

Original Article

VALIDATION OF TWO SPECTROPHOTOMETRIC METHODS FOR FLUOXETINE QUANTIFICATION

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ABSTRACT

Objective: This paper describes the development and validation of two UV-Visible spectrophotometric analysis methods, using absolute ethanol (method A) and HCl 0.1 M (method B) as solvents, to quantify fluoxetine (FLX) in its generic and brand name form. The objective is to validate both methods and compare the concentrations of the samples obtained from each one.

Methods: Validation of each method with the determination of linearity, limit of detection and quantification, intermediary precision, robustness, accuracy and uniformity of mass for both samples was performed.

Results: Uniform distribution of FLX in capsules were verified and good linear relationships were found between the readings and the concentrations of FLX, in the ranges of 100 µg/ml to 300 µg/ml and 5 µg/ml to 25 µg/ml, for method A and B, respectively. The limit of quantification obtained was 9.96 µg/ml for method A and 0.87 µg/ml for method B. The limit of detection obtained was 2.988 µg/ml for method A and 0.26 µg/ml for method B. Was also verified robustness, good inter-day precision and accuracy of both methods.

Conclusion: The methods were successfully validated to the determination of FLX in its pharmaceutical formulations.

Keywords: Fluoxetine, UV-Visible spectrophotometry, Quantification, Validation

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INTRODUCTION

Depression is a significant and prevalent, chronic or recurrent illness that affects both economic and social functions of patients and can eventually lead to suicidal behaviors [1, 2].

FLX, (N-Methyl-3-(p-trifluoro-methylphenyl)-3-phenyl-propylamine), is a cyclic secondary amine and is a selective serotonin reuptake inhibitor (SSRI) anti-depressant drug. Is orally used in its hydrochloride form and has the empirical formula of $C_{17}H_{18}F_3NO \cdot HCl$ [3-6]. Fluoxetine Hydrochloride (FLX-HCl) was first patented and commercialized by Eli Lilly under the commercial name of Prozac® in the USA since 1988 and became the most widely prescribed antidepressant drug [7-9].

It is among the most prescribed drugs worldwide for treating major depression and related disorders, such as anxiety (panic disorder, social phobia, obsessive-compulsive disorder, post-traumatic stress disorder), eating disorders (anorexia, bulimia) and personality disorders (borderline personality disorder, attention-deficit hyperactivity disorder). This drug is popular due to the low potential of abuse and the relative absence of adverse reactions [10, 11].

During depression treatment, a quantity of 20–80 mg of FLX per day is commonly used, and its therapeutic level covers the range from 50 to 500 ng/ml [12, 13]. It is well absorbed after oral administration, and it takes 6–8 h to reach the plasma peak. It has a long half-life time that allowed for the introduction of once-weekly dosing as an enteric-coated formulation [3, 14, 15].

Demand for FLX analytical assay is growing with the increased use of the drug in order to monitor both compliance and unexpected toxic concentrations after chronic use of the drug [6, 16, 17]. Thus, reliable, accurate and sensitive analytical procedures are needed to determine drug levels either in quality control or in therapeutic drug monitoring [1, 6, 17]. The major problem, as for other antidepressants, is their great inter-individual variability in clinical response, which makes it difficult to evaluate the correct posology [17-20].

FLX has been determined in its pharmaceutical formulations by titrimetry, nuclear magnetic resonance spectrometry, potentiometry,

thin-layer chromatography, liquid chromatography, gas chromatography, conductometry, fluorimetry, chemiluminescence, voltammetry and capillary electrophoresis. These methods were time-consuming, tedious, and/or dedicated to sophisticated and expensive analytical instruments [5, 6, 14, 19, 21, 22]. In general, spectrophotometry is considered the most convenient analytical technique because of its inherent simplicity, low cost, wide sensitivity, and availability in most quality control laboratories [14, 23].

Method validation is the process of proving that an analytical method is acceptable for its intended purpose. In the pharmaceutical industry, validation of analytical method is required in support of product registration applications. For pharmaceutical methods, guidelines from the United States Pharmacopeia (USP), International Conference on Harmonization (ICH) and the Food and Drug Administration (FDA) provide a framework to perform such validation methods. Validating a method consists of the evaluation of various parameters such as accuracy, precision, linearity, sensitivity, limits of detection and quantification, recovery from the matrix and specificity [20].

In 2001 the exclusivity patent of FLX expired and its generic became available [24, 25]. However despite a large amount of success due to lower prices, a randomized, double-blind, crossover study comparing a branded antidepressant with its generic counterpart involving FLX was made and shocking results were found. In general, it was observed in some patients who started taking the generic FLX an initial lower antidepressant effect, increased rates of side effects, relapses of depression and allergic reactions. In all cases, patients improved after returning to the brand name drug [26-28].

The FDA and European Medicines Agency (EMA) allow generic medications to have a bioequivalence ranging between 80 and 125% of the brand name medication. This means that the generic form of FLX labeled to contain 20 mg could have a decrease to as little as 16 mg or increase to as much as 25 mg [28, 29].

In our research, it was not found any paper comparing the amount of FLX between brand name and generic forms. So this paper describes the development and validation of two spectrophotometric methods,

using absolute ethanol (method A, based on the paper of Fregonezy-Nery [30]) and HCl 0.1 M (method B, based on Brazilian Pharmacopoeia 4th edition [31]) as solvents, to quantify FLX in its capsules form.

MATERIALS AND METHODS

Apparatus

Double beam UV-1603 (Shimadzu, Tokyo, Japan) ultraviolet-visible spectrophotometer with matched 1-cm quartz cells was used for all the absorbance measurements at room temperature. Analytical Balance XR 125SM-FR (Precisa, Moosmattstrasse, Swiss) and ultrasonic bath (Selecta, Barcelona, Spain) were also used in this experiment. Microsoft Excel 2013 and UVProbe 2.10 were the software used to treat data through all the work.

Chemicals and reagents

All reagents used were of analytical grade. Pure Fluoxetine Hydrochloride (Lot: 130927-G3) was obtained from Acofarma Lda. (Águeda, Portugal). Solvents used were absolute ethanol (Merck-Lot: K43665283229 and Fisher-Lot: 1226488) and HCl 0.1 M that was prepared by diluting HCl 37% (Panreac-Lot: 000042795) in purified water (used thorough all the experiment and obtained by reverse osmosis with the system provided from Vidrolab 2, S. A.).

Pharmaceutical dosage forms

Two samples of commercial capsules of FLX-HCl were obtained from a local pharmacy: generic form Fluoxetine Basi® Lot: 3001 (sample 1) and brand name from Prozac® Lot: 44968 (sample 2), both labeled to contain 20 mg of FLX per capsule.

Uniformity of mass

Content determination and uniformity of mass assume a suitable moisture process and distribution of the active principle in the capsules during production, showing good manufacturing practices [32].

In this experiment, twenty capsules of each sample were individually weighed with content. After removing, as completely as possible, the content from each capsule, the empty capsule was weighted, and the content was mixed for use in preparation of the sample solution.

Preparation of fluoxetine stock solution

In order to discover the amount of FLX-HCl to weight it was used the equivalence factor, which indicates that 20 mg of FLX are equivalent to 22.4 mg of FLX-HCl [33, 34].

Method A: 140 mg of FLX-HCl was transferred to a 250 ml calibrated flask and dissolved with absolute ethanol to obtain a FLX stock solution of 500 µg/ml.

Method B: 28 mg of FLX-HCl was transferred to a 100 ml calibrated flask and dissolved with HCl 0.1 M to obtain a FLX stock solution of 250 µg/ml.

Preparation of sample solution of the pharmaceutical dosage forms

Method A: The amount of the average weight (obtained in uniformity of mass) of each sample, was accurately weighed (equivalent to 20 mg of FLX), transferred into two 50 ml volumetric flasks (one for each sample), dissolved in ethanol and sonicated for 15 min. Then, the volumetric flasks were completed with ethanol, and the content was homogenized and filtered. In the end, 15 ml of each filtrate were transferred to two 25 ml volumetric flasks that were completed with ethanol, obtaining a concentration of 240 µg/ml of FLX.

Method B: In order to obtain a concentration of 150 µg/ml, was weighed an amount of each sample (obtained in uniformity of mass) that corresponded to 15 mg of FLX to 100 ml volumetric flasks. Then, the powder was dissolved with 70 ml of HCl 0.1 M and sonicated for 5 min. After removed from the ultrasonic bath, the flasks were mechanically shaken for 15 min and completed with HCl 0.1 M. Once homogenized and filtered, 10 ml of each filtrate were

transferred to two 100 ml volumetric flasks that were filled with the solvent, obtaining a concentration of 15 µg/ml of FLX.

Method validation

The method was validated according to the guidelines of the ICH for analytical validation procedures and Resolution RE899 of the National Health Surveillance Agency (ANVISA) [35, 36].

Linearity

Method A: From the FLX stock solution, five aliquots were diluted to 25 ml using ethanol to get standard solutions with concentrations of 300 µg/ml, 250 µg/ml, 200 µg/ml, 150 µg/ml and 100 µg/ml. It was made three standard solutions of each concentration and their absorbances were measured in triplicate at 276 nm using UV spectrophotometer against blank (absolute ethanol).

Method B: From the stock solution, five aliquots were diluted to 50 ml using HCl 0.1 M to get standard solutions with concentrations of 25 µg/ml, 20 µg/ml, 15 µg/ml, 10 µg/ml and 5 µg/ml. It was made three standard solutions of each concentration, and their absorbance were measured in triplicate at 227 nm using UV Spectrophotometer against blank (HCl 0.1 M).

For each method, a calibration curve was obtained by plotting absorbance values against FLX standard solution concentrations.

Limit of quantification (LOQ) and limit of detection (LOD)

LOD and LOQ were calculated based on standard deviation (SD) of response and the slope of the calibration curve. The formulas to calculate each one of these limits are mentioned above:

$$\text{LOD} = 3\sigma/s, \text{LOQ} = 10\sigma/s$$

Where σ is the SD of intercept and s is the slope of calibration curve [37].

Intermediary precision

The precision of the analytical method is expressed as SD or relative standard deviation (RSD) of a series of, in these experiments, five measurements. The same analyst using the same methods, samples, material and reagents, made in two different days the quantification of FLX in the prepared sample solutions.

Robustness

To verify the robustness of the method A, it was used another absolute ethanol manufacturer to prepare samples solutions. Absorbance measurements were made five times and the FLX concentrations, expressed in µg/ml, were obtained from the calibration curve.

Accuracy

Accuracy was evaluated by the addition and recovery of known quantities of FLX standard in commercial samples.

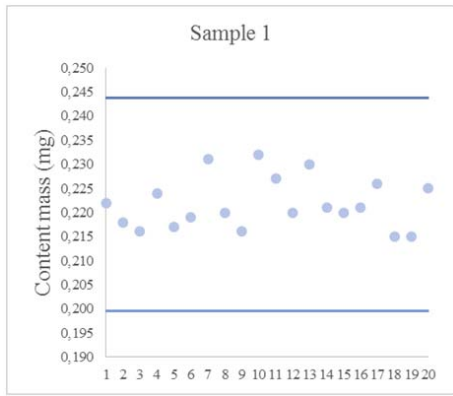
Method A: solutions were prepared in triplicate in 25 ml volumetric flasks containing a mixture of 100 µg/ml sample solution and aliquots from 150 µg/ml standard solution, obtaining concentrations of 102 µg/ml, 104 µg/ml and 106 µg/ml.

Method B: solutions were prepared in triplicate in 25 ml volumetric flasks containing a mixture of 15 µg/ml sample solution and aliquots from 10 µg/ml standard solution, obtaining concentrations of 14.8 µg/ml, 14.6 µg/ml and 14.4 µg/ml.

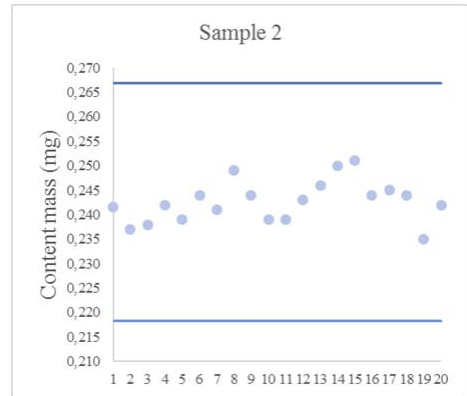
RESULTS AND DISCUSSION

Uniformity of mass

According to Portuguese Pharmacopoeia 8th Edition, no more than 2 of 20 units may differ from the mean mass in superior percentage of 10% and in none of the cases the difference may exceed double that [38]. These requirements were all in conformity with the results for both samples as shown in Graph 1 and 2 (which show the mass of the twenty capsules used of each sample). The percentage difference obtained between the mean mass and maximum quantity of the powder weighed was 4.5% and 3.35%; minimum quantity of the powder weighed was 2.90% and 2.25% in sample 1 and 2, respectively.



Graph 1: Content mass of twenty capsules of sample 1 and limits (10% above and below of the mean mass) according to Portuguese pharmacopeia 8th edition



Graph 2: Content mass of twenty capsules of sample 2 and limits (10% above and below of the mean mass) according to Portuguese pharmacopeia 8th edition

Results indicate a uniform distribution of FLX in capsules, which will ensure effective doses every administration and, therefore, maintenance of therapy concentration.

After registering the measurements, the average mass of the powder in one capsule, SD and RSD were calculated, as showed in table 1.

Table 1: Mean mass of the powder, SD and RSD for both samples

Sample	Mean (mg)	SD	RSD
1	0.2216	0.0053	2.3744
2	0.2426	0.0042	1.7426

Standard solutions spectra

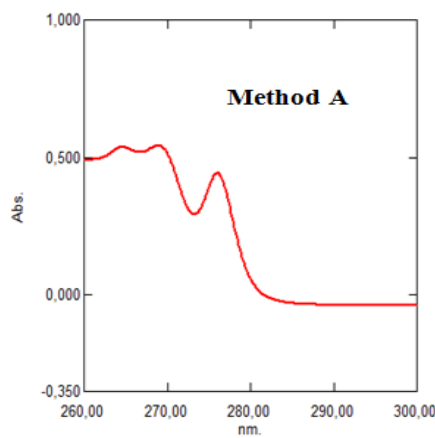


Fig. 1: Spectra of standard solution of FLX with concentration of 200 µg/ml using method A at a range of 260-300 nm

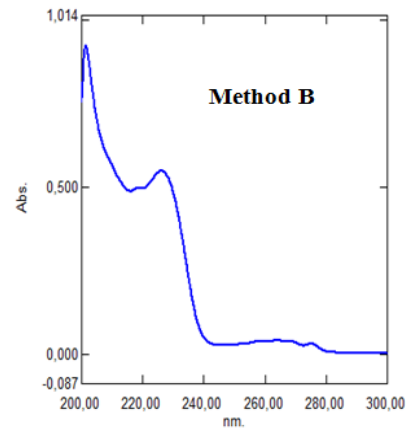


Fig. 2: Spectra of standard solution of FLX with concentration of 15 µg/ml using method B at a range of 200-300 nm

Fig. 1 shows spectra of a standard solution of FLX with a concentration of 200 µg/ml in ethanol at a range of 260-300 nm. Fig. 2 shows spectra of standard solution of FLX with a concentration of 15 µg/ml in HCl 0.1M at a range of 200-300 nm.

The analysis of the spectrum indicates that a peak at 276 nm and 227 nm in fig. 1 and 2, respectively, was obtained, as expected [30, 31].

Table 2: Mean, SD and RSD of absorbance measurements using method A

Concentration (µg/ml)	Mean	SD	RSD
300	0.705	0.002	0.250
250	0.583	0.005	0.860
200	0.454	0.005	1.036
150	0.336	0.002	0.490
100	0.209	0.001	0.646
n=9			

Table 3: Mean, SD and RSD of absorbance measurements using Method B

Concentration (µg/ml)	Mean	SD	RSD
25	0.890	0.006	0.687
20	0.717	0.006	0.793
15	0.547	0.004	0.672
10	0.376	0.007	1.871
5	0.212	0.010	4.798

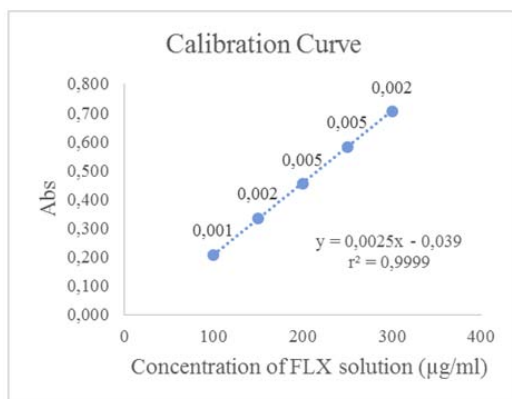
n=9

Method validation

Linearity

After absorbance measurement of standard solutions using method A and B, data was processed statistically, to calculate parameters like mean, SD and RSD presented in table 2 and 3.

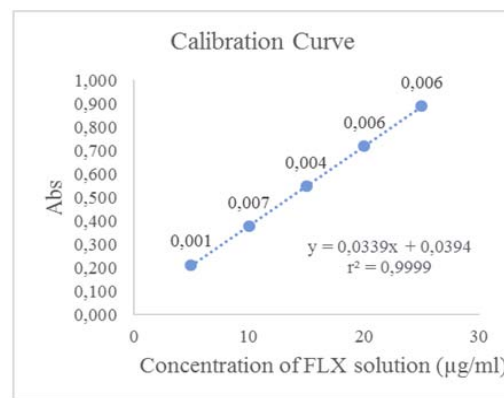
Calibration curves for both methods were obtained from a linear regression method. Standard calibration curve of methods A and B presented linearity between concentration range 100 µg/ml to 300 µg/ml with a determination coefficient (r^2) of 0.9999 and 5 µg/ml to 25 µg/ml with a determination coefficient of 0.9999, respectively, as shown in Graph 3 and 4. The correlation coefficient obtained for both methods indicates good linearity.



Graph 3: Calibration curve of FLX at 276 nm obtained with method A, results of linear regression analysis (regression equation and determination coefficient) and standard deviation of the measurements

Limit of quantification (LOQ) and limit of detection (LOD)

The LOQ obtained was 9.96 µg/ml for method A and 0.87 µg/ml for method B. The LOD obtained was 2.988 µg/ml for method A and 0.26 µg/ml for method B. Once the standard solutions have different concentration range for each method, it was not possible to compare the LOD and LOQ for each method.



Graph 4: Calibration curve of FLX at 227 nm obtained with method B, results of linear regression analysis (regression equation and correlation coefficient) and standard deviation of the measurements

Robustness

Robustness was examined by evaluating the influence of small variation in the method variables on its analytical performance. In these experiments, one parameter was changed whereas the others were kept unchanged. Analyzing and comparing results presented in table 4 it can be seen that the change in ethanol supplier did not induce considerable changes in concentration values of both samples.

Intermediary precision

Intermediary precision was evaluated by the inter-day (between days) study. The results of estimation by proposed methods are shown in table 5.

From obtained results, the concentration of both samples was calculated using as reference the calibration curve obtained from the stock standard solutions that were made in the previous analysis. We made five measurements of each solution at 240 µg/ml and 15 µg/ml (method A and B) and calculated SD and RSD.

Using Related-Samples Wilcoxon Signed Rank Test we verified that there are no statistically significant differences between the concentrations of two days ($p=0.465$), so both methods showed good inter-day precision.

Table 4: Mean of absorbance and concentration values of samples 1 and 2 using two different ethanol manufacturers for method A

Manufacturer	Sample	Mean	Concentration (µg/ml)	SD	RSD
Merck	1	0.587	250.56	0.003	0.533
	2	0.603	256.64	0.004	0.628
Fisher	1	0.570	243.76	0.001	0.096
	2	0.610	259.52	0.006	1.020

n=5

Accuracy

Accuracy results for method A are in the range of 102.75% to 103.46% for sample 1 and 106.27% to 107.55% for sample 2. For method B, accuracy is in the range of 102.76% and 103.36% for sample 1 and 104.80% and 108.75% for sample 2. The theoretical

concentration of standard FLX-HCl added was obtained from each calibration curve resultant from linearity method. Results are shown in table 6.

In United States Pharmacopeia [39] and Brazilian Pharmacopeia [31] recovery percentages for FLX capsules must be between 90-

110%. Once, the obtained results, between 103% and 109%, are in the recommended range, it can be verified that both of the presented methods are valid.

Good percent recovery with a small SD indicates the high accuracy of the developed methods for the quantification of FLX in pharmaceutical preparations.

Table 5: Concentration values of sample 1 and 2 obtained using method A and B in two different days

Day	Method	Sample	Concentration ($\mu\text{g/ml}$)	SD	RSD
1	A	1	249.92	0.006	1.102
		2	259.52	0.002	0.315
	B	1	15.39	0.004	0.791
		2	17.10	0.007	1.183
2	A	1	250.56	0.003	0.533
		2	256.64	0.004	0.628
	B	1	15.58	0.004	0.666
		2	16.30	0.002	0.378

n=5; p=0.465

Table 6: Mean of absorbance and recoveries test of FLX in sample 1 and 2 using standard addition method

Method	Theoretical concentration ($\mu\text{g/ml}$)	Mean of absorbances ($\pm\text{SD}$)		Obtained Concentration ($\mu\text{g/ml}$)		% recovered	
		Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
A	102.00	0.223 (0.0040)	0.232 (0.0021)	104.80	108.40	102.75	106.27
	104.00	0.230 (0.0020)	0.240 (0.0012)	107.60	111.60	103.46	107.31
	106.00	0.234 (0.0038)	0.246 (0.0047)	109.20	114.00	103.02	107.55
B	14.80	0.558 (0.0059)	0.585 (0.0021)	15.30	16.09	103.36	108.75
	14.60	0.548 (0.0049)	0.565 (0.0068)	15.00	15.50	102.76	106.19
	14.40	0.542 (0.0031)	0.551 (0.0050)	14.83	15.09	102.96	104.80

n=3

Application of the methods in sample solutions of the pharmaceutical forms

Using spectrophotometry UV-Visible to measure sample solutions of the pharmaceutical forms absorbance we obtained spectrum representative of each method, presented in fig. 3 and 4.

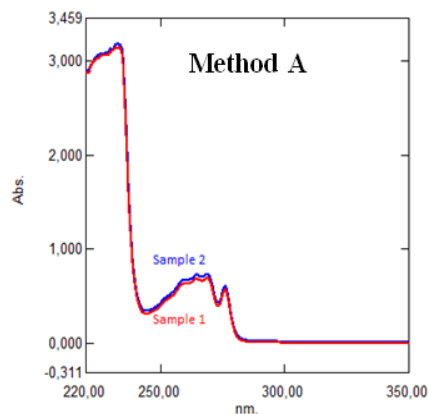


Fig. 3: Spectrum of sample 1 and 2 solutions with concentration of 240 $\mu\text{g/ml}$, using method A at a range of 220-350 nm

Analyzing fig. 3 that shows spectra of sample solution 1 (red line) and 2 (blue line) with a concentration of 240 $\mu\text{g/ml}$, using method A at a range of 220-350 nm, it can be verified that both spectrum are very similar presenting a peak at 276.1 nm. fig. 4 shows spectra of

sample solution 1 (red line) and 2 (blue line) with a concentration of 15 $\mu\text{g/ml}$, using method B at a range of 200-300 nm. The absorbance difference was more visible in method B than in method A, but sample 2 also was the one presenting bigger values.

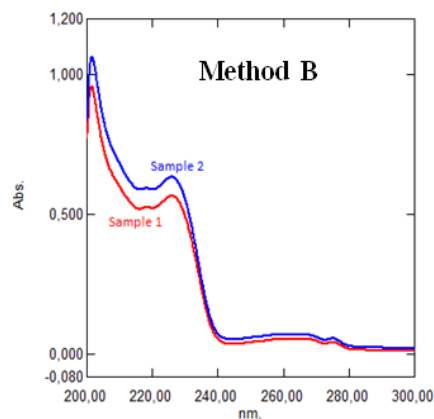


Fig. 4: Spectrum of sample 1 and 2 solutions with concentration of 15 $\mu\text{g/ml}$, using method B at a range of 200-300 nm

Table 7 presents the results of absorbance measurements in sample 1 and 2 using method A and B. These absorbance measurements were applied in the regression equation of each method (presented in graphics 3 and 4) to calculate the concentrations of the samples (presented in table 5-1 d).

Table 7: Results of absorbance measurements and quantity of FLX detected in sample 1 and 2, using Method A and B

Parameter	Method A		Method B*	
	Sample 1	Sample 2	Sample 1	Sample 2
	Absorbance	FLX (mg)	Absorbance	FLX (mg)
Mean	0.586	20.833	0.610	21.633
SD	0.006		0.002	
RSD	1.102		0.315	

n=5, *Using this method the amount of FLX expected was 15 mg instead of 20 mg (method A).

Despite the expected values being different for each of the methods (20 mg of FLX in method A, and 15 mg of FLX in method B), it can be seen that in both the amount of FLX obtained is higher than expected. Analyzing the percentage of active ingredient, for method A, we obtained 104.2% and 108.2% of FLX in sample 1 and 2, respectively; for method B we obtained 102.6% and 114% of FLX in sample 1 and 2, respectively. Comparing samples we verify that sample 2 shows bigger amounts of FLX than sample 1 in both methods.

CONCLUSION

The UV-Visible spectrophotometric methods that we used do not involve boring sample preparation, heating or extraction like other non-spectrophotometric methods. Besides that, they are cheap, sensitive, and fast and use inexpensive, readily available reagents. These great advantages combined with results obtained can contribute to their employment as a quality control tool for the analysis of the FLX in its formulation drug.

The objective of the study was accomplished, since we validated two UV-Visible spectrophotometric methods that were in agreement with the requirements of ICH and Anvisa, in terms of linearity, limits of detection and quantification, robustness, intermediary precision and accuracy. However, robustness was only analyzed in method A due to the lack of different HCl 0.1 M supplier to analyze these parameters in method B. Since we have not found any previous articles in literature comparing generic and brand-name form in terms of quantity of FLX, in these study we decided to analyze a brand name and a generic one, so we could verify which one has the biggest FLX percentage.

In pursuit of an answer to the reports in which an initial lower antidepressant effect in some patients who started taking the generic FLX was observed, this study acknowledges that, as demanded by FDA and EMA, the generic form had bioequivalence ranging between 80 and 125% of the brand name medication. However, the results obtained showed that this generic form of FLX presented the smallest quantity of active substance when compared to Prozac® and both samples presented higher amounts of FLX than we expected. This may help to explain the lower antidepressant effect in some patients when switching from brand name to generic form, although meeting the recommended limits.

Since in this study, each of the samples was restricted to a box of a batch, the samples used were not representative of the global market. Further research can be made to compare FLX quantity in more generic forms with the most used brand name drug in the market, using bigger samples from different batches.

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CONFLICT OF INTERESTS

Declare none

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