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Research Article

Development and Validation of Dissolution Test for Fluconazole Capsules by HPLC and Derivative UV Spectrophotometry

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The purpose of this study is to develop and validate a dissolution test for fluconazole, an antifungal used for the treatment of superficial, cutaneous, and cutaneomucous infections caused by *Candida* species, in capsules dosage form. Techniques by HPLC and UV first derivative spectrophotometry (UV-FDS) were selected for quantitative evaluation. In the development of release profile, several conditions were evaluated. Dissolution test parameters were considered appropriate when a most discriminative release profile for fluconazole capsules was yielded. Dissolution test conditions for fluconazole capsules were 900 mL of HCl 0.1 M, 37 ± 0.5 °C using baskets with 50 rpm for 30 min of test. The developed HPLC and UV-FDS methods for the antifungal evaluation were selective and met requirements for an appropriate and validated method, according to ICH and USP requirements. Both methods can be useful in the registration process of new drugs or their renewal. For routine analysis application cost, simplicity, equipment, solvents, speed, and application to large or small workloads should be observed.

1. Introduction

The dissolution can be defined in a narrow sense as the process by which a solid substance is incorporated into the solvent to form a solution. However, in a broad sense, it is more than a simple measurement of solubility rate and can be better described as physical test to predict the drug release from a dosage form, for a given area for some precise time. Fundamentally, this process is controlled by the affinity between the solvent and the solid substance and the way by which the pharmaceutical system releases the drug [1, 2]. According to Mehta and coworkers [3] dissolution test provides an indication of bioavailability of a drug and, thus, pharmaceutical equivalence from batch to batch. The dissolution test is an important tool in quality control of drugs and it becomes more important for drugs with relatively low water solubility, including broad spectrum antifungal fluconazole. Some characteristics of this drug are not well defined, for instance, its classification as high or low soluble in water or its classification in the biopharmaceutical classification system [4–6]. Well-defined drug features, together with the dissolution studies, can be an important tool to justify the use of in vitro methods instead of in vivo methods when they are requested. The aim of this dissolution study is to contribute to define fluconazole dissolution conditions, what can be the focus for further studies.

A dissolution method should be discriminatory, and it should allow evaluating the performance of the product and possible changes it may suffer from during the stability study. According to Marcolongo [7], many variables can influence the results of a dissolution test. Among these variables we can find solubility, chemical nature of the drug, the dosage form, excipients and manufacturing technology employed, the apparatus used, the stirring speed, the use of devices for floating dosage forms (sinkers), the volume of media

used, pH and temperature of the media, the filtration, and analytical method employed.

To develop a dissolution method the characteristics of the drug and its behavior in the chosen test media should be taken in account. Moreover, the dissolution conditions must follow the sink conditions (final concentration equivalent to 10% of saturation concentration) [7], and the quantitation method should be sensitive, selective, accurate, and precise.

Although there are many published analytical methods for fluconazole, there are few dissolution studies for fluconazole, as it has been well compiled in a review by Corrêa and Salgado [8]. Fluconazole dissolution has not been recommended in any pharmacopeia until December 2010 when it has been incorporated in the capsule monograph in the Brazilian Pharmacopeia, 5th edition [9]. However, FDA [10] has recommended dissolution methods since 2004 for fluconazole suspension and since 2006 for tablets; the FDA-recommended method for tablets was tested in this study without good results. In the Brazilian Pharmacopeia [9], the dissolution method recommended for fluconazole capsule uses 900 mL of 0.1 M HCl at 37°C and baskets with 100 rpm for 30 min with quantitation by spectrophotometry at λ = 261 nm.

The aim of this work is to develop and validate a discriminative dissolution method for fluconazole capsules employing two analytical methods to determine fluconazole by high-performance liquid chromatography (HPLC) and by first-order derivative UV spectrophotometry (FDS). Problems encountered by the UV spectrophotometric method, recommended by the Brazilian Pharmacopeia 5th edition [9] are discussed.

2. Experimental

2.1. Material and Equipment. Fluconazole chemical reference (assigned purity 100%) was purchased from Sigma Aldrich. Bulk drug was kindly donated (EMS, Hortolândia, SP, Brazil) and was standardized against fluconazole chemical reference. Capsules were purchased from local market with 150 mg drug label claim. A Hanson SR 8 *Plus* dissolution system containing six vessels was used for dissolution tests.

LC grade methanol was purchased from Tedia (Fairfield, USA). Purified water was prepared in-house by using Direct-Q water system (Millipore, Billerica, MA, USA). Prior to use, mobile phase solvents were degassed in an ultrasonic bath for 30 min. Purified water (>18 MOhm cm) was used to prepare the mobile phase. Solvents were filtered through a 0.45 μm membrane filter.

An HP 8453 UV-Visible spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA, USA) with photodiode array (PDA) and HP ChemStation software with automatic differentiation was used. A liquid chromatograph (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 in an RP C18 column (Symmetry, 5 μm, 4.6 mm × 250 mm, Waters, Milford, MA, USA).

2.2. Preparation of Standard and Sample Solutions

2.2.1. Dissolution Performance. Fluconazole 150 mg capsules were dissolved in 900 mL of HCl 0.1 M at 37°C for 30 min, using baskets. The collected samples were filtered through quantitative filter paper, when evaluated by FDS, or through 0.45 μm regenerated cellulose membranes, when evaluated by HPLC.

2.2.2. LC and UV Determination Method. A stock solution was prepared by dissolving 50.0 mg of fluconazole in a 100 mL volumetric flask using HCl 0.1 M with 30 min sonication. Five standard solutions were prepared from stock solution in different concentrations (135, 150, 165, 180, and 195 μg/mL) by dilution with the same solvent. The range of concentrations chosen has the used concentration (150 mg of fluconazole in 900 mL of dissolution media) as the central concentration. The samples were filtered using quantitative filter paper, when they were evaluated by FDS, and using membranes of regenerated cellulose, 0.45 μm, when they were evaluated by HPLC. Sample, standard, and placebo-enriched solutions were prepared in the selected central concentration in the same way.

3. Results and Discussion

3.1. Analytical Development

3.1.1. UV Determination Method. The selectivity of the Brazilian-Pharmacopoeia-recommended method was evaluated using the compounding fluconazole and its excipients (capsules and placebo). The calculations of interference were performed in accordance with the recommendations of USP 32 [11], since the Brazilian Pharmacopoeia does not cite this calculation, using the following equation. The interference must not exceed 2%:

$$100C \times \left(\frac{\text{Ap}}{\text{As}}\right) \times \left(\frac{V}{L}\right), \quad (1)$$

where C is the concentration (mg/mL) of standard solution, Ap and As are the absorbances of placebo and standard solutions, respectively, V is the volume of medium (mL), and L is the dosage of the product (mg).

Spectrum results showed a strong interference, caused by both capsule shells and placebo in the analytical result. Using the analytical method recommended by the Brazilian Pharmacopoeia the mean percentage of dissolution for six fluconazole capsules was 115.21% and the mean percentage of response for placebo and capsule was 3.94% and 10.70%, respectively, a total of 14.64%. These results are in accordance with those obtained by Oliveira and coworkers [12], who reported fluconazole capsules dissolution. They have shown a huge interference of the excipients in fluconazole determination by UV.

In order to eliminate the interference of the excipients (placebo and capsule) UV-FDS was tested. In general, the spectral derivation provides simultaneous drugs determinations in association, as well as, increased selectivity. In addition, there are often an increased sensitivity and

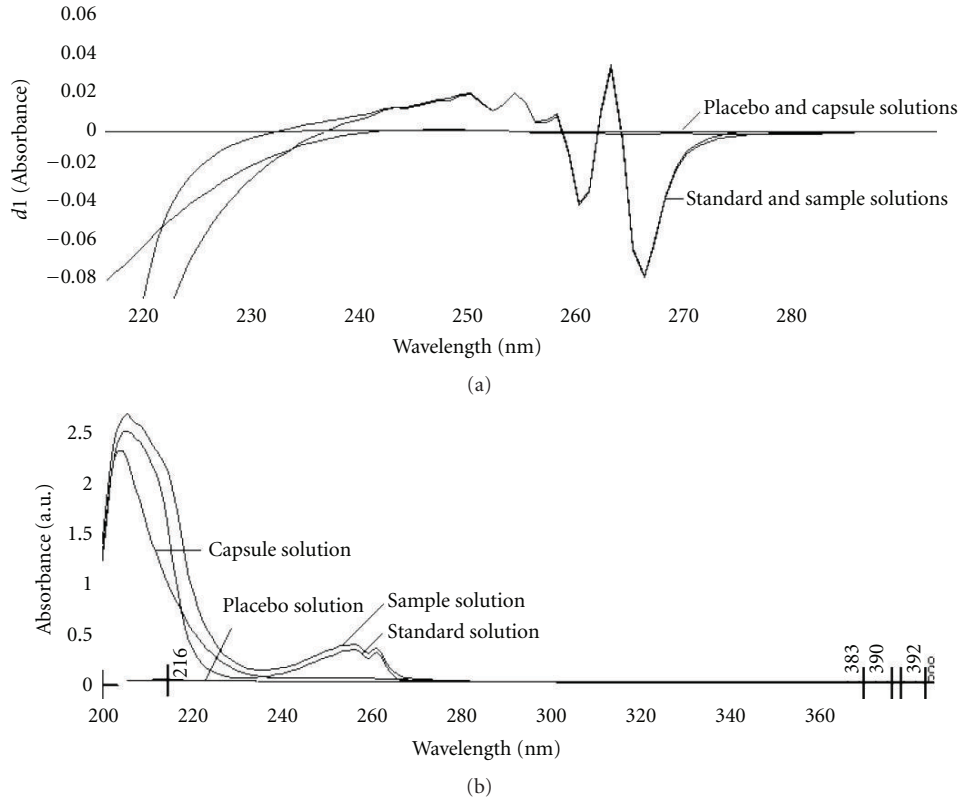

Figure 1: (a) First-order derivative spectra overlay of fluconazole standard, dissolution sample, and placebo and capsule shells solutions. (b) Zero-order derivative spectra overlay of standard solution, dissolution of fluconazole sample, and capsule shells and placebo solutions.

improved detection limits. The increased sensitivity observed in the derivative spectroscopy is based on the observation that the amplitude of the absorbance derivative relative to the wavelength is inversely proportional to the bandwidth of the ordinary spectrum. The order of the derivative must be carefully selected since there is usually an increase in noise level with increasing order of differentiation [13, 14].

Figure 1 shows the first-order derivative of absorbance of fluconazole that has an intense and well-defined valley at 268 nm. It was the wavelength chosen. Fluconazole capsule samples were tested according to the method recommended by the Brazilian Pharmacopeia [9]. The same samples were employed to evaluate the derivative spectrophotometric method. The average interference from placebo and capsules was again calculated in the same way but using the derivative spectrophotometric method. The interference was equal to 0.1% due to placebo and 1.98% due to capsules shells. These values have low analytical significance and are satisfactorily acceptable. Thus, the interference of excipients (placebo and capsule) was considerably reduced by using the differentiation.

3.1.2. HPLC Determination. HPLC is a widely used method of separation with high precision and accuracy. It allows the separation between the drug and excipients, as well as degradation products, which is useful as an indicative stability method.

The analytical development must be developed to obtain a simple and optimal method. Good results were obtained using reversed phase C18 (250×4.6 mm, 5 mm) Water Symmetry endcapped column, 1.0 mL/min, water, and methanol (60 : 40, v/v), injection of 20 μL monitored at $\lambda = 261$ nm.

The samples used to test the method recommended by the Brazilian Pharmacopeia [9] were now employed to evaluate the HPLC method. The results showed that the HPLC method is selective, and it was able to separate the drug from the placebo and capsule (Figure 2).

Figure 2 shows that capsule shells and placebo absorb energy in UV region (peaks at 5.15 min); however, they could be well separated from fluconazole (peak at 3.65 min). The capsule peak was observed in capsule shells and sample solutions. The peak at 2.45 min, present in all samples, refers to the dissolution media (HCl 0.1 M), including the blank solvent.

3.1.3. Dissolution Performance. Fluconazole three different pK_a [15] values, 11.01 ± 0.29, 2.94 ± 0.10, and 2.56 ± 0.12, correspond to the groups alcohol (proton donor) and two nitrogens (proton acceptors), respectively. Thus, the aqueous solubility of fluconazole is greater in solutions with extreme pH values (above 11.01 and below 2.94), situations in which the drug would be fully ionized.

However, dissolution of fluconazole capsules was evaluated in deionized water, as recommended by FDA [10], and

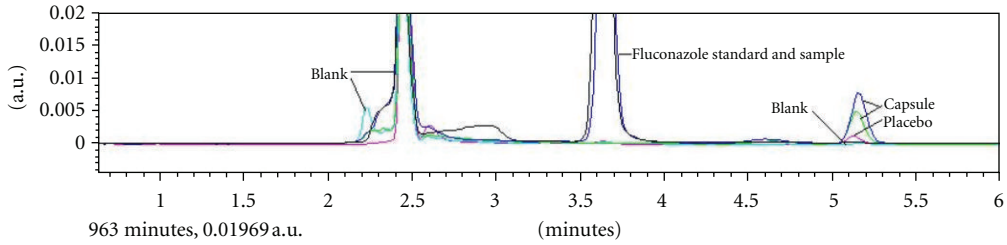

Figure 2: Chromatograms overlay of fluconazole dissolution samples, placebo and capsule shells, at $\lambda = 261$ nm.

in HCl 0.1 M at 37°C. Both baskets and paddles (with and without sinkers) apparatuses were used in a total medium volume of 900 mL in all tests to evaluate dissolution profile. The dissolution media were degassed by sonication for 30 min at 37°C before initiating the dissolution test. The final concentration of fluconazole in 900 mL was nearly 165 mg/mL as capsule products contained 150 mg of the drug. This is in agreement with required sink conditions, desirable to prevent saturation. Thus, the concentration 165 mg/mL was included to be the central point of the range used to validate the method.

In the dissolution profile, samples were collected after 5, 10, 15, 20, 30, 45, 60, and 65 min, filtered through quantitative filter paper, and fluconazole was quantified by UV-FDS. The dissolution medium was used as blank. The replacement of media was performed after each collection using the dissolution media at 37°C. Both mass withdrawn and the dilution made for each replacement of media were taken into account in the calculations for the construction of the dissolution profiles.

Rotation speeds 75 and 100 rpm were used in each test with baskets and 50 and 75 rpm with paddles. During the last 5 min, the speed of rotation was changed to 150 rpm in all tests. The speed can be increased at this point to verify if the drug contained in the capsule was entirely released during the test. The results of dissolution profiles using the described conditions are shown in Table 1 and Figure 3.

Water was used as dissolution medium in Tests 1 and 2, as recommended by FDA [10]. It shows not to be an appropriate medium for fluconazole capsules even after 65 min test at the highest speed because the drug percentage release was below 57%. The HCl 0.1 M dissolution media tested showed better results.

The paddle apparatus was tested at 50 and 75 rpm, with and without sinker. The use of sinker made the dissolution slower, and it is shown comparing Tests 4 and 6 to Tests 3 and 5. Tests 3 and 4, when 75 rpm was employed, showed fast dissolution in the beginning and low discriminatory power. Tests 5 and 6 showed slow dissolution in the beginning; however, at the end the drug was not released from the dosage form to the dissolution media. It could be realized after employing 150 rpm for 5 minutes in the end of test that the concentration of drug increased rapidly.

Baskets were employed in Tests 7 and 8 using HCl 0.1 M as dissolution media and 100 and 75 rpm, respectively. Test

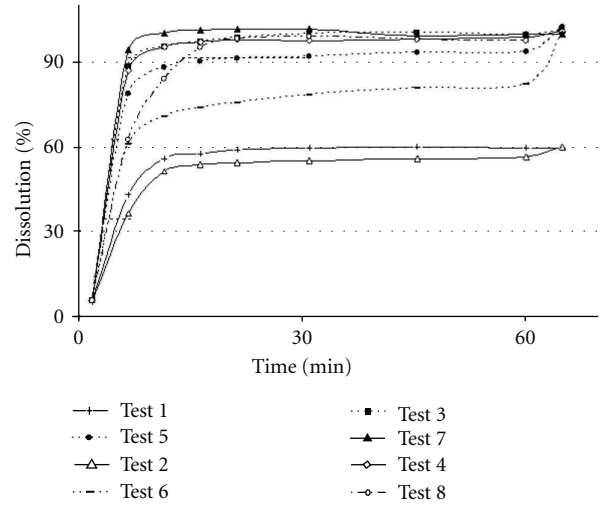

Figure 3: Dissolution profile obtained for testing fluconazole 150 mg capsules by UV-FDS. Conditions: as in tests 1–8 specified in Table 1.

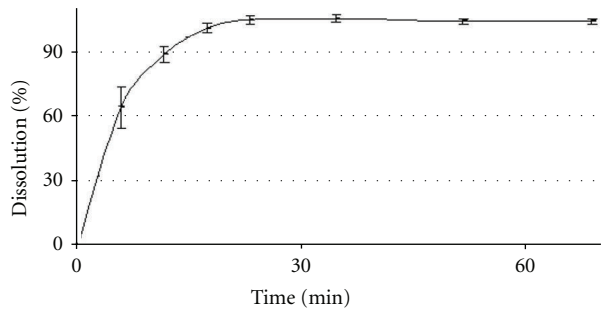

Figure 4: Dissolution profile for fluconazole capsules. Samples analyzed using 0.1 M HCl at 37°C as dissolution media, basket, and 75 rpm. UV method was employed.

7 has shown fast initial dissolution with more than 90% of drug release after 5 min and therefore a low discriminatory power. In Test 8, there were small release increases in fluconazole amount along the test, which has shown a discriminative profile (Figure 4).

Therefore, basket apparatus, 75 rpm, 900 mL of 0.1 M HCl at 37°C as dissolution media, and 30 min of testing

Table 1: Dissolution profiles for fluconazole determination: tested parameters and results.

Test	Time (min)	Medium	Medium volume (mL)	Apparatus	Rotation (rpm)	Average % dissolution	% R.S.D.
	5					39.05	10.58
	10					52.79	3.26
	15					55.73	3.14
1	20	water	900	Basket	100	56.73	1.16
	30					56.65	0.38
	45					56.82	0.31
	60					56.74	0.60
	65				150	56.69	1.11
	5					32.29	0.07
	10					48.09	4.78
	15					50.31	3.21
2	20	water	900	Paddle	75	51.01	2.02
	30					51.77	1.57
	45					52.48	1.25
	60					53.52	1.26
	65				150	56.99	0.89
	5					86.79	6.23
	10					94.14	3.47
	15					95.89	2.72
3	20	0.1 M HCl	900	Paddle	75	97.32	3.43
	30					98.64	2.40
	45					99.19	1.58
	60					98.24	1.68
	65				150	100.24	2.37
	5					85.45	9.69
	10					93.92	1.13
	15					95.70	0.26
4	20	0.1 M HCl	900	Paddle + sinker	75	96.63	0.73
	30					96.34	1.31
	45					97.06	1.71
	60					97.57	1.37
	65				150	100.00	1.43
	5					76.67	5.84
	10					86.41	1.35
	15					88.70	2.58
5	20	0.1 M HCl	900	Paddle	50	89.78	3.47
	30					90.44	3.65
	45					91.79	2.94
	60					92.36	3.81
	65				150	101.24	2.77
	5					58.10	4.62
	10					68.09	6.98
	15					71.54	7.02
6	20	0.1 M HCl	900	Paddle + sinker	50	73.34	5.75
	30					76.28	4.53
	45					78.76	4.70
	60					80.19	4.78
	65				150	99.05	4.55

Table 1: Continued.

Test	Time (min)	Medium	Medium volume (mL)	Apparatus	Rotation (rpm)	Average % dissolution	% R.S.D.
	5					92.96	6.34
	10					98.96	0.32
	15					100.07	0.20
7	20	0.1 M HCl	900	Basket	100	100.56	0.39
	30					100.47	0.38
	45					98.09	0.15
	60					98.66	0.23
	65				150	98.92	0.87
	5					59.50	15.07
	10					82.07	4.45
	15					93.61	2.19
8	20	0.1 M HCl	900	Basket	75	97.00	1.93
	30					97.64	1.36
	45					96.43	1.35
	60					96.38	1.23

were the conditions chosen for the dissolution of fluconazole capsules. This method was validated by FDS and HPLC.

3.2. Method Validation. The quantitative methods were validated according to the ICH [16] and USP [11] guidelines for development and validation of dissolution methods. Because nonuniform drug distribution may affect the dissolution test in the single-dose units, fluconazole capsule samples were evaluated regarding uniformity content (UC). All fluconazol capsules UC results obtained by HPLC (between 91.42–100.2% of labeled value) were within accepted specifications.

The calibration curves were obtained at five fluconazole concentration levels from 135 to 195 μg/mL for HPLC (λ = 261 nm) and UV-FDS (λ = 268 nm). Lambert-Beer law was observed at this concentration range. Linearity was evaluated by the least square method with determinations in triplicate at each concentration level. Both methods were linear with this model. Regression equations were $y = 2.5 \times 106X - 1.1 \times 104$ ($r^2 = 0.996$) for HPLC and $y = 0.51439X - 2.9 \times 10^{-3}$, ($r^2 = 0.998$) for UV-FDS. The standard deviations of the regression were 0.83% for HPLC and 0.62% for UV-FDS. The validity of the assay was verified by means of ANOVA. According to ANOVA there is a statistically significant linear regression ($F_{calculated} > F_{critical}$; $P = 0.05$) and there is no deviation from linearity ($F_{calculated} < F_{critical}$; $P = 0.05$) for both methods.

The precision of the methods was determined by repeatability (intraday) and intermediate precision (interday). For repeatability test three curves were constructed with the established five concentration levels using standard solutions in the same day; for intermediate precision three curves were constructed with three concentration levels (high, intermediate, and low) using standard solutions in a different day. An interval of two days between repeatability and intermediate precision was observed. The results were expressed as percentage of relative standard deviation (R.S.D.). The intraday precision tests showed R.S.D. of 0.81%, HPLC and 0.75%, FDS and interday precision tests showed R.S.D. of 2.29%, HPLC and 2.55%, FDS. These results indicate good precision.

The accuracy was performed in triplicate using the standard addition method (enriched placebo). Known amounts of standard of fluconazole were added to placebo in order to reach five established concentration levels. The mean percentage recovery of fluconazole standard found was 98.56 ± 0.82% for HPLC and 98.35 ± 0.88% for UV-FDS (Table 2). These results indicate an agreement between the true values and found values.

This paper compares the methods to determine fluconazole after dissolution test regarding their precision accuracy, and repeatability (Table 3). Both methods showed to be specific, precise, accurate, and linear in the range of concentration tested.

3.2.1. Dissolution Performance Validation. After several conditions tested for the dissolution test development, appropriate parameters were considered optimized whether provided most discriminatory dissolution profile for fluconazole capsules. That means test conditions must be able to show differences in drug release from batch to batch products, as well as to distinguish possible changes that may occur during stability studies or shelf-life of the product. The optimal parameters for fluconazole capsules dissolution are 900 mL of HCl 0.1 M, 37 ± 0.5°C using baskets with 50 rpm during 30 min.

Validation of dissolution performance was carried out by the two methods of quantification, HPLC and FDS. The concentration of 150 mg of fluconazole in 900 mL of dissolution media is nearly equal to the central concentration (165 mg/mL) at the range established.

The precision was determined by repeatability (intraday) and intermediate precision (interday). The repeatability

Table 2: Recovery data for fluconazole standard solutions added to the placebo by using the proposed HPLC and UV-FDS.

Method	Added amount (μg/mL)	Found[a] amount (μg/mL)	Bias (%)	Recovery[a] (%) ± R.S.D.
HPLC	135	133.92	0.80	99.20 ± 0.77
	150	148.00	1.33	98.67 ± 1.36
	165	161.28	2.25	97.75 ± 0.30
	180	177.30	1.50	98.50 ± 0.20
	195	192.41	1.33	98.67 ± 0.65
FDS	135	134.54	0.34	99.66 ± 1.02
	150	147.51	1.66	98.34 ± 0.17
	165	161.93	1.86	98.14 ± 0.08
	180	175.68	2.40	97.60 ± 0.22
	195	190.96	2.07	97.93 ± 0.15

[a] Average of three replicates.

Table 3: Validation parameters for different analytical methods, UV-FDS and HPLC, to determine fluconazole in capsules.

Parameters	FDS	HPLC
Analytical curve	$0.51439X - 2.9 \times 10^{-3}$	$2.5 \times 10^{6}X - 1.1 \times 10^{4}$
Intercept values	-2.9×10^{-3}	-1.1×10^{4}
Standard error of slope	8.8346×10^{-7}	19.63
Correlation coefficient (r^2)	0.998	0.996
R.S.D. of repeatability (%)	0.75	0.81
R.S.D. intermediate (%)	2.55	2.29
Accuracy (%)	98.35	98.56
R.S.D. of accuracy (%)	0.88	0.82
LOQ	4.9×10^{-3}	1.28×10^{-8}
LOD	1.4×10^{-3}	3.84×10^{-9}

was tested by dissolution of five fluconazole capsules containing 150 mg of drug in duplicate, in the same day; and intermediate test was evaluated by the same way in a different day. An interval of two days between repeatability and intermediate test was observed. The results were expressed as percentage of dissolution and the R.S.D. The repeatability results were 97.96% ± 2.01% for FDS and 97.10% ± 2.44% for HPLC, and the interday results were 96.94% ± 2.48% for FDS and 95.02% ± 2.80% for HPLC. These results indicate good precision.

The accuracy of the dissolution performance was determined using enriched placebos. Known amounts of fluconazole standard were added to placebos in order to obtain three concentrations (high, intermediate, and low) of the range established. The accuracy test was performed in triplicate. The mean recovery was found to be 98.04% ± 0.89% for HPLC and 98.86% ± 1.20% for FDS (Table 4) indicating an agreement between the true values and the values found.

Table 4: Recovery data of dissolution performance obtained by HPLC and UV-FDS methods.

Method	Added amount (μg/mL)	Found[a] amount (μg/mL)	Bias (%)	Recovery[a] (%) ± R.S.D.
HPLC	135	133.55	1.07	98.93 ± 0.23
	165	160.33	2.83	97.17 ± 0.55
	195	191.14	1.98	98.02 ± 0.63
FDS	135	135.11	0.08	100.08 ± 0.42
	165	163.40	0.97	99.03 ± 0.32
	195	190.05	2.54	97.46 ± 0.34

[a] Average of three replicates.

4. Conclusions

A discriminative dissolution test for fluconazole capsules determination was presented in this study. Selective, sensitive, precise, and accurate analytical methods were used for quantitation. The results showed that the determination of fluconazole capsules using direct UV spectrophotometry, recommended in the Brazilian Pharmacopeia, is not enough selective; however, the developed first-order UV derivative spectrophotometry and the HPLC showed to be selective and meet requirements for an appropriate validated method. Both methods are useful for the registration of new drugs or their renewal. The application of each method, as a routine analysis, should be observed considering cost, simplicity, equipment, solvents, speed, and application to large or small workloads.

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