTRODUCTION

Fungi represent a threat for human health, especially for immunocompromised individuals (Lacaz et al., 1984; Rang et al., 2007). Nowadays, the fungal infections are more frequent and antifungal medications are increasingly used, either individually or in conjunction therapies with antibiotics and corticosteroids (Veraldi & Milani, 2008; Gilman et al., 2003; Catalan & Montejo, 2006, Corrêa & Salgado, 2011a).

Fenticonazole, 1-[2-(2,4-dichlorophenyl)-2-[[4-(phenylsulfanyl) phenyl] methoxy]ethyl]-1H-imidazole, Figure 1, is a topic imidazole derivative with antifungal activity. The antifungal activity of drug is described by the inhibition of some enzymes of cytochrome P450 complex. Therefore, the ergosterol synthesis in fungal plasma membrane is deficient as their growth (Sheppard & Lampiris, 2008; Carrillo-Muñoz et al., 2006).

Fenticonazole has an important role in treatment of candidiasis infections. It is among the antifungal therapeutic options for topical use. Beside it shows high efficacy and low incidence of adverse reactions (Gorlero et al., 1994). Hence, fenticonazole has been used widely around the world.

Among all concerns about medications, the stability is counted. Even under various and unfriendly climatic conditions drugs should remain its therapeutically properties and safety until the expiration time (Corrêa et al., 2011b). The stability of fenticonazole has been poorly studied. Studies related to stability analysis of the drug are very scarce. Thus, studies, which evaluate the behavior of the drug under environmental conditions, are needed to give a comprehensive understanding about the drug stability and contribute to improvement of current medications (Gorlero et al., 1994; Corrêa et al., 2011b, 2013). Some researchers have been studied the fenticonazole analyses but the drug stability has been not addressed (Di Pietra et al., 1992; Corrêa et al., 2019). In the same way, analytical methods that allow quantification of fenticonazole in presence of its degradation products were not founded. Pharmacopeial official monograph for fenticonazole raw material is found at British Pharmacopeia (2012), where titration is indicated for drug quantitation (Corrêa et al., 2020). This analytical lack makes clear the importance of this current research, since the stability of fenticonazole cream was studied using a chromatographic separation technique that allows the dosage of the drug in the presence of its degradation products.

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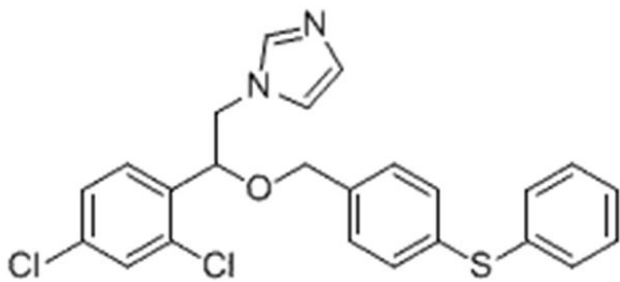


FIGURE 1. Chemical structure of fenticonazole

In current practice, as highlighted by Springall (2018), analytical procedures are typically developed utilizing one factor at a time approach, in which one parameter is optimized in isolation with the others remaining constant. By following this approach for method development, no information is obtained around how method parameters interact with each other and therefore impacts the results obtained (Springall, 2018). The major problem in this kind of practice is that can result in analytical procedures with very narrow robust ranges of operation. An analytical method without a suitable robustness can lead to a method failure after the analytical procedure is transferred from the development environment into an internal or external quality control laboratory (Springall, 2018).

Per ICH guideline, characteristics to be considered during the validation include specificity, range, linearity, accuracy, precision, limit of detection and quantitation, and robustness (ICH, 2005). Following the guidance from International Conference on Harmonization (ICH) the evaluation of robustness was considered during the analytical development phase and it has shown the reliability of an analysis with respect to deliberate variations in method parameters (ICH, 2005). According the ICH guidance, one consequence of the evaluation of robustness should be that a series of parameters is established to ensure that the validity of the analytical procedure is maintained whenever used (ICH, 2005). One of the most important goals with this research is to developing an analytical method that can be applied by any quality control laboratory for the quality control of fenticonazole medicines and contribute to the insertion of fenticonazole monograph in the Brazilian Pharmacopeia. Therefore, this method has to be robust.

In this research, the Design of Experiments (DoE) was applied to assisting the analytical development. According to ICH guidance, a structured, organized method for determining the relationship between factors affecting a process and the output of that process is also known as Design of Experiments (ICH, 2009). The DoE methodology is a test or series of tests in which purposeful changes are made to the input variables (factors) of a process so that we may observe and identify corresponding changes in the output response. In short, DoE is an approach that can be used to determine cause and effect relationships in a stepwise manner (Springall, 2018).

DoE can be utilized for the purposes of demonstrating robustness of the analytical procedure by assessing the capacity

of the method to remain unaffected by small variations in the method parameters, so that it can perform to the requirements and can be successfully transferred to quality control laboratories with a reduction in the risk involved (Springall, 2018). As a DoE tool, a factorial planning was used to assist the analytical method robustness test. It is a useful analytical strategy and its main application lies in the screening of the most relevant variables of a given analytical system (Vicentini et al., 2011). Youden approach, which is a fractional factorial design, is a useful test to avoid the difficult task of performing too many experiments, since it can be necessary a high number of experiments taking into consideration the most important parameters (Karageorgou & Samanidou, 2014).

Therefore, the aim of this work was to study the behavior of fenticonazole under several conditions and to develop a robust stability-indicating LC method to separate the possible degradation products and quantify the drug in the presence of them. To contribute to the development of pharmaceutical sciences and guarantee the quality of medicines, in addition to providing data for the insertion or update the drug monograph in official compendia.

MATERIAL AND METHODS

Chemicals

EMS S/A (Hortolândia, São Paulo, Brazil) gently donated Fenticonazole chemical secondary reference (assigned purity of 99.8%), tubes with 40g of fenticonazole vaginal cream (medicine) and its placebo.

Methanol LC grade was purchased from Synth (Diadema, São Paulo, Brazil). Ultrapurified water (<math><18</math><math>$\mu\text{m}>>$) was prepared in-house by using Direct-QR water system (Millipore Corporation, Billerica, USA). Prior to use, mobile phase solvents were degassed in an ultrasonic bath for 30 min. Glacial acetic acid, sodium hydroxide, hydrochloric acid, and hydrogen peroxide (reagent grade) were purchased from Merck (Darmstadt, Germany).

Apparatus

Liquid Chromatography apparatus (Waters Corporation, Milford, MA, USA), equipped with a Waters 1525 binary pump, a Rheodyne Breeze 7725i manual injector, and a Waters 2487 UV-VIS wavelength detector was used.

HPLC analysis was conducted by using RP C18 column (ChromSpher Agilent, Munich, Germany), with 5 μm particle size, 4.6 mm x 250 mm. For robustness test another column was used in addition, a RP C18 column (Symmetry Waters, Milford, MA, USA and), with 5 μm particle size, 4.6 mm x 250 mm.

LC conditions

Chromatographic analysis were performed in isocratic mode. Mobile phase consisted of methanol-water (85:15, v/v) at a flow rate of 1 mL/min. The injection volume was 20 μL and the detection wavelength was 252 nm.

Standard and Sample Solutions Preparation

To prepare the stock sample solution twenty tubes containing 40 g of fenticonazole vaginal cream were accurately weighed and mixed. An amount of cream equivalent to 12 mg of fenticonazole was weighed and transferred into a 200 mL volumetric flask. Methanol was used as diluent. It was sonicated for 60 min and then the volume was making up with methanol. Working sample solutions were prepared in triplicate by diluting the stock standard solution with methanol to reach six final concentrations: 15, 21, 30, 33, 39 and 45 µg/mL.

The concentration of 30 µg/mL was used to assess the fenticonazole content. Therefore, the linear range (15-45 µg/mL) was prepared covering from 50 to 150% of the working concentration (30 µg/mL). Placebo solution was prepared at the same form. Thus, the same amount of cream used for prepare the stock sample solution was used for prepare the placebo stock solution, but using the placebo of the cream formulation.

A stock standard solution of fenticonazole was prepared by dissolving with methanol 6.0 mg of fenticonazole chemical reference accurately weighted in 100 mL volumetric flask. Diluted standard solutions were prepared in the same way and in the same concentrations as working sample solutions.

All sample and standard solutions were filtered through a 0.45 µm regenerated cellulose membrane filter.

Stability Studies

Acid, alkali, neutral, oxidative and thermal degradations

To evaluate the stability of fenticonazole the drug was challenged by four stressing media and by high temperature (90°C) for a few time (6 hours). It was prepared sample solutions, all containing fenticonazole at 30 µg/mL (work concentration), in 3% hydrogen peroxid (H₂O₂) (v/v), 0.1 M hydrochloric acid (HCl), 0.1 M sodium hydroxide (NaOH) and water (ICH, 2003). These solutions were stored at room temperature for 30 minutes and after they were stored for 2, 4 and 6 hours in heating bath at 90°C. Aliquots were removed and evaluated by LC method.

Specificity

The specificity of the method was demonstrated by stability study samples as well as fenticonazole standard, cream and placebo solutions. The method was validated according to International Conference on Harmonization (ICH) guide to validation of methods (ICH, 2005).

Linearity and precision

The calibration curve was obtained using work standards solutions at six concentrations levels: 15, 21, 30, 33, 39 and 45 µg/mL. The linearity was evaluated with triplicate. The validity of linearity was verified by analysis of variance (ANOVA).

Precision was determined by repeatability (intraday) and intermediate precision (interday). To repeatability test, three curves were constructed with established six concentration

levels using standard solutions in the same day. To intermediate precision (interday) three curves were constructed with three concentrations levels (15, 30 and 45 µg/mL) using standard solution in a different day. It was considered an interval of two days between repeatability and intermediate precision. The results were expressed as percentage of relative standard deviation (R.S.D.) (ICH, 2005).

Accuracy

The accuracy of the method was evaluated by the recovery method, using placebo enriched. In that, recovery method was determined with the addition of known amounts of fenticonazole standard to placebo in order to obtain the six established concentrations levels in triplicate (ICH, 2005). The accuracy test was performed at six levels, the same concentrations used for linearity test, but now applying enriched placebo.

Detection and quantitation limits

The limits of detection and quantitation were inferred using the calibration curves as suggested by ICH guideline (ICH, 2005).

Robustness analysis by design of experiments (DoE)

This parameter was evaluated using the fractional factorial design (2⁸⁻⁵) as a DoE framework proposed by Youden and Steiner method (Youden & Steiner, 1975). Thus, eight analytical runs employing factorial combination could evaluate seven different parameters. The most significant analytical variables was screened so that experiments can be performed allowing refinement and a better knowledge of the developing analytical method (Vicentini et al., 2011)

RESULTS AND DISCUSSIONS

Analytical development

The LC method validated has good separation and symmetry of fenticonazole peak. The peak was obtained by using Agilent™ column and mix of methanol and water (85:15, v/v) as mobile phase. The retention time is 8.26 min. The chromatograms of fenticonazole standard, fenticonazole cream (sample), its placebo and stressed sample under alkaline media at 30 µg/mL for 30 minutes can be observed in Figure 2.

The Table 1 shows the most important chromatographic parameters monitored during the validation of the developed method and the results can be compared with the acceptance criteria widely used in LC (Harris, 2005).

Stability Study

The LC method was able to show the instability of fenticonazole and to separate the peaks from possible degradation products and the drug. This new method was applied as an indicating-stability method. The stress conditions tested were aqueous, alkaline (0.1 M NaOH), acid (0.1 M HCl) and oxidative (3% v/v H₂O₂) at room temperature and under

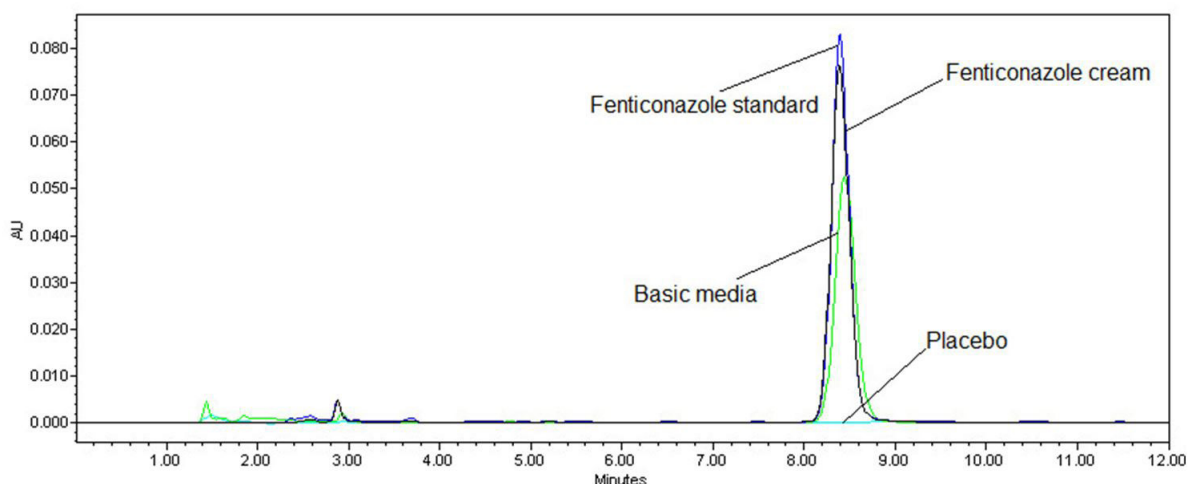


FIGURE 2. Overlay chromatograms for fenticonazole standard, fenticonazole cream (product), its placebo and stressed sample under alkaline media at 30 µg/mL for 30 minutes, all samples at room temperature.

Table 1. Chromatographic parameters and the acceptance criteria for fenticonazole determination by developed LC method.

Chromatographic parameters	Results	Acceptance Criteria
t_R (retention time, min)	8.26	-
T_0 (column dead time, min)	1.50	-
k' (retention factor)	4.16	$2 < k' < 10$
Tail factor	1.2	0.8–1.5
N (column efficiency)	2227	$N > 2000$

heating (90°C) for, 2, 4 and 6 hours. Fenticonazole showed to be unstable under all tested conditions with high-speed degradation under heating, even under neutral medium (aqueous). After 2 hours, the drug was consumed 85%, 18% and 40% under aqueous, alkaline and oxidative condition, respectively. Under acidic conditions, some peaks emerged along stress test, which were considered degradation products. Over time, under acid plus heating conditions, fenticonazole was completely consumed. It is possible to note in Figure 3 the chromatograms obtained from aqueous, acid, alkaline and oxidative stress.

The developed stability-indicating method was able to show the drug degradation and to separate completely the peaks of fenticonazole standard, cream and degradation products, without any placebo interferences. Placebo preparations were evaluated at the same way of product and chemical secondary reference preparations and none of any substances that make up the cream matrix has shown peak's retention time equal or even close to that observed for the drug substance.

Therefore, it can be applied in stability studies and quality control of fenticonazole cream to monitor the drug and its degradation products. This monitoring is a recent and global concern about drugs and medicines (Brasil, 2019).

Validation of the method

The method was validated by means of the ANOVA. According to ANOVA there is a statistically significant linear regression in tested concentration range, 15 to 45 µg/mL

(F calculated $> F$ critical). The method precision (Table 2) was evaluated by repeatability (intraday) and intermediate precision (interday). The average of R.S.D. (%) obtained for repeatability and for interday precision, calculated from assays on three days, indicate a very good precision method. Regarding the accuracy (Table 3), it was determined through the mean recovery. It was evaluated over the entire linearity range in triplicate but using placebo enriched samples. Table 3 shows the results for accuracy test, which indicate a very good agreement between the true value and the value found.

Robustness testing is so important to prove if some small changes in the procedure produce a different result. If the analytical method being susceptible to tested procedures variations, this should be controlled using precautions described in the method (Brasil, 2017)

A method can be considered as robust if small analytical variations produce no significant changes in its results. The Youden & Steiner robustness test (Youden & Steiner, 1975) permit to evaluate deliberated changes in seven parameters on the fenticonazole content. The follow parameters were evaluated: column (A/a), wavelength (B/b), flow rate (C/c), mobile phase (D/d), reagent brand (E/e), room temperature (F/f) and sonication time of sample, standard and placebo solutions (G/g) (Karageorgou & Samanidou, 2014).

The factorial combination of these analytical parameters evaluated can be observed in the Table 4 and the Table 5 shows the analytical parameters and its variations (Karageorgou & Samanidou, 2014).

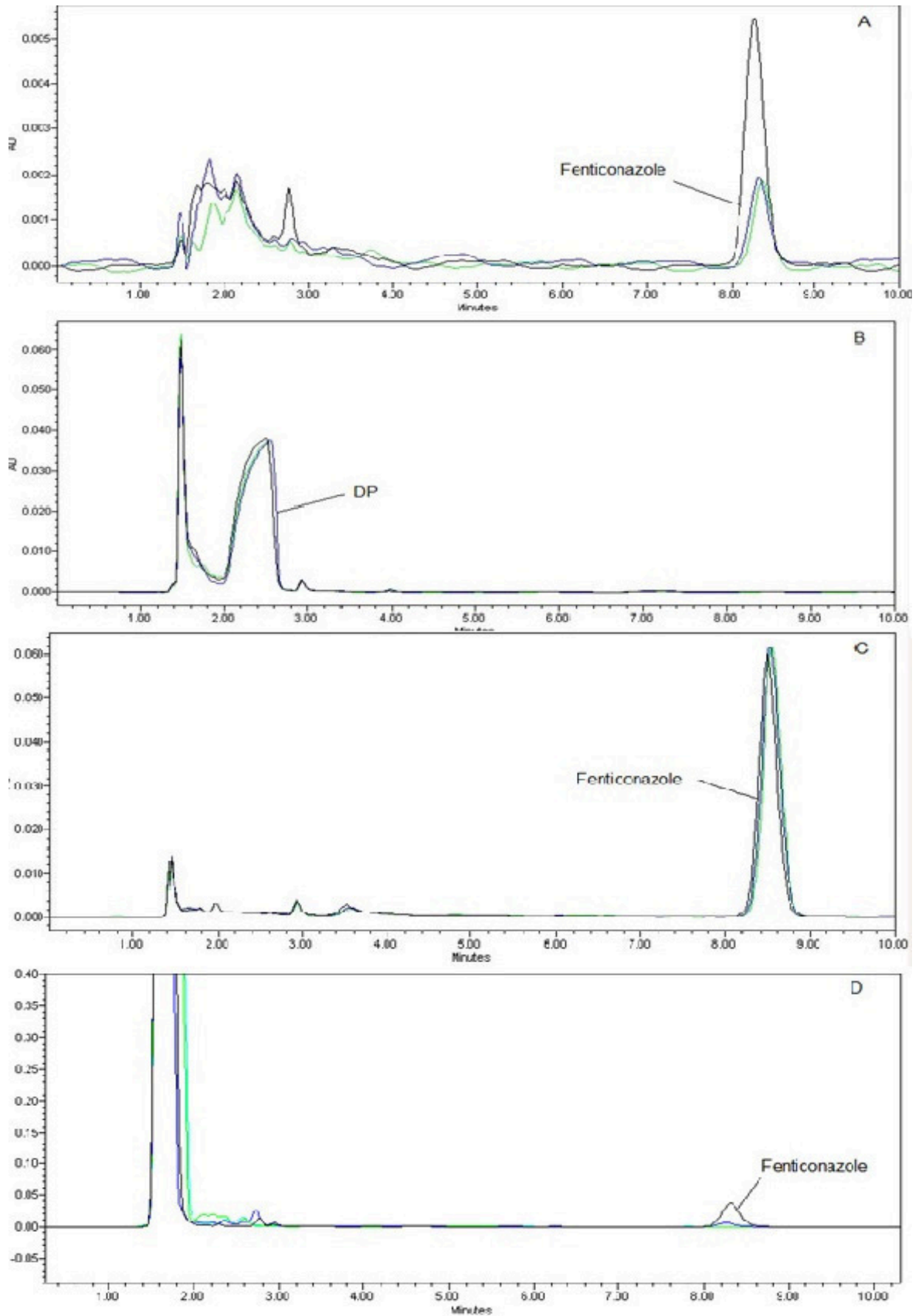


FIGURE 3. The overlaid chromatograms obtained from aqueous (A), acid (B), alkaline (C) and oxidative (D) stress for fenticonazole cream over the heating time: 2h (black line), 4h (blue line) and 6h (green line). The possible degradation product (DP) is highlighted on acid stress (B).

Table 2. Validation parameters for chromatographic method.

Parameters	Acceptance Criteria
Useful concentration, µg/mL	30
Analytical curve	33370.79x-12686.1
Coefficient of correlation (r)	0.9997
R.S.D. for repeatability ¹ (%; n = 54)	0.30
R.S.D. of intermediate precision (%; n= 27)	0.51
Detection limit, µg/mL	1.89
Quantitation limit, µg/mL	6.30

¹ measured at the six concentration levels

Table 3. Accuracy results by recovery method.

Concentration of placebo enriched samples (µg/mL) ^a	Recovery (%)	R.S.D. (%)
15	101.71	0.05
21	99.37	0.15
30	98.75	0.29
33	99.95	0.36
39	98.97	0.35
45	99.70	0.27
Average	99.74	0.25

^a prepared in triplicate

Table 4. Factorial combination of parameters according to Youden & Steiner test.

Parameter	Factorial combination							
	1	2	3	4	5	6	7	8
A,a	A	A	A	A	a	A	a	a
B,b	B	B	b	B	B	B	B	b
C,c	C	c	C	C	C	C	C	c
D,d	D	D	d	D	d	D	D	D
E,e	E	e	E	E	e	E	e	E
F,f	F	F	f	F	F	F	f	F
G,g	G	G	g	G	g	G	G	g
Results	s	t	u	v	w	x	y	z
	98.83	91.01	92.32	98.85	92.04	98.90	98.95	92.49

The follow equation was used to evaluate the effect of each variation on the fenticonazole assay (César & Pianetti, 2009):

$$A, a(x + y + w + z) / 4 - (s + t + u + v) / 4 \text{ (Equation of parameter } A, a) \quad (1)$$

The robustness results are shown in tables 5 and 6. The standard deviation (s) value calculated for the analytical results of the eight analysis (s to z) is equal to 3.72. Moreover, the criterion $s\sqrt{2}$ was used to evaluate the results. Effect values higher than the criterion $s\sqrt{2}$ were considered significant, which means that the method is sensitive to changes in the concerned variable (Bonfilio et al., 2011; Youden & Steiner, 1975). The major calculated effect was for the sonication time parameter (6.92); it is higher than the criterion (5.26). In addition, the sonication time variation was able to cause a deviation in the drug content analysis greater than acceptable variation in the pharmaceutical product assay (5%), when under stability study (Brasil, 2019). This result indicate that is necessary to describe in the method that the sonication time has to be rigorously observed during method perform to ensure complete extraction of drug from the pharmaceutical form.

Table 5. Analytical parameters and its variations to robustness test.

	Parameters	Variations
A	Chromatographic column	A: Agilent ^a a: Waters ^a
B	Wavelength	B: 252 nm b: 242 nm
C	Flow rate	C: 1,0 mL/min c: 0,8 mL/min
D	Mobile phase	D: 85:15 ^b d: 90:10 ^b
E	Methanol brand	E: Synth e: Qhemis
F	Room temperature	F: 18.5°C f: 27.0°C
G	Sonication time	G: 60 min g: 55 min

a: C18, 250 x 4,6mm, 5 µm; b: methanol:water

Table 6. Robustness test results.

Factor	Effects
Chromatographic column	-0.34
Wavelength	-0.45
Flow rate	0.22
Mobile phase	-0.21
Methanol brand	0.42
Room temperature	0.26
Sonication time	6.92 ^a

^a Effect value higher than the criterion $s\sqrt{2}$

The analytical method robustness was evaluated by assisting of the DoE and it is now well know, so that it can be applied by any quality control laboratory and contribute to insertion of fenticonazole monograph in Brazilian Pharmacopeia.

CONCLUSION

Fenticonazole shows to be unstable when under all stressed test conditions, and it was completely degraded under acid medium. The drug is used in a cream formulation therefore, to guarantee the drug stability; the composition and pH of the product formulation have to be carefully chosen. In addition, strict care must be taken with regard to the temperature to which the formulation is subjected, since the degeneration of the drug was observed under heating even when in a neutral medium.

The new developed and validated LC method shows to be able to reveal the fenticonazole instability and to separate its degradation product without any interference of placebo cream formulation. It is a reliable and robust analytical method; therefore it can be applied to stability studies and quality control of fenticonazole cream formulations and can contribute to insertion of the fenticonazole monograph in the Brazilian official compendia.

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RESUMO

Desenvolvimento de método cromatográfico (LC) indicativo de estabilidade empregando planejamento experimental para análise de fenticonazole creme na presença de produtos de degradação

O fenticonazol é um medicamento antifúngico amplamente utilizado na forma de creme, inclusive como medicamento genérico. Os estudos de estabilidade do fenticonazol em na forma de creme são muito escassos. Nesta pesquisa, pretendemos contribuir com o controle de qualidade de medicamentos genéricos e fornecer dados confiáveis buscando a inserção da monografia com fenticonazol em compêndios oficiais. Portanto, neste trabalho, estudou-se o comportamento do fenticonazol sob várias condições e foi desenvolvido um método analítico indicativo de estabilidade por cromatografia a líquido (LC) para separar os produtos de degradação e quantificar o fármaco na presença daqueles, usando o planejamento experimental como ferramenta para obter um método robusto e de fácil transferência. A estabilidade do fenticonazol foi avaliada em meio aquoso, alcalino (NaOH 0,1 M), ácido (HCL 0,1 M) e oxidativo (3% v / v, H₂O₂) à temperatura ambiente e sob aquecimento a 90° C, durante 6 horas. O fármaco mostrou-se instável em todas as condições ensaiadas. Foi completamente degradado em meio ácido com o surgimento de produtos de degradação. O método analítico robusto e indicativo de estabilidade foi validado. O método desenvolvido é capaz de revelar a instabilidade do fenticonazol e separar seu produto de degradação com exatidão e precisão (RSD ± 2%) e sem interferências do placebo.

Palavras-chave: Cromatografia a Líquido. Planejamento Experimental. Produtos de Degradação. Fenticonazol. Estudo de Estabilidade. Controle de Qualidade.

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