# UNIVARIATE AND CHEMOMETRICS-ASSISTED SPECTROPHOTOMETRIC METHODS FOR DETERMINATION OF FLIBANSERIN IN A RECENTLY RELEASED DOSAGE FORM**

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*Five simple, selective, and rapid spectrophotometric methods are developed for the determination of flibanserin (FL) in the presence of its oxidative degradation products (ODPs). Stress studies are performed according to the International Conference on Harmonization (ICH) guidelines to assess the behavior of FL against oxidative, thermal, and acidic conditions. FL was stable against thermal and acidic conditions, while it was susceptible to oxidative degradation. Three of the spectrophotometric methods were univariate methods, namely, third derivative ($D^3$), ratio difference of ratio spectra (RD), and first derivative ratio of spectra ($D^1R$), while the other two methods were multivariate methods, namely, partial least square (PLS) and principal components regression (PCR). No preliminary separation steps were required in these methods. The chemical structures of the ODPs were confirmed using mass spectrometry and $^1$H NMR spectroscopy. The proposed methods were developed and validated according to the ICH guidelines. The linearity, accuracy, and precision were determined, and the selectivity was assessed by analyzing synthetic laboratory mixtures containing different ratios of FL and its ODPs. Statistical comparisons were applied to the five spectrophotometric methods, and no significant differences were found. The validated methods could be applied for routine testing and quality control.*

***Keywords:*** *flibanserin, degradation study, $^1$H NMR, LC-MS/MS, spectrophotometry, chemometrics.*

# ОДНОФАКТОРНЫЕ И ХЕМОМЕТРИЧЕСКИЕ ВСПОМОГАТЕЛЬНЫЕ СПЕКТРОФОТОМЕТРИЧЕСКИЕ МЕТОДЫ ОПРЕДЕЛЕНИЯ ФЛИБАНСЕРИНА В ЛЕКАРСТВЕННОЙ ФОРМЕ

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*Разработаны пять простых, селективных и быстрых спектрофотометрических методов для определения флибансерина (FL) в присутствии продуктов его окислительного разложения (ODP). Стресс-исследования проведены в соответствии с руководящими принципами Международной конференции по гармонизации (ICH) для оценки поведения FL в отношении окислительных, термических и кислотных условий. FL оказался устойчив к термическим и кислотным условиям, в то время как ранее он был подвержен окислительной деструкции. Три спектрофотометрических метода одномерные: третья производная ($D^3$), разность отношений спектров отношения (RD) и отношение первой производной спектров ($D^1R$). Два других метода многомерные: частичный метод наименьших квадратов (PLS) и регрессия главных компонент (PCR), в них не требовалось предварительных этапов разделения. Химическая структура ODP подтверждена с помощью масс-спектрометрии и*

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*спектроскопии ЯМР $^1$H. Определены линейность, точность и прецизионность. Селективность оценена путем анализа синтетических лабораторных смесей, содержащих различные соотношения FL и его ODP. Статистическое сравнение пяти спектрофотометрических методов не обнаружило никаких существенных различий.*

***Ключевые слова:*** *флибансерин, исследование деструкции, $^1$H ЯМР-спектроскопия, LC-MS/MS, спектрофотометрия, хемометрия.*

**Introduction.** Flibanserin (FL) [2-(1,3-dihydro-2-oxobenzimidazol-1-yl)ethyl]piperazine [1] is a serotonergic agent and has been shown to have dual action as an agonist of 5-$HT_{1A}$ receptors and/or an antagonist of 5-$HT_{2A}$ receptors [2]. Because of its high affinity for serotonin receptors, after clinical trials it was approved by the US Food and Drug Administration (FDA) in 2015 as the first drug for the treatment of hyposexual desire disorder (HSDD) for premenopausal women [3]. Different approaches, such as psychological treatment and taking hormonal medications, were developed for the treatment of HSDD before FDA approval of this drug [4, 5]. Increasing sexual desire in premenopausal females using FL is initiated by the activation of dopamine and noradrenaline neurotransmitters and the inhibition of serotonin [6]. Additionally, the cited drug can be used as an antidepressant, but because its pharmacological activity as an antidepressant is mild, this indication was discontinued [7].

FL is rapidly absorbed after oral administration and then primarily metabolized by CYP3A4 in the liver and reaches its maximum plasma concentration (419 ng/mL) at 0.75 h after administration [7–9].

Pharmaceutical drugs can undergo degradation during storage and/or transportation, which results in the formation of toxic degradation products or affects their pharmacological activity. Therefore, stability studies should be performed following the guidelines set by the International Conference on Harmonization (ICH) to study the behavior of drugs under different conditions, which includes hydrolysis, oxidation, and dry heat [10–12].

A literature survey reported that only four different chromatographic methods for the estimation of FL as adulterants in supplements marketed towards women [13, 14] and in plasma using high-performance liquid chromatography (HPLC) with different detectors have been published [1, 15]. No spectrophotometric methods for FL have been reported for the study of its spectral characteristics or stability, which indicates that methods to illustrate and specify the conditions that impact its stability would be of great importance. Therefore, the aim of the current study is to establish an economical, rapid, simple, and comprehensive spectrophotometric method, which uses univariate and multivariate techniques, for the determination of FL in the presence of its degradation products in its pharmaceutical dosage forms for quality control purposes and to assess its stability.

**Experimental.** Spectrophotometric measurements were performed on a Shimadzu double beam spectrophotometer (model: 1800 PC, Japan) connected to a computer running Shimadzu software UV probe 2.10. Scans were carried out in the range of 200–400 nm at 0.1-nm intervals using a 1-cm quartz cell. The computations were performed using SOLO 8.7 software powered by a partial least squares (PLS) toolbox to carry out PLS and principal components regression (PCR) routine work. All pH measurements were performed on a Jenway pH meter 3510 (Cole-Parmer, Staffordshire, UK).

FL (99.98%) was obtained from Hikma Pharmaceutical Industries (Cairo, Egypt). Veroxeserin® tablets (Batch 001) were supplied by Hikma Pharmaceutical Industries, and each tablet contained 100 mg of flibanserin. Methanol (HPLC grade) was purchased from Sigma Aldrich (St. Louis, MO, USA). Hydrochloric acid, sodium hydroxide, and hydrogen peroxide (30% w/v) were purchased from El-Nasr Pharmaceutical Chemicals Co. (Egypt).

*Preparation of a standard solution of FL.* A stock standard solution of FL was prepared by dissolving 10 mg of the drug in methanol in a 100-mL volumetric flask to reach a concentration of 100 μg/mL. Working standard solutions were prepared by further dilution of the FL stock solution to reach concentrations in the range of 2.0–30.0 μg/mL.

*Preparation of the acid degradation products of FL.* Acidic degradation was performed by dissolving 10 mg of FL in methanol and treating the solution with 10 mL of 1 M HCl. The solution was heated for 4 h in a water bath at 100°C. The solution was cooled and neutralized with 1 M NaOH and then diluted with methanol. The degradation process was confirmed by using an HPLC method developed in-house.

*Preparation of the thermal degradation products of FL.* Thermal degradation was conducted on 10 mg of FL by placing the sample in a thermostatic oven at 100°C for 8 h. The material was transferred to a 100-mL volumetric flask and then dissolved in methanol to a concentration of 100 μg/mL. The degradation process was confirmed using an HPLC method developed in-house.

*Preparation of the oxidative degradation products of FL.* Oxidative degradation was carried out using 30% w/v hydrogen peroxide. In a 100-mL volumetric flask, 10 mg of FL was dissolved in 10 mL of hydrogen peroxide, and the prepared solution was immersed in a boiling water bath for 5 h at 100°C. After cooling, the solution was diluted with methanol to obtain a degradation product concentration of 100 μg/mL. The complete degradation of FL was confirmed using an HPLC method developed in-house, and the peak of the parent drug was absent from the chromatogram.

*Spectral characteristics of FL and its oxidative degradation products (ODPs).* The zero-order UV spectra of FL and its ODPs were separately recorded over the range of 200–400 nm using methanol as a blank, and the spectra were stored in a computer.

*Construction of the calibration curves.* Aliquots of the FL standard solution were accurately measured and transferred to a series of 10-mL volumetric flasks and brought to volume with methanol to accurately obtain solutions with concentrations in the range of 2.0–30.0 μg/mL.

*Third derivative method ($D^3$).* The third derivative of the stored zero-order spectra of FL was obtained using a third derivative transformation at $\Delta\lambda = 8$ and a scaling factor of 10. Then, the peak amplitudes were measured at 265 nm. A calibration curve was constructed by plotting the peak amplitudes of FL at 265 nm against the corresponding concentrations, and the regression equation was obtained.

*Ratio difference (RD) method.* The ratio difference of the ratio spectra was obtained by measuring the amplitudes of the ratio spectra at 291 and 238 nm and calculating the difference in the corresponding amplitudes ($\Delta P = 291–238$). A calibration curve was constructed by plotting the differences in amplitudes ($\Delta P = 291–238$) against the corresponding concentrations, and the regression equation was computed.

*First derivative ratio ($D^1R$) method.* The recorded zero-order spectra of FL were divided by the UV spectrum of 20 μg/mL ODPs. The obtained ratios were transformed using the first derivative at $\Delta\lambda = 8$ and a scaling factor of 10, and the peak amplitudes were measured at 251 nm. A calibration curve was constructed by plotting the peak amplitudes at 251 nm against the corresponding concentrations, and the regression equation was calculated.

*Experimental design for the chemometrics methods.* A five-level 2-factor experimental design was used to construct 25 mixtures of different concentrations of FL and ODPs for calibration and validation sets. In the design, 6.0 and 0.6 μg/mL were chosen as the center points for FL and ODPs, respectively. Eight samples were chosen randomly and used as the validation set, while the rest of the samples were used as the calibration set to build the PLS and PCR models. Analyses were performed to test the predictability of the developed models.

*Assay of a laboratory-prepared mixture of FL and ODPs.* Accurately measured aliquots of FL and its ODPs were transferred from the standard solutions used to prepare mixtures of different ratios. The spectra of the prepared mixtures were recorded and stored, and the procedures described above for each method were followed.

*Assay of FL in tablet dosage form.* Ten tablets of Veroxeserin® were weighed and finely powdered. Appropriate amounts equivalent to 10 mg of FL were accurately weighed and transferred to 100-mL volumetric flasks. The powder was sonicated for 30 min in 50 mL of methanol, and the solutions were brought to volume with methanol to obtain final concentrations of 100 μg/mL. The solutions were filtered, and accurately measured aliquots of the filtrate were transferred into a set of 10-mL volumetric flasks and then brought to volume with methanol to obtain concentrations within the linear range. The procedures of the proposed methods were used to confirm the reported concentration of FL in the tablets using the specified regression equations.

**Results and discussion.** *Confirmation of the ODPs.* The degradation behavior of FL was studied according to the ICH guidelines, and the drug was subjected to hydrolysis under oxidative stress conditions, which yielded complete degradation and the formation of two degradation products (ODP1 and ODP2) after refluxing with 30% $H_2O_2$ for 5 h. No degradation products were formed upon acidic or thermal hydrolysis. These results indicated that the degradation of FL arose from N-oxide formation and cleavage of the NH-CO fragment (Fig. 1). The ODPs were identified, characterized, and confirmed using mass spectrometry and $^1$H NMR spectroscopy.

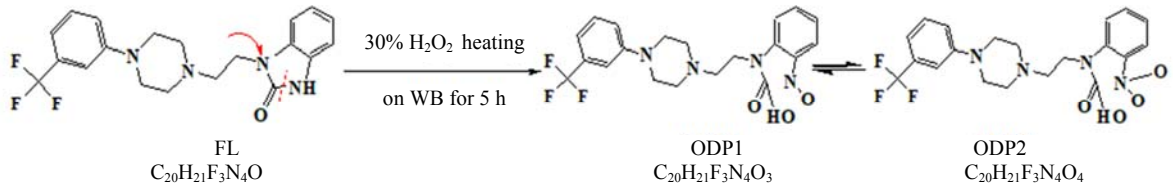


Fig. 1. Degradation pathway of FL to its ODPs.

The mass spectrum of the parent drug (Fig. 2a) showed an abundant peak for $C_{20}H_{21}F_3N_4O$ at *m*/*z* 391 in positive mode, while the mass spectrum of the degradation products (Fig. 2b,c) showed no peak for the parent compound, and two abundant peaks, which corresponded to $C_{20}H_{21}F_3N_4O_3$ and $C_{20}H_{21}F_3N_4O_4$ at *m*/*z* 423 and *m*/*z* 439, respectively, were observed instead.

In the $^1$H NMR spectrum of FL, as shown in Fig. 3a, the proton of the amine group appeared at 3.96 ppm, and this signal was not present in the NMR spectrum of the degradation products. As shown in Fig. 3b, the NMR spectrum of the ODPs was characterized by a singlet resonance at 11.64 ppm, which indicated the cleavage of the NH-CO functional group and the formation of a carboxylic acid derivative.

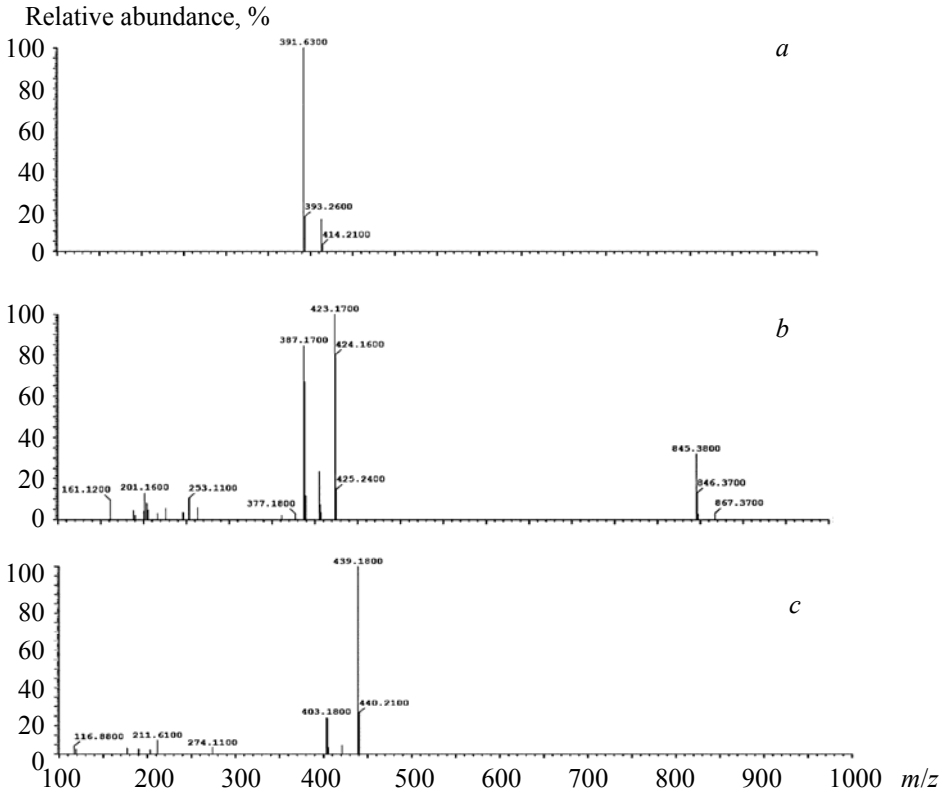


Fig. 2. Mass spectra of (a) intact FL (100 µg/mL), (b) ODP1 (100 µg/mL), and (c) ODP2 (100 µg/mL).

No spectrophotometric methods for the determination of FL alone, with its metabolites, or with its degradation products have been reported. Therefore, the aim of this study is to design novel methods for resolving the overlapping spectra of FL and its ODPs to assess the stability and to allow a comparative study between univariate and multivariate techniques through the analysis of synthetic mixtures of FL and its ODPs, and analysis of FL in its recently released pharmaceutical dosage form.

Structure elucidation and identification of the ODPs indicated that the parent drug and its degradates are structurally similar, which results in substantial overlap between their spectra (Fig. 4). Therefore, the overlapped spectra should be resolved using indirect spectrophotometric methods through transformation of the zero-order UV spectra and applying multivariate approaches.

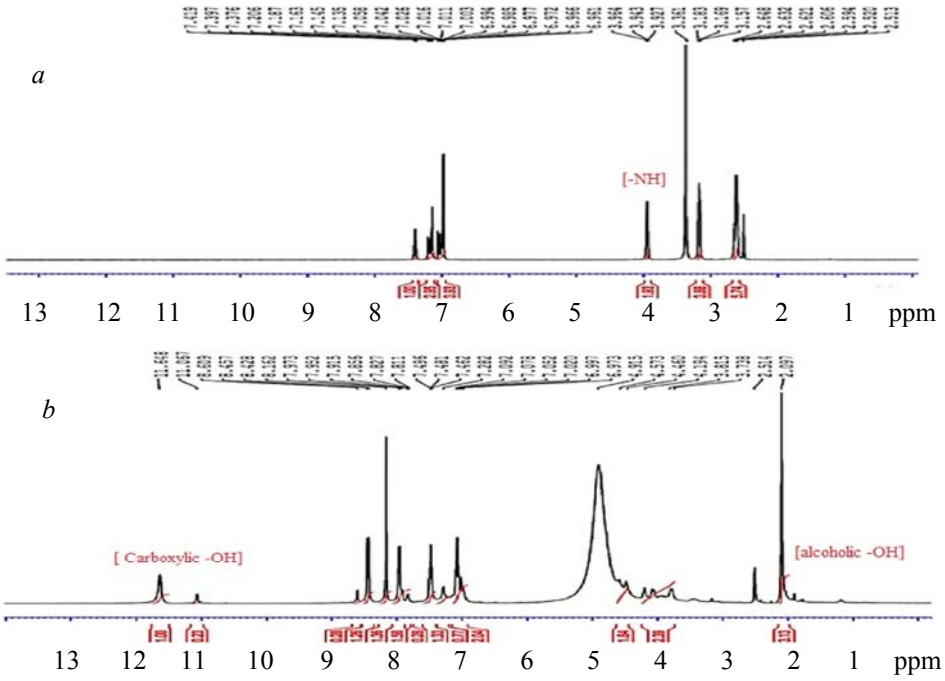


Fig. 3. $^{1}$H NMR spectra of (a) intact FL and (b) ODP.

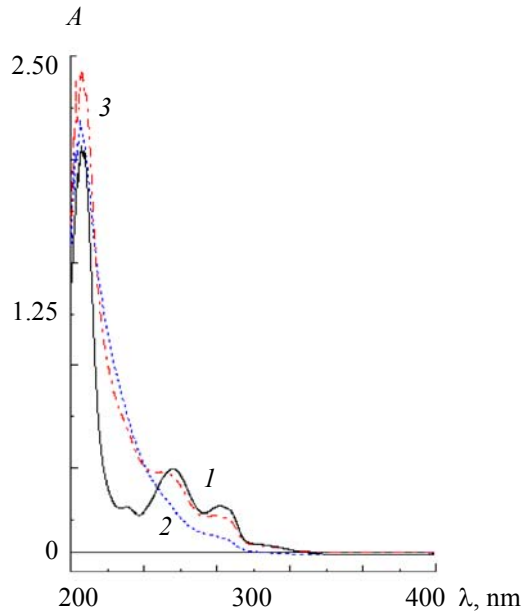


Fig. 4. Zero-order spectra of (1) 10 μg/mL FL, (2) 2 μg/mL ODP, and (3) mixture of FL and its ODPs.

*Univariate techniques. Third derivative method ($D^3$).* The third derivative technique was proposed as a stability-indicating method for the determination of FL, owing to its simplicity in resolving the overlapped spectra and decreasing the signal-to-noise ratio. The third derivative of the obtained zero-order spectra was recorded using $\Delta\lambda$ = 8 nm and a scaling factor of 10. The peak amplitudes were measured at 265 nm at different concentrations of FL, which showed zero crossing points of the ODP spectrum (Fig. 5). The calibration curve was constructed at this wavelength, and the regression equation was calculated as follows:

$$D^3 = 0.0780X + 0.0263,$$

where $D^3$ is the peak amplitude and $X$ is the concentration of FL in μg/mL.

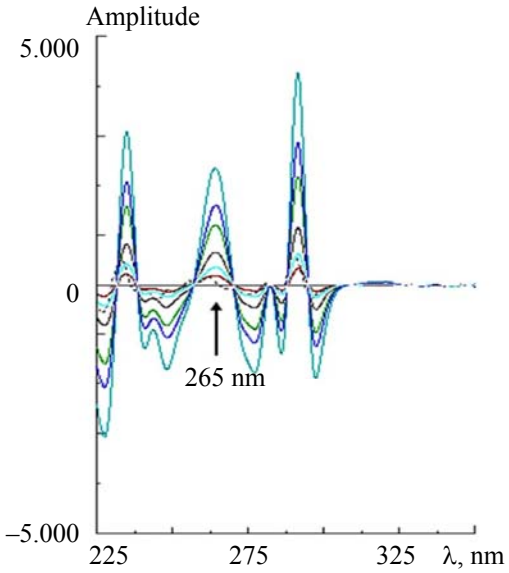


Fig. 5. Third derivative spectra of FL (2–30 μg/mL).

*Ratio difference (RD) method.* The ratio difference method was found to be suitable for resolving the overlapping spectra without prior separation. The method was developed by selecting a divisor concentration of ODP of 20 μg/mL, which provided reliable results. The obtained ratio spectra of FL in the range of 2–30 μg/mL exhibited two signals, at 291 and 238 nm, with significant amplitudes, whereas the ODP ratio spectrum showed constant amplitudes at these wavelengths (Fig. 6). The concentration of FL was calculated from the regression equation using the difference in the amplitudes of the constructed ratio spectra at the two selected wavelengths, and the obtained values were proportional to the corresponding concentrations. The regression equation was computed as follows:

$$RD = 0.0359X - 0.0231,$$

where RD is the difference peak amplitude and $X$ is the concentration of FL in μg/mL.

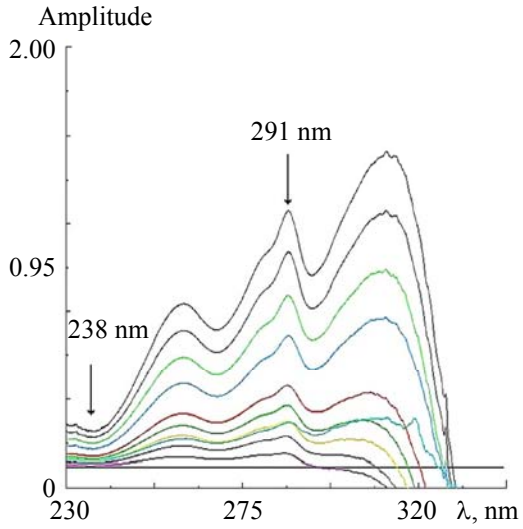


Fig. 6. Ratio spectra of FL (2–30 μg/mL) using 20 μg/mL ODP as a divisor.

*First derivative ratio method ($D^1R$).* This method depends on using the ratio spectra of FL. The obtained ratio spectra were then derivatized to determine the wavelength at which ODP zero crossing occurred. The concentration at which the divisor spectrum was obtained was optimized to 20 μg/mL ODP, and this curve was used to obtain the ratio spectra. Then the third derivative was computed, which resulted in a good reso-

lution of the overlapped spectra with minimal noise. The peak amplitude was measured at 251 nm, as presented in Fig. 7, and the calibration curve was prepared by plotting the relationship between the peak amplitudes at the selected wavelength and the corresponding concentrations. The regression equation was computed as follows:

$$D^1R = 0.1195X - 0.0007,$$

where $D^1R$ is the peak amplitude and $X$ is the concentration of FL in μg/mL.

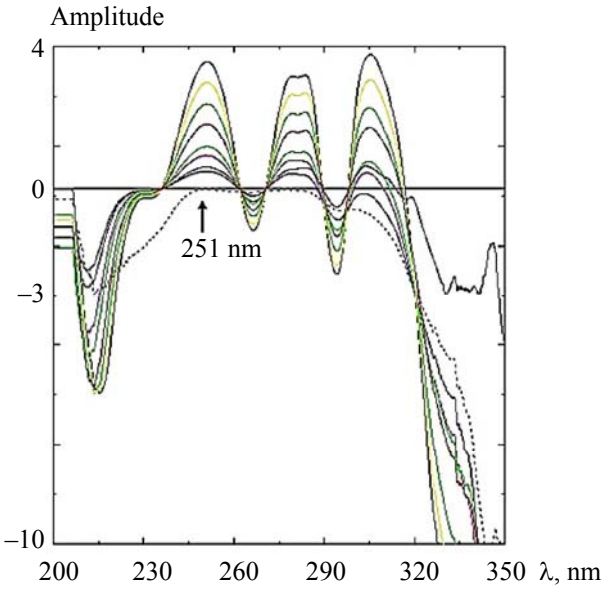


Fig. 7. First derivative ratio of FL (2–30 μg/mL) using 20 μg/mL ODP as a divisor.

*Multivariate methods.* PLS and PCR chemometric techniques were successfully applied for the determination of FL in the presence of its ODPs. Using the whole spectral wavelength in multivariate techniques, compared with a single wavelength in univariate techniques, is useful to improve and enhance the precision and prediction capabilities of a given method [16]. The calibration models were constructed using 17 samples of synthetic mixtures of FL and ODP at different ratios, as shown in Table 1. The experimental data were mean centered as a pre-processing step, and a cross validation procedure was performed leaving one sample out at a time using SOLO software. The selected model with the smallest number of latent variables and minimum root mean square error in the calibration curve (RMSECV) was accepted. Two latent variables were suitable for the PLS model, while three were suitable for PCR (Fig. 8a,b).

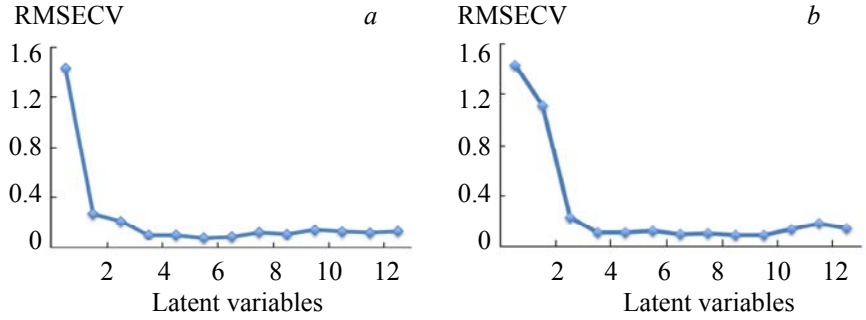


Fig. 8. RMSECV plots of the calibration set of FL as a function of the latent variables; used to construct the PLS (a) and PCR (b) calibration models.

TABLE 1. The Five-level, Two-factor Experimental Design of the Calibration and Validation Set Shown as Concentration of the Mixture Combination in μg/ mL

| Mix No. | FL | ODP | Mix No. | FL | ODP |
|---|---|---|---|---|---|
| 1 | 6 | 0.6 | 14 | 6 | 1 |
| 2 | 6 | 0.2 | 15 | 10 | 1 |
| 3 | 2 | 0.2 | 16 | 10 | 0.2 |
| 4 | 2 | 1 | 17 | 2 | 0.8 |
| 5 | 10 | 0.4 | 18 | 8 | 0.2 |
| 6 | 4 | 1 | 19 | 2 | 0.6 |
| 7 | 10 | 0.6 | 20 | 6 | 0.8 |
| 8 | 6 | 0.4 | 21 | 8 | 0.8 |
| 9 | 4 | 0.4 | 22 | 8 | 0.4 |
| 10 | 4 | 0.8 | 23 | 4 | 0.2 |
| 11 | 8 | 1 | 24 | 2 | 0.4 |
| 12 | 10 | 0.8 | 25 | 4 | 0.6 |
| 13 | 8 | 0.6 | | | |

N o t e. The shaded rows represent the validation set.

*Method validation.* All the proposed methods were validated according to the ICH guidelines [17]. The linearity of the proposed methods was evaluated by preparing solutions at six different concentrations in the range of 2–30 μg/mL from the FL standard solution (three replicates at each concentration). A calibration curve for each method was constructed by plotting the concentration and the corresponding response. Good linearities were achieved with regression coefficient values of 1, 0.9993, and 0.9998 for $D^3$, RD, and $D^1R$, respectively, as shown in Table 2.

The Limit of detection (LOD) and limit of quantitation (LOQ) were investigated using the formula LOD = 3σ/*s* and LOQ = 10σ/*s,* where σ is the standard deviation of the response, and *s* is the slope of the standard curve. The LOD and LOQ were calculated, and the results are summarized in Table 2.

The accuracy of the proposed methods was evaluated by analyzing solutions of FL at three different concentrations within the linear range. The concentrations were calculated from the corresponding regression equations, and then the percentage recoveries and standard deviations were determined. Satisfactory results were found (between 98.10 and 101.30%), which indicated that the proposed stability-indicating method was sufficiently accurate, as shown in Table 2.

Three solutions of pure FL at different concentrations (5.0, 10.0, and 20.0 μg/mL) were analyzed on the same day and on three sequential days to determine the intraday and inter-day precision, respectively, to evaluate the precision of the proposed methods. The relative standard deviations were calculated, and the precision of the method was confirmed to be satisfactory (Table 2).

TABLE 2. Results of Validation Parameters of Proposed Univariate Spectrophotometric Methods

| Parameter | $D^3$ | RD | $D^1R$ |
|---|---|---|---|
| Wavelength (nm) | 265 | 291–238 | 251 |
| Range (μg/mL) | | 2–30 | |
| Slope | 0.0780 | 0.0359 | 0.1195 |
| Intercept | 0.0263 | –0.0231 | –0.0007 |
| Regression coefficients | 1 | 0.9993 | 0.9998 |
| LOD | 0.58 | 0.67 | 0.52 |
| LOQ | 1.93 | 2.23 | 1.73 |
| Accuracy (%Recovery ±SD) | | | |
| QCL (5 μg/mL) | 99.94 ± 1.33 | 98.11 ± 1.04 | 100.74 ± 0.83 |
| QCM (10 μg/mL) | 99.53 ± 0.85 | 98.86 ± 0.36 | 99.21 ± 0.75 |
| QCH (20 μg/mL) | 100.05 ± 0.52 | 100.79 ± 0.12 | 101.26 ± 0.86 |

*Continue Table 2*

| Parameter | $D^3$ | RD | $D^1R$ |
|---|---|---|---|
| Precision (RSD %) | | | |
| Repeatability | | | |
| QCL (5 μg/mL) | 1.49 | 0.16 | 0.17 |
| QCM (10 μg/mL) | 0.75 | 0.43 | 0.99 |
| QCH (20 μg/mL) | 0.32 | 0.07 | 1.14 |
| Intermediate precision | | | |
| QCL (5 μg/mL) | 0.44 | 0.06 | 0.3 |
| QCM (10 μg/mL) | 0.57 | 0.07 | 0.29 |
| QCH (20 μg/mL) | 0.48 | 0.02 | 0.14 |

The specificity of the proposed method was evaluated by separately analyzing three laboratory-prepared standard solutions of FL with its ODPs with FL at known concentrations within the linear range. No interference was found from the ODPs upon analyzing the prepared mixtures and calculating the percentage recoveries of FL, which indicated the specificity of the proposed method (Table 3).

*Application to pharmaceutical dosage form.* The proposed validated method was successfully used to determine FL in its pharmaceutical dosage form. The calculated results were found to be satisfactory, and good recoveries of the labelled amount of FL were achieved, which indicated the suitability of the proposed method for quality control and routine analysis. A standard addition technique was applied to evaluate interference from excipients, and the obtained recoveries revealed no interference, as shown in Table 3.

TABLE 3. Determination of Flibanserin in Laboratory Synthetic Mixture, Pharmaceutical Dosage Forms, and Standard Addition Techniques by Proposed Methods

| Method | Laboratory prepared mixture (mean recovery± SD) | Application in pharmaceutical dosage form $n = 5$ (mean recovery ± SD) | Application of standard addition techniques |
|---|---|---|---|
| $D^3$ | 99.44±1.14 | 98.48±0.73 | 99.50±0.23 |
| RD | 100.69±0.64 | 99.01± 0.86 | 100.87±0.77 |
| $D^1R$ | 99.55± 0.91 | 99.79±0.58 | 99.75±0.39 |
| PLS | 100.83±0.30 | 98.80±0.57 | 99.98±1.71 |
| PCR | 101.24±0.50 | 98.73±0.73 | 99.69±0.64 |

*Validation of the proposed chemometrics method.* The chemometrics method was validated using different diagnostic tools. Root mean square error of cross validation (RMSECV) and root mean square error of prediction (RMSEP) were calculated and measured to determine the error and prediction ability of the model. Additionally, the error variation in the validation set was examined by plotting the predicted concentration and actual concentration. Then, linear equations were computed, and all the obtained results are summarized in Table 4.

TABLE 4. Results of Assay Validation Obtained by the Proposed Chemometrics Techniques for the Determination of FL in the Presence of ODP

| Parameter | PLS | PCR |
|---|---|---|
| Slope | 0.934 | 1.002 |
| Intercept | 0.489 | 0.021 |
| Correlation | 0.9985 | 0.9983 |
| RMSECV | 0.276 | 0.235 |
| RMSEP | 0.273 | 0.221 |

*Statistical analysis.* A statistical analysis was performed using Student's *t*-test and the *F*-test to compare the results obtained from the estimation of FL in its dosage forms using the proposed methods with those obtained from an HPLC method developed in-house. The calculated *t*-values and *F*-values were found to be less than the theoretical values, which indicated no significant differences between the proposed method and the HPLC method, as shown in Table 5.

TABLE 5. Statistical comparison of the results obtained by the proposed methods and in-house method for determination of FLB in Veroxeserin ® Tablets

| Method | Mean ± SD | Variance | *N* | *t*-test (2.33) | *F*-value (6.33) |
|---|---|---|---|---|---|
| $D^3$ | 98.48 ± 0.73 | 0.53 | 5 | 0.34 | 3.64 |
| RD | 99.01 ± 0.86 | 0.78 | 5 | 1.56 | 5.04 |
| $D^1R$ | 99.79 ± 0.58 | 0.14 | 5 | 0.78 | 1.02 |
| PLS | 98.80 ± 0.57 | 0.33 | 5 | 1.45 | 2.23 |
| PCR | 98.73 ± 0.73 | 0.54 | 5 | 0.99 | 3.68 |
| In-house method* | 98.36 ± 0.38 | 0.15 | 5 | – | – |

* An Eclipse XDB $C_{18}$ column (150 mm × 4.6, 5 μm) using the mobile phase consisted of methanol:0.05M ammonium acetate buffer (pH 4.0) 90:10. The elution was performed using isocratic mode with flow rate 1ml/min, and FLB was detected at 237 nm.

*Comparative study.* The efficiencies of the proposed univariate and multivariate spectrophotometric methods were studied and compared with each other. Based on the proposed $D^3$ method, only one step was needed for derivatization of the zero-order spectra, unlike the derivative ratio method, which resulted in fewer manipulation steps being required. Similar to the RD method, only one manipulation step was performed, and it was a division step without derivatization. One more advantage of RD is an improvement of the signal-to-noise ratio by measuring the difference in the amplitudes at two wavelengths, while $D^1R$ was found to be the most sensitive method with a lower LOD value compared with those of the other two univariate methods.

**Conclusions.** In this work, binary mixtures of FL and ODPs were successfully resolved using univariate and multivariate methods using basic instruments, which indicates the power of these techniques for indicating stability. These novel, inexpensive, and simple spectrophotometric methods are the first spectrophotometric methods for the determination of FL in its pure form and its pharmaceutical dosage form. FL was susceptible to oxidative degradation, and their chemical structures elucidated and confirmed using mass spectrometry and $^1$H NMR spectroscopy. Generally, these proposed methods could be applied in quality control laboratories, and they do not require sophisticated instruments.

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## REFERENCES

1. M. A. Sultan, A. K. Attia, M.J. Eissa, *Biomed. Chromatogr.*, **33**, 1–9 (2019).
2. R. W. Invernizzi, G. Sacchetti, S. Parini, S. Acconcia, R. Samanin, *Br. J. Pharmacol.*, **139**, 1281–1288 (2003).
3. A. H. Clayton, S. A. Kingsberg, I. Goldstein, *Sex. Med.*, **6**, 59–74 (2018).
4. M. Géonet, P. De Sutter, E. Zech, *Sexologies*, **22**, e9–e15 (2013).
5. J. Warnock, *CNS Drugs*, **16**, 745–753 (2006).
6. C. Johnson-Agbakwu, L. Brown, J. Yuan, R. Kissling, D. J. Greenblatt, *Clin. Ther.*, **40**, 64–73 (2018).
7. E. D. Deeks, *Drugs*, **75**, 1815–1822 (2015).
8. H. A. Croft, *J. Sex. Med.*, **14**, 1575–1584 (2017).
9. R. Baid, R. Agarwal, *Ind. Psychiatry J.*, **27**, 154–157 (2018).
10. S. Sonawane, S. Jadhav, P. Rahade, S. Chhajed, S. Kshirsagar, *Scientifica (Cairo)*, **2016**, 1–9 (2016).
11. M. Blessy, R. D. Patel, P. N. Prajapati, Y. K. Agrawal, *J. Pharm. Anal.,* **4**, 159–165 (2014).
12. P. N. Vaingankar, P.D. Amin, *Anal. Chem. Insights*, **2016**, 13–20 (2016).
13. M. Y. Low, L. Li, X. Ge, C. L. Kee, H. L. Koh, *J. Pharm. Biomed. Anal.,* **57**, 104–108 (2012).
14. M. Poplawska, A. Blazewicz, P. Zolek, Z. Fijalek, *J. Pharm. Biomed. Anal.*, **94**, 45–53 (2014).
15. M. Iqbal, E. Ezzeldin, N. L. Rezk, A. A. Bajrai, K. A. Al-Rashood, *Bioanalysis*, **10**, 1087–1097 (2018).
16. P. K. Sahu, N.R. Ramisetti, T. Cecchi, S. Swain, C. S. Patro, J. Panda, *An Overview of Experimental Designs in HPLC Method Development and Validation*, Elsevier B.V. (2018).
17. *FDA Guidance for Industry: Q1A(R2) Stability Testing of New Drug Substances and Products,* U.S. Department of Health and Human Services, Food and Drug Administration, 1–22 (2003).