J Fluoresc (2014) 24:1745–1756
DOI 10.1007/s10895-014-1463-3

ORIGINAL PAPER

Convolution of Emission Derivative Ratio Curves of Closely Related Fluorescent Reaction Products Using Discrete Fourier Functions and Non-Parametric Linear Regression Method

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Received: 27 June 2014 / Accepted: 16 September 2014 / Published online: 16 October 2014

Abstract A spectrofluorimetric method was used for the estimation of closely related fluorescent reaction products, Fluoxetine and Olanzapine, in their mixture after derivatization of both drugs using 4-chloro-7- nitrobenzo - 2 -oxa-1,3 - diazole (NBD-Cl) in borate buffered medium (pH 9.5) to form highly fluorescent products. The method based on the use of first and second derivative ratio of the emission data along with their convolution using 8-points sin x_i or cos x_i polynomials (discrete Fourier functions). The proposed method facilitates their simultaneous determination despite the presence of a minor component (Olanzapine) and strong overlapped spectra of the two NBD-Cl fluorescent products of fluoxetine and olanzapine. The accurate and precise estimation of the minor component was achieved after the convolution of the derivative ratio curves. Moreover, the obtained data were subjected to non-parametric linear regression analysis (Theil's method). The work combines the advantages of convolution of derivative ratio curves using discrete Fourier functions together with the reliability and efficacy of the non-parametric analysis of data.

Keywords Chemometrics · Overlapped emission curves · Convoluted derivative ratio curves · Discrete Fourier function · Theil's method · NBD-Cl

Introduction

Fluorescence spectroscopy is commonly used in quantitative analysis because of its high sensitivity and selectivity. Even if the analyte of interest possesses a weak fluorescence signal, the signal could be amplified to enable its determination [1]. The amplified signals may not be only attributed to the analyte of interest. Interference with the signal after amplification may occur from different sources as background fluorescence from the solvents, emission from the optical components, light leaks in the instrumentation, light scattered by turbid solutions and stray light passing through the optics [1]. Molecular fluorescence bands tend to be broad (25 nm or more) and featureless. As a result, fluorescence spectra of the various sample components may overlap during multi-component analysis. Background fluorescence and other types of additive interference from other sample components or contaminants can be a major problem, especially in multi-component analysis [2]. Different approaches may be applied to overcome such problem. Mathematical (chemometric) technique is one of these approaches used to decompose spectra that consist of overlapping bands into contributions from the individual sample components [2]. Examples of this technique, synchronous [3] and derivative [4] fluorescence spectrometry are the simplest and most widely used.

In certain cases, at which the spectra are strongly overlapped as in closely related drugs, these spectrofluorimetric methods cannot cope with the high level of interference. Derivative of ratio spectra has been developed by Salinas [5] to resolve mixtures of overlapped spectra. This method has been extended to the spectrophotometric or spectrofluorimetric determination of binary and ternary mixtures [6–8].

Even with the use of derivative ratio of emission spectra method, major problems could encounter during spectrofluorimetric analysis of mixtures. These problems are attributed to the strong spectral overlap especially in the presence of minor components in the mixtures and the high level of background noise. As a result, curve convolution using discrete Fourier transform method was investigated to solve such problems.

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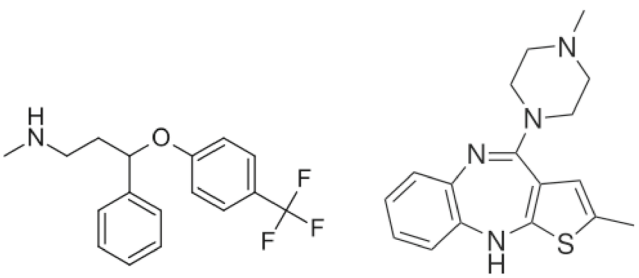
A chemometric method based on non-parametric linear regression of derivative/discrete Fourier transform convoluted chromatographic peak responses was first developed by Korany et al. The method was used in non-ideal conditions in the chromatographic or polarographic analysis [9–12]. Recently, the method of derivative curve convolution along with the non parametric treatment of data was extended to handle common non-ideal cases in spectrofluorimetric analysis [13]. A Spectrophotometric method based on convolution of the ratio curve itself was developed to the analysis of drugs in ternary mixtures [14].

A new hybrid chemometric method was presented in this work. The proposed method depends on applying first and second derivative ratio method followed by the convolution of the resulting first and second derivative ratio curves with discrete Fourier functions method.

The survey in the literature reveals that this proposed chemometric hybrid was not used to handle spectrofluorimetric problems up till now. Furthermore, the spectrofluorimetric data in such case are mostly affected by the incidence of outliers and the lack of any evidence that the examined data are normally distributed especially in case of small data set frequently used in analytical work. As a result the application of non-parametric regression methods (Theil's "incomplete" method) as a statistical method of analysis is an important alternative against the parametric one [15].

Fluoxetine (FLX, N-methyl-c-[4-(trifluoro methyl)phenoxy]benzenepropanamine, Fig. 1) has been approved worldwide in the therapy of major depression [16]. Olanzapine (OLZ, 2-methyl-4-(4-methyl-1-piperazinyl)-10H-thieno [2, 3-b][1,5] benzodiazepine, Fig. 1) is a typical antipsychotic drug. It is used for the treatment of schizophrenia and other psychotic symptoms [16].

Novel combination of FLX and OLZ is available as capsule dosage form in the ratio of 25:12. It is used for the treatment of depressive episodes associated with bipolar disorder. The literature revealed few methods for simultaneous determination of FLX and OLZ in dosage forms, these methods includes; spectrophotometry [17, 18], HPLC [19–21] and HPTLC [20, 22] but it lacks any spectrofluorimetric method for their simultaneous determination. However, FLX alone was assayed after derivatization with NBD-Cl using spectrofluorimetric method [23].



FLX OLZ

Fig. 1 Chemical structures of fluoxetine (FLX) and olanzapine (OLZ)

4-Chloro-7-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) is a highly sensitive and selective chromogenic and fluorogenic reagent used in the determination of aliphatic thiols and primary and secondary aliphatic amines with the formation of colored products which can be measured spectrophotometrically or spectrofluorimetrically. Several pharmaceutical compounds have been determined through this approach [23, 24]. In the present work, NBD-Cl was used to develop highly fluorescent products with FLX and OLZ (being non fluorescent) in borate buffered medium.

The aim of this work was to investigate the application of the new hybrid method on the emission data of the two fluorescent products of FLX and OLZ with NBD-Cl. The extensive overlap of the emission spectra of the two NBD-Cl fluorescent products of the drugs with the presence of a weakly fluorescent product and low concentration drug (OLZ) hinders its accurate and precise determination in its pharmaceutical formulation. The chemometric treatment along with the non-parametric regression method facilitates the simultaneous determination of such mixture in its dosage form with good accuracy and precision. The method combines the advantages of derivative ratio method and convolution using discrete Fourier functions together with the reliability and efficacy of the non-parametric analysis of data.

Experimental

Materials and Reagents

FLX (99.92 %) and OLZ (99.80 %) were kindly supplied as a gift sample by EIPICO pharmaceutical industries company, Egypt. NBD-Cl (Sigma Chemical Co., St. Louis, USA); boric acid, sodium hydroxide (El-Nasr Chemical Ind. Co., Egypt), hydrochloric acid and methanol (BDH Laboratory Suppliers, Poole, England) were used. The pharmaceutical formulation analyzed was Symbyax capsules (label claim: 25 mg FLX and 12 mg OLZ per capsule, Eli Lilly Company – USA). All solvents and materials used throughout this study were of analytical grade.

Apparatus

Fluorescence measurements were carried out using a Shimadzu (Kyoto, Japan) RF-1501 version 3.0 spectrofluorophotometer equipped with a 150 W xenon lamp and 1-cm quartz cells. A KERN & SOHN balance used was (GmbH Balingen, Germany). For sonication, J.P. SELECTA, S.A. sonicator was used (Spain).

Solutions

Stock solutions (1 mg mL^{-1}) were prepared by dissolving appropriate amounts of FLX or OLZ in methanol. Working solutions of 50 and 100 μg mL^{-1} of FLX and OLZ, respectively, were prepared by diluting the stock solutions with the same solvent. A solution of 0.4 mg mL^{-1} of NBD-Cl was freshly prepared in methanol. Solutions of borate buffer (pH 9.5) and 2 M hydrochloric acid were prepared.

Derivatization Reaction

Accurate volumes (100–800 μL) of the working solutions of FLX and OLZ were transferred into 10-mL volumetric flasks and the volume is completed to 1 mL with methanol. Two milliliters of borate buffer followed by 1 mL NBD-Cl solution were added and mixed well. The flasks were placed in a thermostatically controlled water bath at 50±2 °C for 20 min. The reaction was stopped by cooling under tap water then 0.5 ml of 2 M hydrochloric acid solution was added to each flask and the solutions were diluted to volume with methanol to give final concentrations of 0.5–4 and 1–8 μg mL^{-1} of FLX and OLZ, respectively. Blank solution was prepared similarly but without the drugs.

Construction of Calibration Curves

One milliliter aliquots of blank solution, FLX or OLZ reaction mixtures previously prepared in the section of Derivatization Reaction, were transferred into 10-mL volumetric flasks and the volume is completed with methanol. The prepared solutions were covering the concentration range of 0.05–0.4 μg mL^{-1} for FLX and 0.1–0.8 μg mL^{-1} for OLZ. These FLX and OLZ solutions were stable for at least 2 h at room temperature. The emission fluorescence spectrum was scanned for each drug under the following operating conditions.

- Emission wavelength, 480–660 nm, at 1.0 nm intervals.
- Excitation wavelength, fixed at 460 nm.

The emission data were processed using Excel software. For determination of FLX, the emission spectra of FLX were divided by the emission spectrum of OLZ (0.1 μg mL^{-1}) as a divisor. Derivative technique (DD method) was applied to the resulted ratio curves, first (1DD) and second (2DD) derivative data at Δ λ=10 nm were calculated. Then convolutions of the two types of derivative data were made using discrete Fourier functions of 8-points sin x_i polynomials (DD /FF method) at Δ λ=10 nm to get convoluted first derivative ratio curves; 1DD /FF and convoluted second derivative ratio curves; 2DD /FF according to equation 1:

$$t = [(0)D_0 + (+0.707)D_1 + (+1)D_2 + (+0.707)D_3 + (0)D_4 + (-0.707)D_5 + (-1)D_6 + (-0.707)D_7]/4 \quad (1)$$

where D_0 to D_7 stand for eight derivative values; at 10 nm interval. The numbers in brackets are values of the selected Fourier function. The DD values (peak to peak or peak to zero) and the DD/FF values (peak to peak or peak to zero) were measured at the selected wavelengths for FLX to be assayed. For determination of OLZ, an analogous procedure was done for its assay by the proposed chemometric methods using FLX (0.05 μg mL^{-1}) as a divisor. The DD method was applied to the resulted ratio curves at Δ λ=10 nm. Then convolutions of the derivative data were made using discrete Fourier functions of 8-points cos *xi* polynomials (DD /FF method) at Δ λ=2 nm using equation 2:

$$t = [(1)D_0 + (+0.707)D_1 + (0)D_2 + (-0.707)D_3 + (-1)D_4 + (-0.707)D_5 + (0)D_6 + (+0.707)D_7]/4 \quad (2)$$

Preparation of Synthetic Mixtures

Different aliquots of FLX and OLZ working solutions were transferred into 10-mL volumetric flasks to prepare synthetic mixtures of both drugs on which the derivatization reaction was completed as under the section of Derivatization Reaction. One milliliter aliquots of the prepared reaction mixtures were transferred into 10-mL volumetric flasks and the volume is completed with methanol to give synthetic mixtures at the concentration levels of (0.4: 0.1), (0.25:0.12), (0.1:0.1) and (0.15:0.4) μg mL^{-1} for FLX and OLZ, respectively. The emission fluorescence spectra of the mixtures were scanned and processed as under the operating conditions discussed in section of Construction of Calibration Curves.

Assay of Tablets

The contents of 20 capsules were weighed, and finely powdered. An accurately weighed quantity of the powder equivalent to two capsules (50 mg FLX and 24 mg OLZ) was weighed and transferred to a 50-mL volumetric flask. Thirty milliliters of methanol were added and the flask

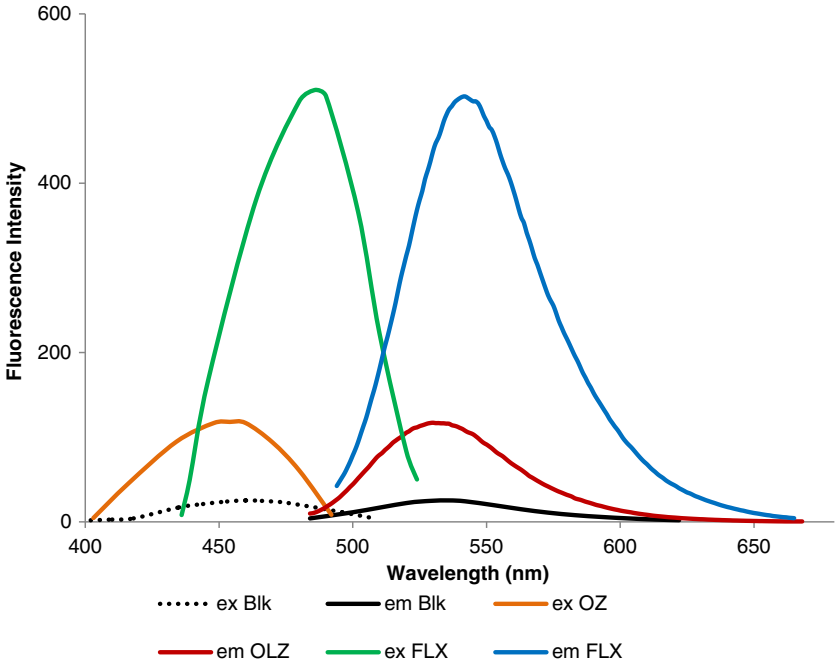


Fig. 2 Excitation and emission spectra of NBD-Cl fluorescent products of 0.25 μg mL^{-1} fluoxetine (FLX), 0.12 μg mL^{-1} olanzapine (OLZ) and blank solutions (ratio of dosage form)

was sonicated for 30 min., completed to the volume with methanol and filtered. An aliquot of 0.5 mL of the filtrate was transferred into 10-mL volumetric flask and the volume was completed with methanol to give concentration of 50 and 24 μg mL^{-1} for FLX and OLZ, respectively. Finally, portions of 0.5 mL of this solution were transferred into 10-mL volumetric flasks and the procedure was completed as under derivatization reaction in section of Derivatization Reaction. One milliliter portions of the reaction mixtures were transferred into 10-mL volumetric flasks and completed to the mark with methanol to give final concentrations of 0.25 and 0.12 μg mL^{-1} for FLX and OLZ, respectively. The emission fluorescence spectra of the diluted solutions were scanned and processed as under the operating conditions discussed in the section of Construction of Calibration Curves.

Results and Discussion

Derivatization Reaction

Both FLX and OLZ do not have native fluorescence, thus derivatization with fluorogenic reagent was necessary for their fluorimetric determination. NBD-Cl forms highly fluorescent product with secondary amines using relatively mild reaction conditions [23, 24] and it was used as derivatizing reagent for the fluorimetric determination of FLX [23]; therefore, it was chosen as a derivatizing reagent for both FLX and OLZ in the present study. It was found that both FLX and OLZ react with NBD-Cl and forms yellow-colored fluorescent products. FLX fluorescent product exhibited maximum fluorescence intensity (λ_{em}) at 542 nm after its excitation at wavelength (λ_{ex}) of 486 nm, while OLZ fluorescent product exhibited maximum

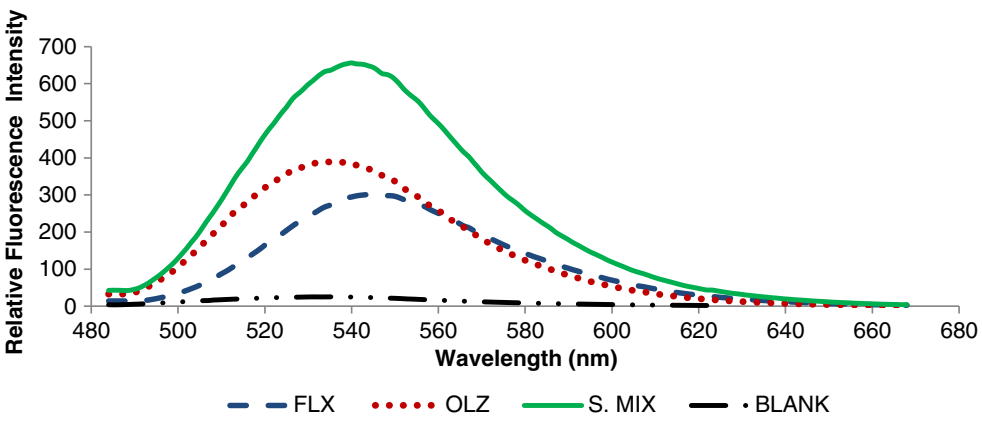


Fig. 3 Emission spectra of 0.15 μg mL^{-1} fluoxetine (FLX), 0.4 μg mL^{-1} olanzapine (OLZ) and their synthetic mixture (S. MIX) with the blank (BLANK)

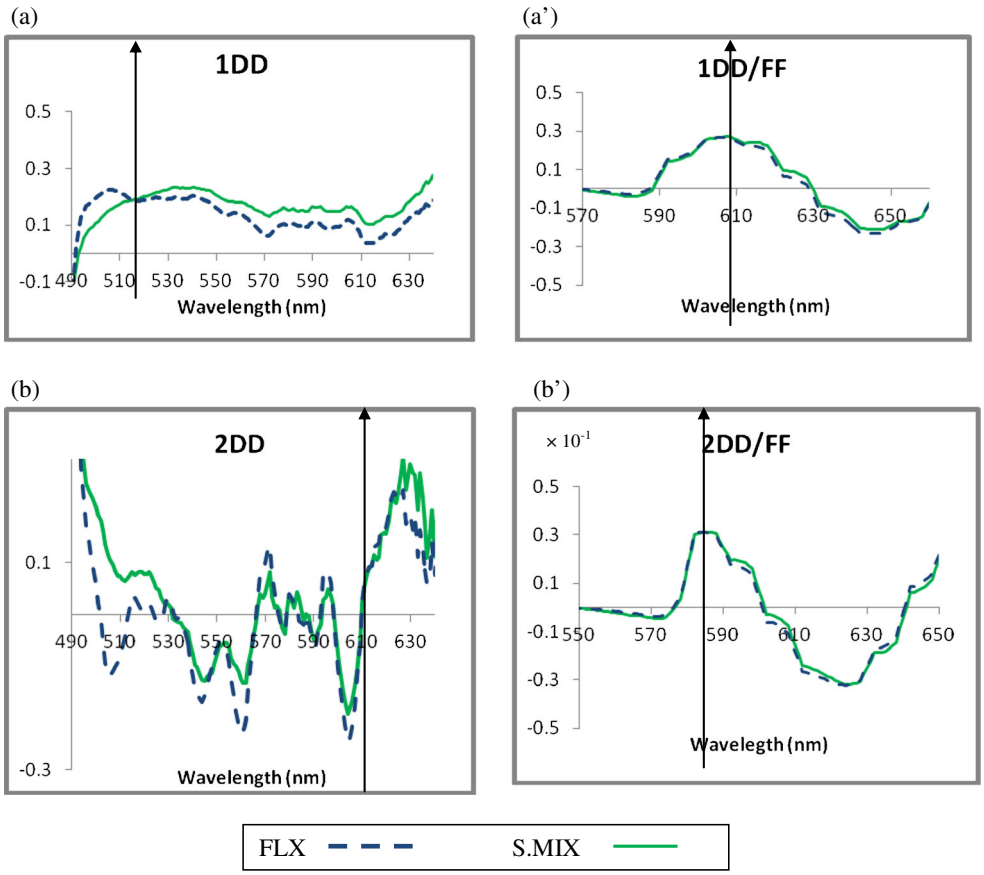


Fig. 4 The first derivative ratio (**a**) and second derivative ratio (**b**) of 0.15 μg mL^{-1} fluoxetine (FLX) and its synthetic mixture (S. MIX) with olanzapine (OLZ) in a ratio of (0.15 FLX: 0.4 OLZ), respectively, and their corresponding Fourier function curves (**a′**) and (**b′**) respectively, using 0.1 μg mL^{-1} (OLZ) as divisor, the *arrows* indicate the selected points (all curves are smoothed using Excel software)

fluorescence intensity (λ_{em}) at 533 nm after its excitation at wavelength (λ_{ex}) of 460 nm. The excitation and emission spectra for the reaction products of FLX and OLZ with NBD-Cl are given in Fig. 2. For the simultaneous determination of FLX and OLZ, λ_{ex} of OLZ (the minor component) was chosen as an optimum excitation wavelength.

Different experimental parameters affecting the derivatization reaction and the stability of the formed fluorescent derivatives were studied and optimized. The effect of concentration of NBD-Cl was studied. It was found that increasing the reagent concentration increased the fluorescence intensity up to 0.4 mg mL^{-1} for both FLX and OLZ, after which no more increase in fluorescence was obtained. The effect of pH change was studied by using borate buffer in the pH range 5–11. The results revealed that both drugs, especially OLZ, have difficulty to react with NBD-Cl in acidic media. Increasing the pH, higher fluorescence values were obtained and pH 9.5 was found optimum for their simultaneous determination. At higher pH values, the background fluorescence of the reagent increased resulting in a net decrease in fluorescence of the drug solutions. The effect of temperature on the reaction was studied by carrying out the reaction at different temperatures (25–90 °C) over a period of 1 h. An increase in the fluorescence intensities of the fluorescent products was achieved upon increasing the temperature up to 50 °C. However, heating at temperatures higher than 50 °C caused a decrease in the fluorescence of OLZ (minor component in the mixture). In order to investigate the effect of heating time at the selected temperature, 50 °C, optimum fluorescence intensities of the fluorescent products were obtained upon heating for 20 min. Heating for more than 20 min caused no further increase in the fluorescence intensity of both drugs. Thus, it was found that heating at 50±2 °C for 20 min optimum for their simultaneous determination.

Treatment of Analytical Data

Derivative ratio calculations were applied to emission data of the scanned standard solutions, the overlapped synthetic mixtures and the dosage form final solutions. Direct measurement of the emission curves shows serious level of overlap (Fig. 3) as the fluorescent products of both drugs are closely related to each other. Moreover, as was mentioned before, OLZ/NBD-Cl product is weakly fluorescent compared to FLX/NBD-Cl product. Constant interferences could be eliminated by calculating the first derivative of emission ratio (1DD), while second derivative of emission ratio (2DD) can eliminate any linear

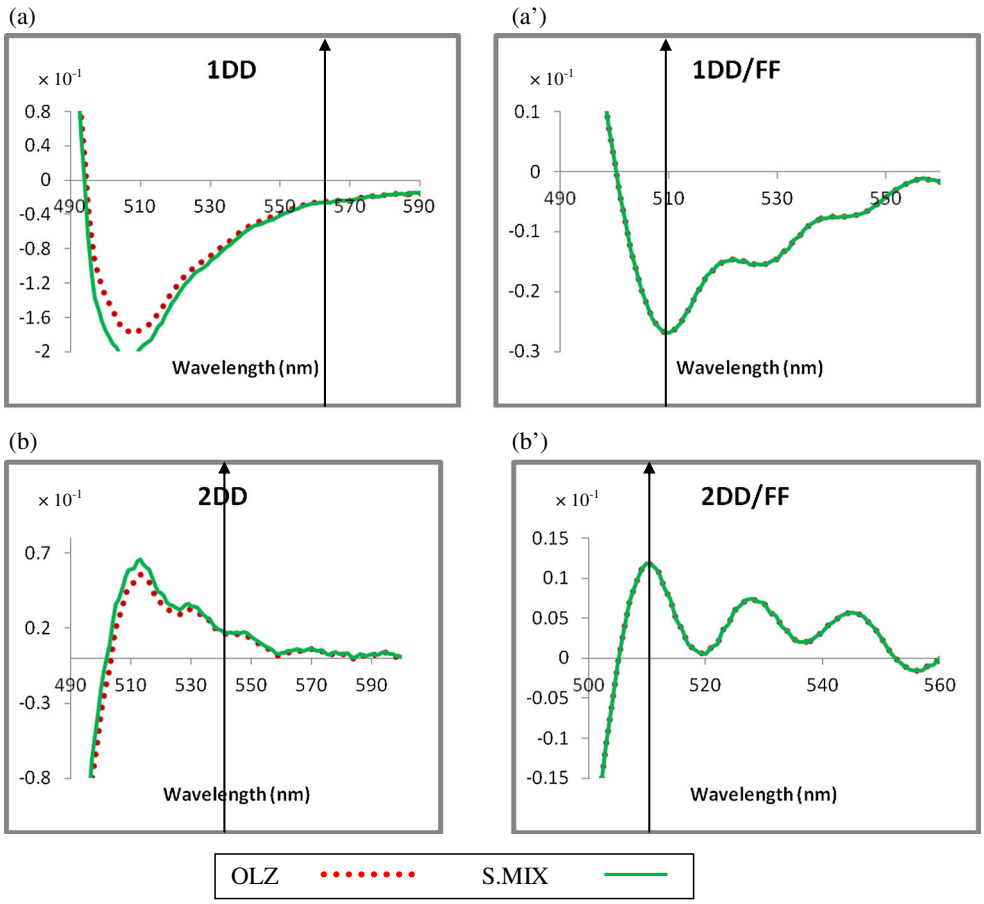


Fig. 5 The first derivative ratio (**a**) and second derivative ratio (**b**) of 0.4 μg mL^{-1} olanzapine (OLZ) and its synthetic mixture (S. MIX) with fluoxetine (FLX) in a ratio of (0.15 FLX:0.4 OLZ), respectively, and their corresponding Fourier function curves (**a′**) and (**b′**) respectively, using 0.05 μg mL^{-1} FLX as divisor, the *arrows* indicate the selected points (all curves were smoothed using Excel software)

Table 1 Parametric linear regression and statistical parameters for the determination of fluoxetine (FLX) by the proposed fluorimetric method

	r	a	b	$S_{y/x}$	S_a	S_b	F	LOD μg mL^{-1}	LOQ μg mL^{-1}
Derivative ratio technique (*DD* method)									
First derivative ratio (*1DD*) at 515 nm	0.9935	-0.682×10^{-2}	1.133	0.102×10^{-1}	0.837×10^{-2}	0.036	229.25	0.266	0.878
Second derivative ratio (*2DD*) at 612 nm	0.9972	-0.679×10^{-3}	0.267	0.319×10^{-2}	0.261×10^{-2}	0.112×10^{-1}	534.71	0.037	0.121
Derivative ratio under Fourier functions (*DD*/FF method)									
First derivative ratio under Fourier functions (*1DD*/FF) at 607 nm	0.9988	1.274×10^{-2}	1.867	0.794×10^{-2}	0.487×10^{-2}	0.021	1252.32	0.013	0.042
Second derivative ratio under Fourier functions (*2DD*/FF) at 585 nm	0.9992	0.999×10^{-3}	0.201	0.593×10^{-3}	0.651×10^{-3}	0.278×10^{-2}	1938.51	0.009	0.029

r: Correlation coefficient. a: Intercept b: Slope

$S_{y/x}$: Standard deviation of residuals

S_a: Standard deviation of intercept

S_b: Standard deviation of slope

F: Variance ratio, equals the mean of squares due to regression divided by the mean of squares about regression (due to residuals)

LOD limit of detection, *LOQ* limit of quantitation

Table 2 Parametric linear regression and statistical parameters for the determination of olanzapine (OLZ) by the proposed fluorimetric method

	r	a	b	$S_{y/x}$	S_a	S_b	F	LOD μg mL^{-1}	LOQ μg mL^{-1}
Derivative ratio technique (*DD* method)									
First derivative ratio (*1DD*) at 564 nm	0.993	0.124×10^{-2}	0.628×10^{-1}	0.813×10^{-3}	0.652×10^{-3}	0.134×10^{-2}	216.39	0.039	0.129
Second derivative ratio (*2DD*) at 538 nm	0.996	0.492×10^{-3}	0.452×10^{-1}	0.309×10^{-3}	0.248×10^{-3}	0.509×10^{-3}	346.28	0.021	0.068
Derivative ratio under Fourier functions (DD/FF method)									
First derivative ratio under Fourier functions (*1DD/FF*) at 510 nm	0.999	0.144×10^{-2}	0.650×10^{-1}	0.302×10^{-3}	0.643×10^{-3}	0.132×10^{-2}	648.42	0.014	0.046
Second derivative ratio under Fourier functions (*2DD/FF*) at 510 nm	0.999	0.116×10^{-3}	0.277×10^{-1}	0.112×10^{-3}	0.897×10^{-6}	0.184×10^{-3}	759.17	0.012	0.040

r: Correlation coefficient. a: Intercept b: Absolute value of Slope

$S_{y/x}$: Standard deviation of residuals

S_a: Standard deviation of intercept

S_b: Standard deviation of slope

F: Variance ratio, equals the mean of squares due to regression divided by the mean of squares about regression (due to residuals)

LOD limit of detection, *LOQ* limit of quantitation

interference [10, 11]. For choosing the optimum divisor concentration, different divisor concentrations were studied and the optimum one was chosen based on the best accuracy and precision obtained for the assay of each drug in the prepared mixtures. For choosing the optimum Δ λ, different wavelength intervals were studied and Δ λ= 10 nm was chosen as it gave the best curves shape (smoothed) with reasonable responses of the obtained curves. The 1DD and 2DD values at the selected points shown in Figs. 4 and 5, for each of the two compounds were correlated to the concentration. These selected points were based on that, best recovery and RSD % were obtained for each drug assay in its prepared mixtures at these points. As will be discussed later, OLZ assay needed further treatment of the resulting derivative ratio curves to enable its assay with good accuracy and precision.

For the DD/FF method, the suitable function for convolution of the derivative ratio curves was selected according to the shape of the resulted derivative ratio curves, discrete Fourier functions of 8-points sin x_i polynomials was found to be optimum for first and second derivative ratio curves in FLX assay [10, 11]. While for OLZ assay, discrete Fourier functions of 8-points cos x_i

Table 3 Comparison between parametric and non-parametric regression models for the determination of Fluoxetine (FLX) by the proposed Fluorimetric method

	|a|		|b|		Percentage change in |a|	Percentage change in |b|
	Parametric	Non-parametric	Parametric	Non-parametric		
Derivative ratio technique (*DD* method)						
First derivative ratio (*1DD*)	0.682×10^{-2}	0.141×10^{-2}	1.133	1.078	−79.326	−4.854
Second derivative ratio (*2DD*)	0.679×10^{-3}	0.660×10^{-3}	0.267	0.259	−2.798	−2.996
Derivative ratio under Fourier functions (*DD*/FF method)						
First derivative ratio under Fourier functions (*1DD/FF*)	1.274×10^{-2}	1.270×10^{-2}	1.867	1.842	−0.314	−1.339
Second derivative ratio under Fourier functions (*2DD/FF*)	0.999×10^{-3}	0.992×10^{-3}	0.201	0.199	−0.701	−0.995

|*a*| Modulus of intercept

|*b*| Modulus of slope

Percentage change in |*a*| means percentage change in |*a*| of NP vs. |*a*| of P = [(|*a*| of NP − |*a*| of P)/|*a*| of P] × 100

Percentage change in |*b*| means percentage change in |*b*| of NP vs. |*b*| of P = [(|*b*| of NP − |*b*| of P)/|*b*| of P] × 100

Table 4 Comparison between parametric and non-parametric regression models for the determination of olanzapine (OLZ) by the proposed fluorimetric method

	|a|		|b|		Percentage change in |a|	Percentage change in |b|
	Parametric	Non-parametric	Parametric	Non-parametric		
Derivative ratio technique (*DD* method)						
First derivative ratio (*1DD*)	0.124×10^{-2}	0.438×10^{-3}	0.628×10^{-1}	0.654×10^{-1}	−64.677	4.140
Second derivative ratio (*2DD*)	0.492×10^{-3}	0.171×10^{-3}	0.452×10^{-1}	0.466×10^{-1}	−65.244	3.097
Derivative ratio under Fourier functions (*DD*/FF method)						
First derivative ratio under Fourier functions (*1DD/FF*)	0.144×10^{-2}	0.571×10^{-3}	0.650×10^{-1}	0.664×10^{-1}	−60.347	2.154
Second derivative ratio under Fourier functions (*2DD/FF*)	0.116×10^{-3}	0.299×10^{-4}	0.277×10^{-1}	0.282×10^{-1}	−74.224	1.805

|a| Modulus of intercept

|b| Modulus of slope

Percentage change in |a| means percentage change in |a| of NP vs. |a| of P = [(|a| of NP − |a| of P)/|a| of P] × 100

Percentage change in |b| means percentage change in |b| of NP vs. |b| of P = [(|b| of NP − |b| of P)/|b| of P] × 100

polynomials was found to be optimum for the resulted first and second derivative ratio curves [10, 11]. The obtained responses after convolution using the selected function were reasonable to allow determination of minor component and the selection of this function was confirmed by assessment of the accuracy and precision. Different wavelength intervals were studied and Δ λ= 10 nm and 2 nm were chosen for FLX and OLZ assay, respectively, as they gave reasonable responses with the best recovery% and RSD %, especially in the assay of the minor component. Also, the values at selected points for each drug shown in Figs. 4 and 5 were related to concentration. As shown in Figs. 4 and 5, further treatment of the derivative ratio curves by their convolution using discrete Fourier functions was necessary as it filters the whole derivative ratio curve from any kind of interferences. The standard and mixture curves after convolution, Figs. 4a′, b′ and 5a′, b′, are totally coincide unlike their derivative curves before convolution. This allows choosing the selected points for the assay of each drug at the points with maximum response of the drug (peak to zero) with minimum contribution of the other.

Since convolution using Fourier functions corrects all types of interferences except for linear interference, application of Fourier functions on derivative ratio data would eventually lead to removal of all types of interference producing high degree of purity of the analytical peaks at the selected points [10, 11]. This would be

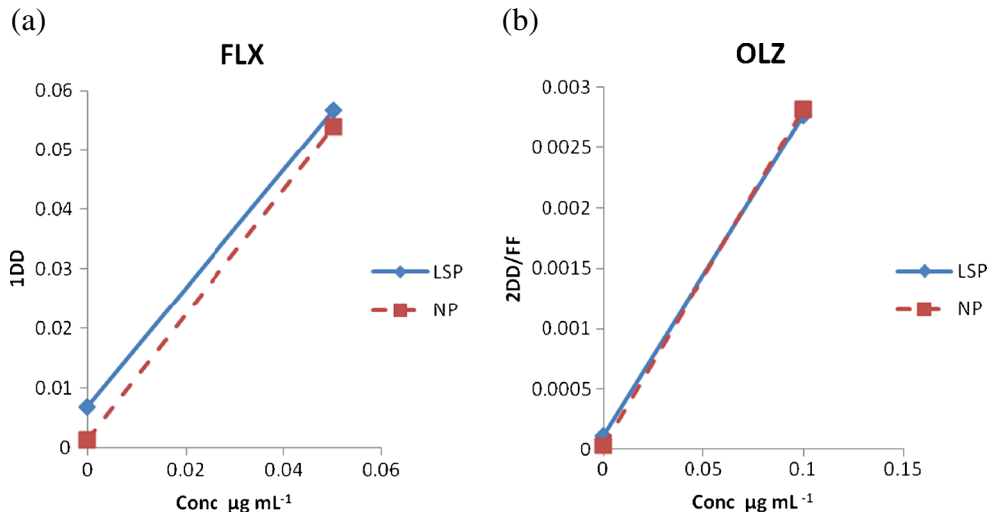


Fig. 6 Regression lines calculated by Theil's method, non-parametric (– – ·), and by the least squares method, parametric (———), for the determination of fluoxetine (FLX) (**a**), and olanzapine (OLZ) (**b**), using 1DD and 2DD/FF methods, respectively, as representative example

Table 5 Parametric and non parametric evaluation of the precision and accuracy for the determination of Fluoxetine (FLX) in different synthetic mixtures with Olanzapine (OLZ) by the proposed fluorimetric method

	Recovery %							
	Parametric				Non parametric			
FLX nominal conc μg mL^{-1} (FLX: OLZ)	1DD	2DD	1DD/FF	2DD/FF	1DD	2DD	1DD/FF	2DD/FF
(0.4: 0.1)	95.00	98.00	100.00	101.50	97.00	99.00	101.00	101.00
(0.25: 0.12)	96.00	100.00	100.90	99.00	97.50	99.80	100.70	100.00
(0.1: 0.1)	104.00	102.00	98.00	99.00	103.00	102.20	99.00	99.00
(0.15: 0.4)	104.00	103.00	99.00	100.00	105.00	102.70	100.00	99.90
Mean %	99.75	100.75	99.43	99.88	100.63	100.93	100.18	99.98
E_r %	−0.25	0.75	−0.53	−0.13	0.63	0.93	0.175	−0.03
SD	4.92	2.217	1.25	1.181	3.99	1.80	0.89	0.82
RSD (%)	4.94	2.200	1.26	1.18	3.96	1.79	0.89	0.82
% change in E_r %	150.00	24.00	−66.67	−80.00				
% change in RSD (%)	−19.75	−18.82	−29.55	−30.85				

Recovery% is the mean recovery of three determinations at each concentration level

The mean of all recoveries of different concentrations in the same method

Percentage relative error

Standard deviation of the recoveries of different concentration in the same method

Percentage relative standard deviation

% change in Er% of NPR versus that of LSPR = [(|Er%| of NPR − |Er%| of LSPR)/|Er%| of LSPR]*100

% change in RSD (%) of NPR versus that of LSPR = [RSD (%) of NPR − RSD (%) of LSPR] /RSD (%) of LSPR]*100

beneficial in case where high incidence of interference could be found from different sources as background noise, instrumental and other mixture components, as in the simultaneous assay of FLX and OLZ. At which the selected points would represent the pure compound and neglect the other interferences.

Table 6 Parametric & Non parametric evaluation of the precision and accuracy for the determination of olanzapine (OLZ) in different synthetic mixtures with fluoxetine (FLX) by the proposed fluorimetric method

	Recovery %							
	Parametric				Non parametric			
OLZ nominal conc μg mL^{-1} (FLX: OLZ)	1DD	2DD	1DD/FF	2DD/FF	1DD	2DD	1DD/FF	2DD/FF
(0.4: 0.1)	93.00	95.00	99.30	100.50	95.00	104.00	100.00	100.00
(0.24:0.12)	94.30	97.00	98.70	99.50	97.00	103.00	99.00	99.90
(0.1:0.1)	97.60	100.70	100.70	99.10	98.00	99.00	99.00	100.00
(0.15:0.4)	100.00	101.00	99.10	98.60	101.00	99.00	100.00	101.50
Mean %	96.23	98.43	99.45	99.43	97.75	101.25	99.50	100.35
E_r %	−3.78	−1.58	−0.55	−0.58	−2.25	1.25	−0.50	0.35
SD	3.18	2.92	0.87	0.81	2.50	2.63	0.58	0.77
RSD (%)	3.30	2.97	0.88	0.81	2.56	2.60	0.58	0.77
% change in E_r%	−40.47	−20.88	−9.09	−39.66				
% change in RSD (%)	−22.46	−12.47	−33.71	−5.55				

Recovery% is the mean recovery of three determinations at each concentration level

The mean of all recoveries of different concentrations in the same method

Percentage relative error

Standard deviation of the recoveries of different concentration in the same method

Percentage relative standard deviation

% change in Er% of NPR versus that of LSPR = [(|Er%| of NPR − |Er%| of LSPR)/|Er%| of LSPR]*100

% change in RSD (%) of NPR versus that of LSPR = [RSD (%) of NPR − RSD (%) of LSPR]/RSD (%) of LSPR]*100

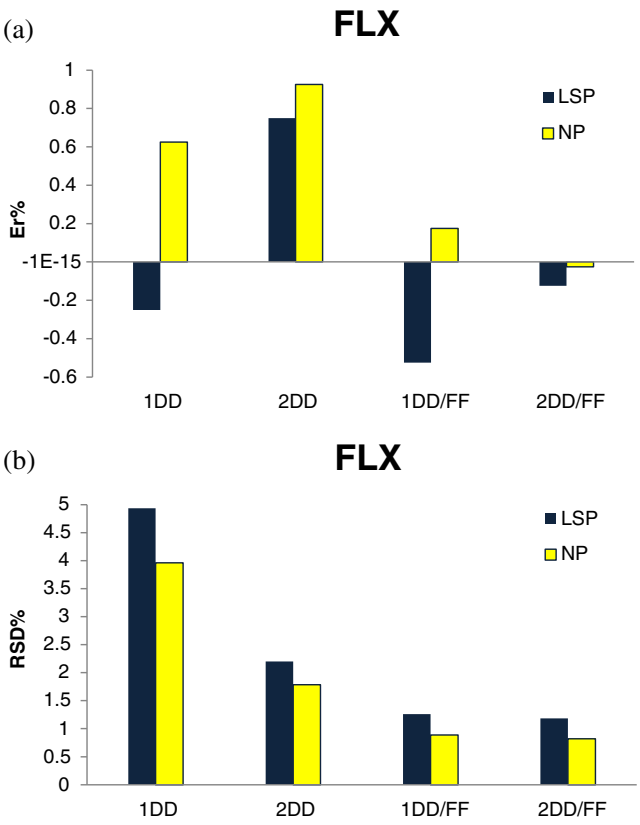


Fig. 7 Comparison between E_r (%), (**a**), and RSD (%), (**b**), calculated for fluoxetine (FLX), in the different chemometric methods of analysis, 1DD, 2DD, 1DD/FF and 2DD/FF, using the two types of regression models, parametric (LSP) and non-parametric (NP)

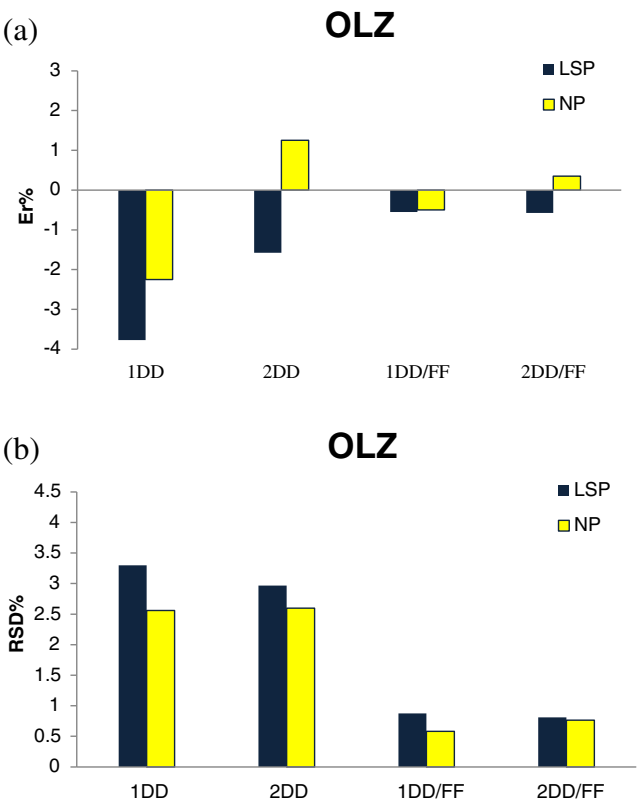


Fig. 8 Comparison between E_r (%), (**a**), and RSD (%), (**b**), calculated for olanzapine (OLZ), in the different chemometric methods of analysis, 1DD, 2DD, 1DD/FF and 2DD/FF, using the two types of regression models, parametric (LSP) and non-parametric (NP)

Validation

ICH guidelines [25] for method validation were followed for the developed spectrofluorimetric method. All validation parameters will be discussed below in details.

Parametric Calibration Graphs and Statistical Data

The linearity of the proposed methods was evaluated by analyzing series of different concentrations of each of FLX and OLZ. Under the experimental conditions described, the graphs obtained by plotting derivative ratio and convoluted derivative ratio data versus concentration for each of the two compounds, show various degrees of linearity (Tables 1 and 2). As convolution using Fourier functions corrects all types of interferences except for linear interference. Thus, application of Fourier functions on derivative ratio data would be beneficial giving the best linearity parameters (Tables 1 and 2). Good regression lines show high values for both (r) and (F) values [26].

Application of Non-Parametric Regression Methods

The assumption in the parametric method is that data being examined follow the normal (Gaussian) distribution. However, this theorem is not really valid for the very small data sets used in analytical work [15]. If there is no evidence that the data follow normal distribution and presence of outliers is suspected, the non-parametric statistics as Theil's "incomplete" method is the best alternative for the parametric one. The calculations depend on the use of the median instead of the mean. The method was applied to the data of chromatographic, polarographic and spectrophotometric methods [9–12, 14]. Recently, the non parametric regression method was also applied to emission data [13].

For the different chemometric methods used, the emission response data were handled using Theil's method. The best-fit straight line obtained using Theil's method was compared with the least squares best fit line. Comparing the intercepts and slopes of the two statistical methods used indicates that, the non-parametric regression method could be considered superior over the parametric one (Tables 3 and 4 and Fig. 6). Using the Second derivative Ratio under Fourier functions (*2DD /FF*) for the determination of OLZ as an example, the intercept decreases from 0.116×10^{-3} to 0.299×10^{-4} and the slope slightly increases from 0.277×10^{-1} to 0.282×10^{-1}, upon applying the non-parametric method relative to the parametric one.

Table 7 Parametric and non- parametric evaluation of the precision and accuracy for the determination of fluoxetine (FLX) and olanzapine (OLZ) in pharmaceutical preparation

		Parametric				Non-parametric			
Nominal value μg mL^{-1}		1DD	2DD	1DD/FF	2DD/FF	1DD	2DD	1DD/FF	2DD/FF
FLX 0.25	Recovery	96.00	102.20	98.60	99.50	97.00	101.80	99.50	100.00
	RSD (%)	3.15	1.91	1.34	1.18	2.27	1.88	0.93	0.84
OLZ 0.12	Recovery	95.20	97.00	99.70	99.10	96.50	103.20	99.90	100.70
	RSD (%)	3.10	2.06	1.67	0.998	2.02	1.87	0.68	0.77

Recovery % is the mean recovery of five determinations (n=5)

Percentage relative standard deviation

Generally, it was found that the intercept decreases and the slope slightly increases (Tables 3 and 4). Figure 6, as representative example for comparing the two lines, clearly show the improvement of the regression lines by using two points which are the intercept and the first point of the linearity range.

Detection and Quantitation Limits

Limit of detection (LOD) and limit of quantitation LOQ were calculated for each compound according to Miller [15]. Their values were lower than those obtained before the treatment of data. Moreover, among the different chemometric methods used, the 2DD/FF gave the least values for LOD & LOQ. Taking the determination of FLX as an example, upon applying the 2DD/FF relative to the 1DD, the LOD and LOQ decreased from 0.266 and 0.878 to 0.009 and 0.029 μg mL^{-1}, respectively. This indicated that the linearity ranges of each drug could be expanded to lower limits of quantitation after the chemometric treatment of data permitting its determination at lower levels, Tables 1 and 2.

Precision and Accuracy

For the assessment of precision and accuracy, triplicate determinations were carried out on the prepared synthetic mixtures. The data shown in Table 5 indicate that good accuracy and precision were obtained for FLX determination following DD and DD/FF methods.

For OLZ determination as a minor component, Table 6 showed bad precision and accuracy for the synthetic mixtures at which OLZ is of low concentrations, due to its extensive overlap of FLX. However after applying DD/FF method, the (RSD %) and mean percentage recovery became in the accepted ranges of each indicating good precision and accuracy, respectively. As application of Fourier functions on derivative ratio data is capable of removing all types of interference, it produces analytical peaks with high degree of purity at the selected points. The significance of the proposed method (convolution of the derivative ratio curves) is demonstrated by assaying synthetic mixtures containing OLZ (gives weakly fluorescent product with NBD-Cl) in low concentrations relative to FLX. For the assay of OLZ in synthetic mixtures with ratio of 0.4:0.1 (lowest conc. of OLZ) and 0.25:0.12 (dosage form ratio), FLX: OLZ, respectively, the recovery obtained upon applying DD method alone was in the range from 93 to 97 %. However upon convolution of these derivative ratio curves with suitable discrete Fourier functions polynomials, the obtained recovery of OLZ in these mixtures was nearly 100 %.

For the non-parametric regression method, the same was done except that the (RSD %) and the mean percentage recovery calculations were based on the intercepts and slopes obtained by the non-parametric method. The percentage change in E_r% and RSD % in the non-parametric method of regression compared with the parametric one indicates that good accuracy and precision were achieved after the non-parametric treatment of the data, Tables 5 and 6 and Figs. 7 and 8.

Analysis of Pharmaceutical Formulations

For the two proposed statistical methods of analysis, the data shown in Table 7 indicated that agreement of the found results with the label claim was attained after the chemometric treatment of data using DD and DD/FF method for FLX assay. However, OLZ was successfully assayed after the convolution of the derivative ratio curves with discrete Fourier functions.

Conclusion

Convolution of derivative ratio curves with discrete Fourier functions has been successfully used in purifying overlapped emission spectra of closely related drugs in spectrofluorimetry. This is highly needed in spectrofluorimetry at which different sources could interfere with the broad emission curves obtained. Theil's method which depends on the median not the mean is highly advantageous in cases when outliers are suspected. In

this paper, different chemometric and statistical regression methods were compared by analyzing synthetic mixtures and pharmaceutical formulation of closely related NBD-Cl fluorescent products of FLX and OLZ. It was found that the combination of non-parametric method along with the DD/FF method was superior for the determination of weakly OLZ-NBD-Cl fluorescent product in presence of the highly fluorescent one of FLX.

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