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ORIGINAL PAPER

Second-Derivative Synchronous Fluorescence Spectroscopy for the Simultaneous Determination of Fluphenazine Hydrochloride and Nortriptyline Hydrochloride in Pharmaceutical Preparations

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Abstract A rapid, simple, and highly sensitive second-derivative synchronous fluorimetric (SDSF) method has been developed for the simultaneous analysis of binary mixtures of fluphenazine hydrochloride (FLZ) and nortriptyline hydrochloride (NTP) in their co-formulated tablets. The method is based upon measurement of the native fluorescence of these drugs at constant wavelength difference ($\Delta\lambda$)=120 nm in acetic acid. The different experimental parameters affecting the fluorescence intensity of the studied drugs were carefully studied and optimized. The fluorescence-concentration plots were rectilinear over the range of 0.25–3.0 and 1–10 μg/ml for FLZ and NTP respectively, with lower detection limits (LOD) of 0.05 and 0.18 μg/ml and quantitation limits of 0.15 and 0.53 μg/ml for FLZ and NTP respectively. The proposed method was successfully applied for the determination of the studied compounds in their synthetic mixtures and in commercial co-formulated tablets. The results obtained were in good agreement with those obtained by the reference methods.

Keywords Second derivative synchronous spectrofluorimetry · Fluphenazine · Nortriptyline · Simultaneous determination

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Introduction

Fluphenazine (FLZ) is a phenothiazine antipsychotic agent that is co-formulated with nortriptyline in its tablets. Fluphenazine hydrochloride, 2-[4-[3-(2-(trifluoromethyl) phenothiazine-10-yl) propyl]piperazine-1-yl]ethanol dihydrochloride (Fig. 1), is used to treat psychiatric disorders such as schizophrenia, mania, severe anxiety, and behavioural disturbances [1].

Nortriptyline hydrochloride (NTP), 3-(10, 11 –dihydro- 5H-dibenzo-[a,d] cyclohepten-5-ylidene)-N-methylpropylamine hydrochloride (Fig. 1), is a tricyclic antidepressants used to treat depression, nocturnal enuresis, some anxiety disorders, and neuropathic pain [1].

Several analytical methods were reported for the determination of FLZ, either in pure form or pharmaceutical preparations and biological fluids. These methods included spectrophotometry [2, 3], spectrofluorimetry which is based on oxidation of FLZ with Ce (IV) and measuring the fluorescence intensity of the produced Ce (III) [4], voltammetry [5–8], High Performance Liquid Chromatography (HPLC) [9–14], and Densitometric High Performance Thin Layer Chromatography HPTLC [15].

As for NTP, various reports were published concerning its determination in pure form, formulations and biological fluids including: spectrophotometry [16–20], spectrofluorimetry where derivatization with 4-chloro-7-nitrobenzofurazan was performed with a detection limit of 0.18 μg/ml and a calibration graph linear up to 60 μg/ml [20], voltammetry [21], HPLC [11, 22–38], TLC [35], and capillary electrophoresis [39].

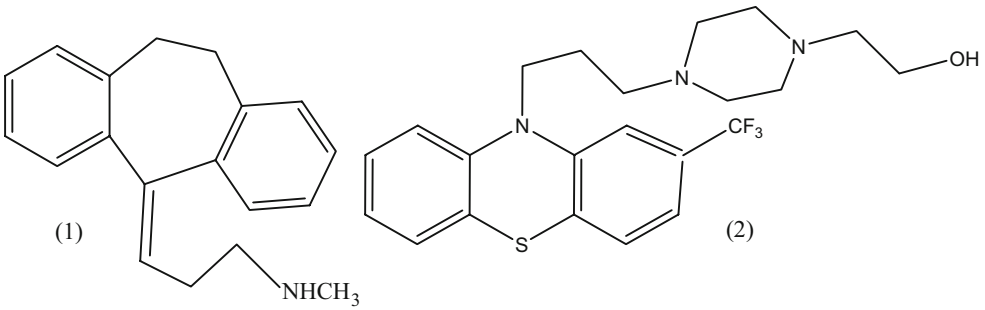

Fig. 1 Structural formula of nortriptyline NTP (1), and fluphenazine FLZ (2)

The British Pharmacopoeia (BP) [40] recommended a non aqueous potentiometric method for the determination of FLZ and NTP using perchloric acid and sodium hydroxide as a titrant for FLZ and NTP respectively.

The United States Pharmacopoeia (USP) [41] on the other hand, described an HPLC for the determination of FLZ in pure form using mobile phase consisting of phosphate buffer: acetonitril: methanol (40: 30: 30) containing 0.2% triethylamine, adjusted to pH 2.5 with UV detection at 254 nm. A similar approach was used for the determination of FLZ injection using mobile phase consisting of methanol: acetonitril: 0.05 M ammonium acetate (2:2:1). Moreover, the USP recommended a non aqueous potentiometric method for the determination of NTP in a mixture of acetic acid and mercuric acetate (5:1) using perchloric acid as a titrant.

A good guide to the work published for FLZ and NTP up to 1973 and 1972 is found in their monographs written by K. Florey and J. L. Hale respectively on the Analytical Profiles of Drug Substances and Excipients [42, 43].

FlZ and NTP are formulated in a binary mixture for the treatment of patients with mild to moderate mixed anxiety depression states. HPLC was reported for the simultaneous determination of these two drugs in pharmaceutical preparations and biological fluids [11, 44].

In fluorometric methods, high sensitivity and selectivity are generally expected. However, problems of selectivity can occur in multicomponent analysis because of the overlap of their spectra that could be observed. Specificity is a particular problem in the determination of fluorescent drugs. Synchronous fluorescence spectroscopy (SFS) has been found to have several advantages [45], such as simple spectra, high selectivity, low interference, etc. Because of its sharp, narrow spectrum, SFS serves as a very simple, effective method of obtaining data for quantitative determination in a single measurement [46]. It has attracted the attention of many researchers and developed rapidly since it was firstly proposed by LIoyd [47].

Synchronous fluorescence spectroscopy techniques are classified according to different scanning modes of monochromators into constant wavelength, variety angle, and constant energy. At present, the constant wavelength method, in which a constant difference between the emission and excitation wavelengths is maintained, is used most extensively.

The combination of SFS and derivative is more advantageous than differentiation of the conventional direct spectrofluorimetry in terms of sensitivity, because the amplitude of the derivative signal is inversely proportional to the bandwidth of the original spectrum [48].

Recently, derivative synchronous fluorometry (DSF) has been utilized for the determination of different mixtures in their co formulated dosage forms and biological fluids, such as metacycline and oxytetracycline [49], aspirin with caffeine [50] or aspirin with salicylic acid [51], diflunisal and salicylic acid [52], carvedilol and ampicillin [53], oxytetracycline in medicated premixes and feeds [54], mixtures of non steroidal anti-inflammatory drugs [55], and cinnarizine with either domperidone or nicergoline [56, 57].

FLZ and NTP are co formulated in tablets. Because of the high overlap of their synchronous spectra, it was difficult to determine their contents by direct synchronous fluorometry. The problem is most aggravated in the analysis of biological fluids. This problem was resolved by using second derivative SFS (SDSFS). The synchronous spectrum at constant wavelength difference ($\Delta\lambda$)=120 nm between the emission and excitation wavelengths was selected as optimum to perform the analysis. This method was applied to the simultaneous determination of FLZ and NTP in synthetic mixtures and in their co-formulated pharmaceutical preparations (Motival® tablets). The aim of the present work was to develop a simple, sensitive, and rapid method for the simultaneous determination of FLZ and NTP using SDSFS based on their synchronous fluorescence. To the best of our knowledge, up till now neither direct nor synchronous spectrofluorometry has been reported for the analysis of FLZ and NTP in binary mixtures.

Experimental procedures

Apparatus

Spectrofluorometer: Perkin Elmer LS 45 Luminescence Spectrometer, equipped with a 150 W Xenon arc lamp,

grating excitation and emission monochromators, and a recorder. Slit widths for both monochromators were set at 10 nm. A 1 cm quartz cell was used. Derivative spectra were evaluated using Fluorescence Data Manager (FLDM) software, Perkin Elmer Buck i.e. FL WINLAB, version 400.02.

Materials and reagents

All reagents and solvents were of Analytical Reagent grade.

a) Fluphenazine hydrochloride (FLZ) and nortriptyline hydrochloride (NTP) were kindly provided by Bristol-Myers Squibb Pharmaceutical Company, Cairo, Egypt. Their purities were checked according to BP [40] and were found to be 99.2, and 100.8% for the two drugs respectively.
b) Pharmaceutical preparations:
   * Modecate® injection (Batch # B80219), labeled to contain 50 mg fluphenazine decanoate/2 ml, Bristol-Myers Squibb, Cairo, Egypt.
   * Motival® tablets (Batch # B80280), labeled to contain 0.5 mg fluphenazine hydrochloride and 10 mg nortriptyline hydrochloride, Bristol-Myers Squibb, Cairo, Egypt.
d) Acetic acid 65% (BDH, Poole,UK).

Standard solutions

Stock solutions of FLZ and NTP were prepared by dissolving 100.0 mg of each of the studied compounds in 100 ml of distilled water and were further diluted with the same solvent as appropriate. The working standard solutions were stable for 7 days when kept in refrigerator.

General procedures

Aliquots of FLZ and NTP standard solutions over the concentration range of 0.25–3.0, 1–10 μg/ml for FLZ and NTP respectively were transferred into a series of 10 ml volumetric flasks, diluted to the mark with acetic acid and mixed well. The synchronous fluorescence spectra of the solutions were recorded by scanning both monochromators at ($\Delta\ \lambda$)=120 nm and a scan rate of 600 nm/min using 10 nm excitation and emission windows. The second-derivative fluorescence spectra of FLZ and NTP were derived from the normal synchronous spectra using FLDM software. For best resolution and smoothing 99 points were used for deriving the second-derivative spectra. The fluorescence intensities of the second-derivative spectra were estimated at 259 and 356 nm for FLZ and NTP respectively. A blank experiment was performed simultaneously. The peak amplitude of the second derivative technique was plotted *versus* final concentration of the drug (μg/ml) to obtain the calibration graphs. Alternatively, the corresponding regression equations were derived.

Applications

(a) Procedure for the synthetic mixture

Aliquots of FLZ and NTP standard solutions in the ratio of 1:20 (according to the dosage form), or in a ratio of (1:1, 1:2, 2:1, 3:4, 4:3) for FLZ and NTP respectively were transferred into a series of 10 ml volumetric flasks, diluted to the mark with acetic acid and mixed well. Then, the steps described under "General Procedures" were proceeded. The peak amplitude of the second derivative technique was plotted *versus* the final concentration of the drug (μg/ml) to obtain the calibration graph. Alternatively, the corresponding regression equation was derived.

(b) Procedure for the co-formulated preparation

Twenty tablets were weighed and pulverized. A weighed quantity of the powder equivalent to 0.5 mg of FLZ and 10 mg of NTP was transferred a into a small conical flask, extracted three successive times each with 30 ml of distilled water. The extract was filtered into 100 ml volumetric flask. The conical flask was washed with few mls of distilled water and the volume was completed to mark with the same solvent. Aliquots covering the concentration range were transferred into 10 ml volumetric flasks. The steps described under "General Procedures" were followed. The nominal content of the tablets was determined either from the calibration graphs or from the corresponding regression equations.

(c) Procedure for injection

An accurately measured quantity of the injection, equivalent to about 25 mg of fluphenazine decanoate, was transferred to 50 ml volumetric flask. 20 ml of isopropyl alcohol were added, the stopper was inserted and the solution was shaken vigorously for at least 1 min. 20 ml more of isopropyl alcohol were added and the vigorous shaking was repeated. Dilution with isopropyl alcohol to volume and mixing was performed. The resulting solution was quantitatively diluted with acetonitrile (1:5) to obtain a solution having a concentration of fluphenazine decanoate of about 0.1 mg/ml. Aliquots covering the concentration range were transferred into 10 ml volumetric flasks. The steps described under "General Procedures" were followed. The nominal content of the injection was determined either from the calibration graphs or from the corresponding regression equations.

(d) Procedure for preparation of degradation product

For the kinetic study, aliquot volumes of NTP standard stock solution (400 µg/ml) were transferred into a series of 25 ml volumetric flask to obtain a final concentration of 40 µg/ml, the volume was completed with 0.5 M sodium hydroxide to prepare the alkaline degradation product. The solution was left in a boiling water bath for a fixed time interval (10 min). Aliquot volumes of the degraded solution were transferred to a series of 10 ml volumetric flasks, neutralized with 0.5 M hydrochloric acid, and the steps described under "General procedures" were followed. The synchronous fluorescence spectra intensity of the resulting degraded solutions was recorded at 348 nm. Log a/a–x was plotted versus time (minutes) to get the reaction rate constant and the half life time. Complete degradation was attained by following the same procedure using 2 M sodium hydroxide and boiling for 2 h then neutralizing with 2 M hydrochloric acid.

Results and discussion

It is necessary to record first the normal synchronous spectra of FLZ and NTP to derive the second-derivative synchronous spectra. Figure 2 shows the synchronous fluorescence spectra of different concentrations of FLZ at 302 nm, and NTP at 348 nm. Whereas, Fig. 3 illustrates the synchronous fluorescence spectra of different concentrations of NTP at 348 nm and FLZ at 302 nm. There is still a great overlapping of the spectra of both drugs in normal synchronous spectroscopy; this encouraged us to perform SDSFS technique for the simultaneous determination of FLZ and NTP in their coformulated preparation without prior extraction or separation step.

The fluorescence spectra of FLZ and NTP were separated entirely using SDSFS with a zero crossing technique of measurement. Figure 4 shows that FLZ could be separated at 259 nm in the presence of NTP. While Fig. 5 shows that NTP could be separated at 356 nm in the presence of FLZ.

Optimization of experimental conditions

Different experimental parameters affecting the stability of the fluorescence of the studied compounds were carefully studied and optimized. Such factors were changed individually, while others were kept constant. These factors include Δ λ selection, type and volume of the diluting solvent, and stability time.

Selection of optimum Δ λ

The synchronous fluorescence spectra of FLZ and NTP were recorded using different Δ λ (Fig. 6a, b). The optimum Δ λ value is very important for performing the synchronous fluorescence scanning technique concerning resolution, sensitivity, and features. It can directly influence spectral shape, bandwidth, and signal value. For this reason, a wide range of Δ λ (40, 60, 80, 100, and 120) was examined. When Δ λ was less than 120 nm, poor separation of the two peaks was obtained in addition to lower fluorescence intensities. Therefore, Δ λ of 120 was chosen as optimal for separation of a mixture of FLZ with NTP, since it resulted in two distinct peaks with good shape, and to minimize the spectral interferences caused by each compound in the mixture.

Effect of diluting solvent

Dilution with different solvents such as water, methanol, ethanol, isopropanol, dimethylsulfoxide (DMSO), dimethylformamide, and acetic acid was performed. The results are shown in Table 1. As can be seen, the fluorescence intensity of both FLZ and NTP increased in acetic acid

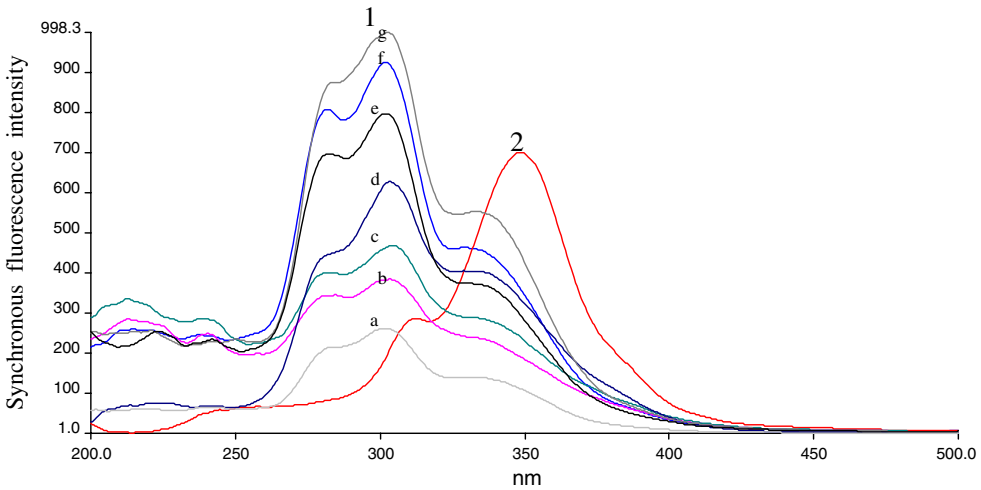

Fig. 2 Synchronous fluorescence spectra of FLZ at 302 nm in the presence of NTP at 348 nm: (1) spectra of FLZ: a) 0.25 µg/ml, b) 0.50 µg/ml, c) 1.0 µg/ml , d) 1.5 µg/ml, e) 2.0 µg/ml, f) 2.5 µg/ml, and g) 3.0 µg/ml. (2) spectrum of NTP (8.0 µg/ml)

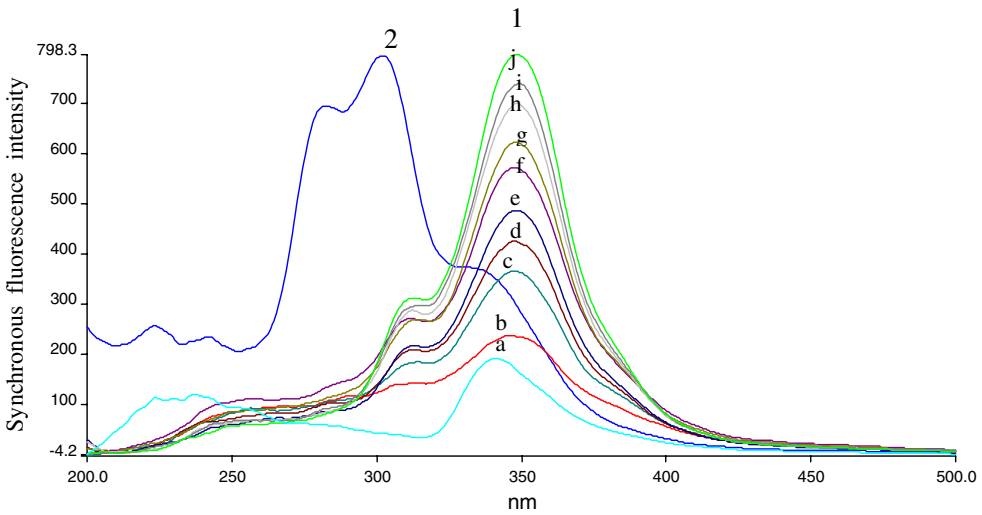

Fig. 3 Synchronous fluorescence spectra of NTP at 348 nm in the presence of FLZ at 302 nm: (1) spectra of NTP: a) 1.0 μg/ml, b) 2.0 μg/ml, c) 3.0 μg/ml , d) 4.0 μg/ml, e) 5.0 μg/ml, f) 6.0 μg/ml, g) 7.0 μg/ml, h) 8.0 μg/ml, i) 9.0 μg/ml ,and j) 10.0 μg/ml. (2) spectrum of FLZ (2.0 μg/ml)

more than the other solvents; therefore, acetic acid was selected as the best solvent for the dilution.

Effect of volume of acetic acid

The effect of the volume of acetic acid was also studied, and it was found that the synchronous fluorescence intensity of FLZ and NTP increased gradually by increasing the volume of acetic acid, then it remained constant after the addition of 5, and 6 ml for FLZ and NTP respectively. Therefore the volume was completed with acetic acid (Fig. 7).

Effect of time

The effect of time on the synchronous fluorescence of the drugs was also studied. It was found that the fluorescence emission developed instantaneously and remained stable for more than 2 h.

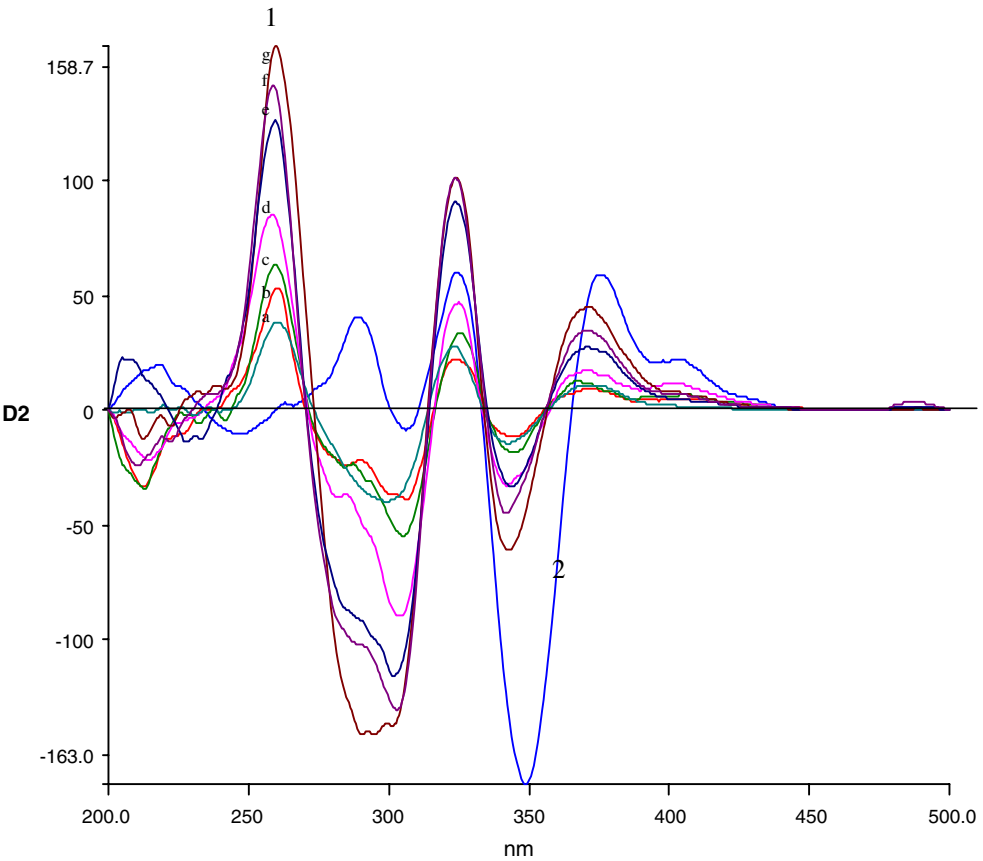

Fig. 4 Second-derivative synchronous fluorescence spectra of different concentrations of FLZ at 259 nm in the presence of NTP: (1) spectra of FLZ: a) 0.25 μg/ml, b) 0.50 μg/ml, c) 1.0 μg/ml , d) 1.5 μg/ml, e) 2.0 μg/ml, f) 2.5 μg/ml, and g) 3.0 μg/ml. (2) spectrum of NTP (8.0 μg/ml)

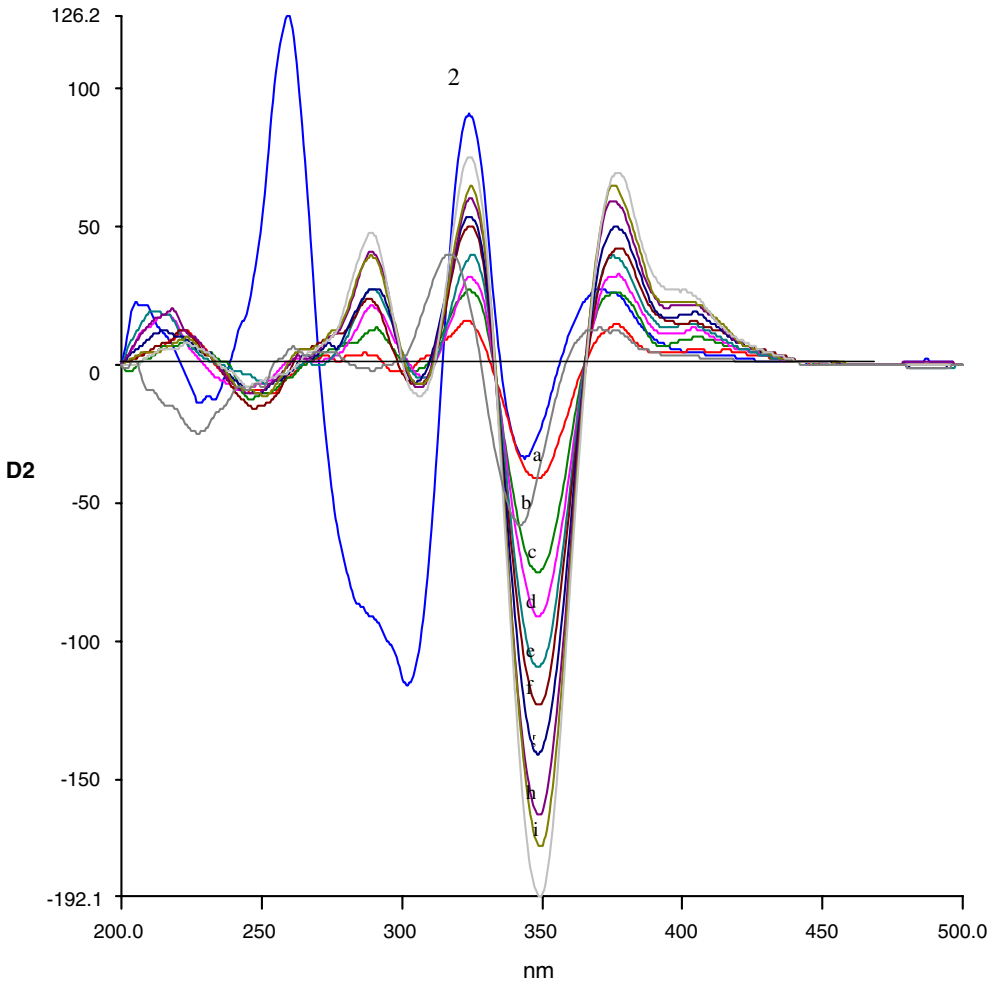

Fig. 5 Second-derivative synchronous fluorescence spectra of different concentrations of NTP at 356 nm in the presence of FLZ: 1) spectra of NTP: a) 1.0 μg/ml, b) 2.0 μg/ml, c) 3.0 μg/ml , d) 4.0 μg/ml, e) 5.0 μg/ml , f) 6.0 μg/ml, g) 7.0 μg/ml, h) 8.0 μg/ml, i) 9.0 μg/ml ,and j) 10.0 μg/ml. (2) spectrum of FLZ (2.0 μg/ml)

Analytical performance

The fluorescence-concentration plots for the studied drugs by SDSFS were linear over the concentration ranges cited in Table 2. Linear regression analysis of the data gave the following equations:

$^2D = 0.44 + 47.84C$ $(r = 0.9998)$ for FLZ at 259 nm with $S_a = 0.73$ and $S_b = 0.43$

$^2D = 0.61 + 14.85C$ $(r = 0.9997)$ for NTP at 356 nm with $S_a = 0.79$ and $S_b = 0.13$

Where ^2D is the peak amplitude in the second derivative mode, C is the concentration of the drug (μg/ml), and r is correlation coefficient. S_a is the standard deviation of the intercept. S_b is the standard deviation of the slope.

The limit of quantification (LOQ) was calculated according to ICH Q2B recommendations [58], and it was found to be 0.15 and 0.53 μg/ml for FLZ and NTP respectively.

LOQ was calculated according to the following equation [58]:

$$LOQ = 10\sigma/S$$

Where σ: the standard deviation of the intercept of the regression line.

S: slope of the calibration curve.

The limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected, and it is calculated according to the following equation [58]:

$$LOD = 3.3\ \sigma/S$$

LOD was found to be 0.05 and 0.18 μg/ml for FLZ and NTP respectively.

The proposed procedure was evaluated by studying the accuracy as percent relative error and precision as percent relative standard deviation (RSD %); the results are abridged in Table 3.

Analysis of synthetic mixture sample

The proposed method was applied to the simultaneous determination of FLZ with NTP in synthetic mixtures containing different concentrations of both drugs in a ratio of 1:20 as present in their co-formulated dosage

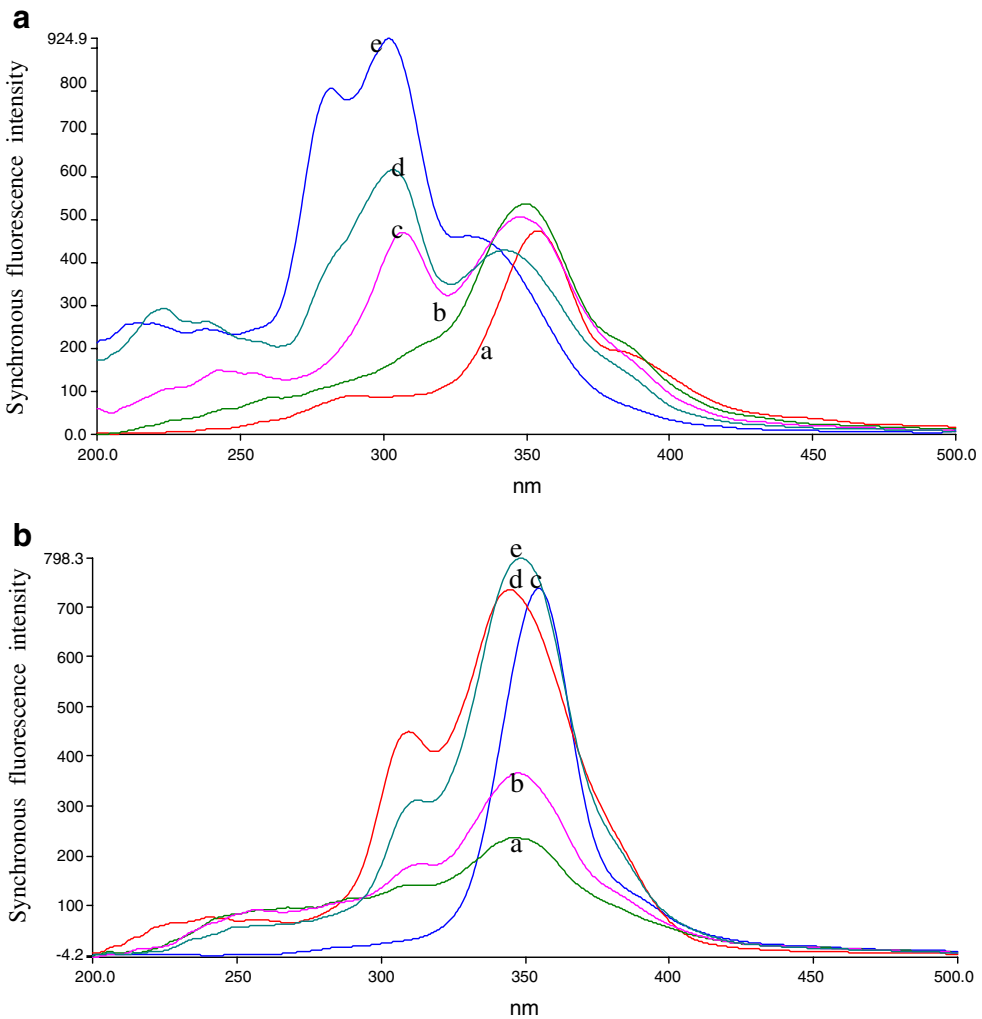

Fig. 6 **a** Effect of Δλ on the intensity of the synchronous fluorescence spectra of FLZ (2.5 μg/ml) where Δλ=a)40 nm, b)60 nm, c)80 nm, d)100 nm, e) 120 nm. **b** Effect of Δλ on the intensity of the synchronous fluorescence spectra of NTP (10 μg/ml) where Δλ=a)40 nm, b)60 nm, c)80 nm, d)100 nm, e) 120 nm

forms (Fig. 8), and in other ratios such as 1:1, 1:2, 2:1, 2:3, 3:2 and 3:4 (Fig. 9).The synchronous fluorescence intensities of second-derivative technique were measured for both drugs. The second-derivative signal of FLZ was measured at 259 nm, which is considered as zero-crossing point for NTP, and the second-derivative signal of NTP was measured at 356 nm, which is zero-crossing point for FLZ. The concentrations of both drugs in the synthetic mixture were calculated according to the linear regression equation of the calibration graphs. The results

Table 1 Effect of different diluting solvents on the readings of the SFS of the studied drugs

Solvent	Synchronous fluorescence intensity	
	FLZ	NTP
Water	250	334
Methanol	446	366
Ethanol	490	346
Isopropanol	440	345
DMSO	420	361
Dimethylformamide	330	240
Acetic acid	550	480

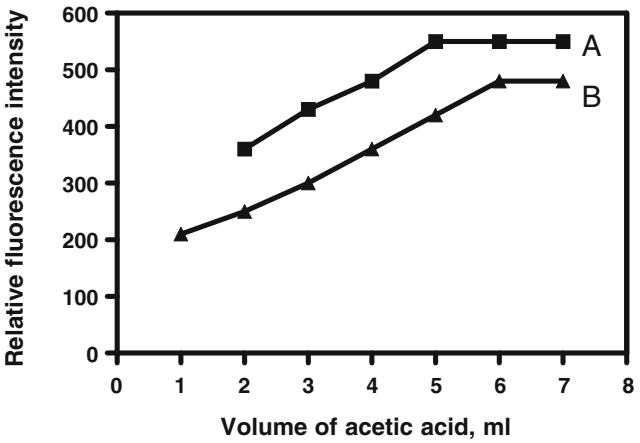

Fig. 7 Effect of volume of acetic acid on the intensity of the synchronous fluorescence spectra of FLZ (**a**), and NTP (**b**)

Table 2 Performance data of the proposed SDSFS method

Parameter	Proposed method	
	FLZ	NTP
Concentration range (μg/ml)	0.25–3	1–10
LOD (ng/ml)	0.05	0.18
LOQ (ng/ml)	0.15	0.53
Correlation coefficient (r)	0.9998	0.9997
Slope	47.8	14.9
Intercept	0.44	0.60
Standard deviation of the residuals, $S_{y/x}$	1.11	1.15
Standard deviation of the intercept of the regression line, S_a	0.73	0.79
Standard deviation of the slope of the regression line, S_b	0.43	0.13
% Error(RSD%/ √n)	0.24	0.34
%RSD	0.54	0.76

obtained regarding RSD and relative error were compared with those obtained using a reference method. The results indicate high accuracy of the proposed method as shown in Table 4.

Validation of the method

The method was tested for linearity, specificity, accuracy and precision. Linear regression equations were obtained.

Table 3 Application of the proposed SFS & SDSFS methods to the determination of FLZ and NTP in pure form

Compound	Amount taken (μg/ml)	Amount found (μg/ml)		Found %		Reference methods [41]
Fluphenazine hydrochloride		SFS	SDSFS	SFS	SDSFS	
	0.25	0.249	0.253	99.60	101.20	
	0.5	0.498	0.507	99.60	101.40	
	0.75	0.745	0.746	99.33	99.47	100.25
	1.0	1.014	0.998	101.40	99.80	101.23
	1.5	1.511	1.497	100.73	99.80	99.84
	2.0	2.012	1.99	100.60	99.50	
	2.5	2.488	2.49	99.52	99.60	
	3.0	3.016	2.98	100.53	99.33	
$\bar{X}$±SD				100.16 ±0.75	100.01±0.83	100.44±0.71
Student's t test				0.29 (2.37)	0.11(2.37)	
Variance ratio F test				1.12(4.35)	1.37(4.35)	
Nortriptyline hydrochloride	1	1.012	0.99	101.20	99.05	Reference methods [33]
	2	1.98	2.03	99.0	101.50	
	3	3.04	2.98	101.33	99.33	
	4	3.99	4.06	99.75	101.50	
	5	5.06	5.01	101.20	100.20	
	6	5.98	6.07	99.67	101.12	100.35
	7	7.09	6.97	101.29	99.57	99.98
	8	7.97	8.03	99.63	100.38	100.98
	9	9.10	8.96	101.11	99.56	
	10	10.06	10.05	100.60	100.50	
$\bar{X}$±SD				100.48±0.88	100.27±0.89	100.44±0.51
Student's t test				0.09 (2.26)	0.25 (2.26)	
Variance ratio F test				2.98 (5.14)	3.05 (5.14)	

Figures between parentheses are the tabulated t and F values respectively at P=0.05 [59]

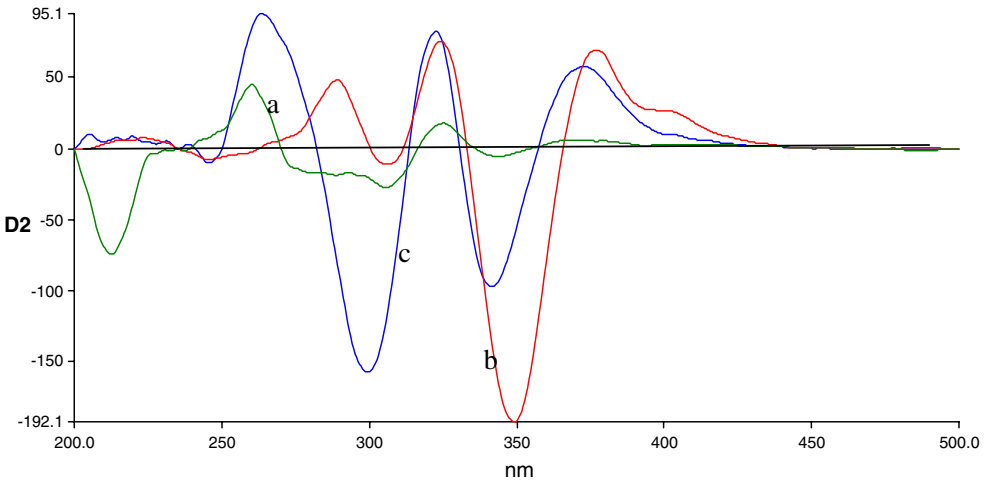

Fig. 8 Second-derivative synchronous fluorescence spectroscopy of a mixture of 0.5 μg/ml FLZ and 10 μg/ml NTP: **a** 0.5 μg/ml FLZ, **b** 10 μg/ml NTP, **c** the mixture

The regression plots showed that there was a linear dependence of peak amplitude values on the concentration of the drug over the range cited in Table 2. The validity of the proposed method was evaluated by statistical analysis of the regression data regarding the standard deviation of the residual ($S_{y/x}$), the standard deviation of the intercept (S_a), and standard deviation of the slope (S_b) [59]. The results are shown in Table 2. The small values of the figures point to the low scattering of the points around the calibration graph and high precision of the proposed method.

Accuracy

The accuracy of the proposed method was evaluated by analyzing standard solutions of the studied drugs. The results obtained by the proposed method were favorably compared with those obtained by the official method [41] for FLZ and a reference method [33] for NTP. Statistical analysis [59] of the results obtained by the proposed and reference methods using student's t-test and variance ratio F- test, revealed no significant difference between the

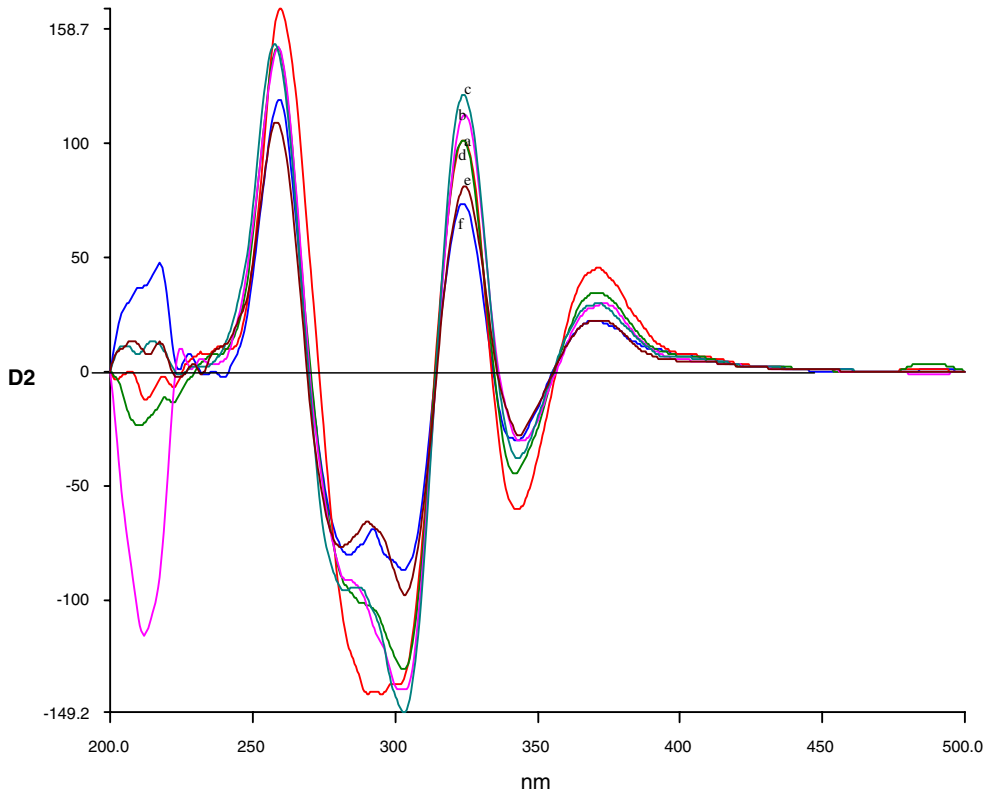

Fig. 9 Second-derivative synchronous fluorescence spectroscopy of a mixture of FLZ and NTP in different ratios: a)1:1 (2 μg/ml each), b) 1:2 (2,4 μg/ml) , c) 2:1 (2,1 μg/ml), d) 2:3 (2,3 μg/ml) , e) 3:2 (3,2 μg/ml) , and f) 3:4 (3,4 μg/ml) for FLZ and NTP respectively

Table 4 Application of the SDSFS method for the determination of FLZ and NTP in their synthetic mixtures

Sample	Concentration taken (μg/ml)		Concentration found (μg/ml)		% found		Reference method [44]	
FLZ and NTP synthetic mixture (1:20)	FLZ	NTP	FLZ	NTP	FLZ	NTP	FLZ	NTP
	0.25	5.0	0.251	5.06	100.40	101.20	100.32	101.23
	0.40	8.0	0.405	7.98	101.25	99.75	99.05	101.56
	0.50	10.0	0.495	9.92	99.00	99.20	101.56	100.07
$\bar{X}$					100.22	100.05	100.31	100.95
± SD					1.14	1.03	1.26	0.78
%RSD					1.14	1.03	1.26	0.78
% Error					0.57	0.59	0.73	0.45
t-test					0.92	0.77		
F-test					1.22	1.74		
(1:1)	2.5	2.5	2.49	2.51	99.60	100.40	100.62	101.63
(1:2)	2.5	5	2.51	5.03	100.40	100.60	101.23	99.45
(1:10)	1	10	0.99	9.98	99.00	99.80	101.23	100.89
(2:1)	3	1.5	2.98	1.52	99.33	101.30	99.23	100.62
(3:4)	3	4	2.97	4.03	99.00	100.75	99.87	99.45
(4:3)	4	3	4.06	2.97	101.50	99.00	100.56	99.63

Table 5 Validation of the proposed method to the determination of the studied drugs in pure and dosage forms

Preparation	% found repeatability	% found intermediate precision
Fluphenazine hydrochloride (pure form)	Fluphenazine (2.0 μg/ml)	Fluphenazine (2.5 μg/ml)
	100.35	101.35
	99.65	101.45
	99.32	100.23
	100.45	100.64
Mean found %	99.94	100.92
± SD	0.55	0.58
% RSD	0.55	0.58
Nortriptyline hydrochloride (pure form)	Nortriptyline (4.0 μg/ml)	Nortriptyline (7.0 μg/ml)
	101.06	99.65
	100.89	99.12
	100.34	99.23
	99.91	100.95
Mean found %	100.55	99.74
± SD	0.53	0.84
% RSD	0.53	0.84
Modecate® injection (50 mg fluphenazine decanoate/2 ml)	100.32	100.45
	101.62	99.85
	100.56	99.04
	101.65	100.25
Mean found %	101.04	99.89
± SD	0.69	0.62
% RSD	0.69	0.62

Table 6 Application of SDSFS method to the determination of fluphenazine and nortriptyline in their co-formulated tablets

Preparation	Concentration taken (μg/ml)		Concentration found(μg/ml)		% found		Reference method [44]	
	FLZ	NTP	FLZ	NTP	FLZ	NTP	FLZ	NTP
	0.25	5.0	0.254	5.02	101.60	100.40	100.25	101.23
	0.40	8.0	0.398	7.98	99.50	99.75	101.05	100.32
	0.50	10.0	0.502	10.03	100.40	100.30	100.69	101.66
$\bar{X}$					100.50	100.15	100.66	101.07
± SD					1.05	0.35	0.40	0.68
%RSD					1.05	0.35	0.40	0.68
% Error					0.61	0.20	0.23	0.39
t-test					0.53 (2.78)	0.58 (2.78)		
F-test					6.89 (19.0)	3.78 (19.0)		

Figures between parentheses are the tabulated t and F values respectively at P=0.05 [59]

performance of the two methods regarding the accuracy and precision, respectively (Table 3). The USP Pharmacopoeia [41] recommended an HPLC for the determination of FLZ in raw material using a mobile phase consisting of phosphate buffer: acetonitril: methanol (40: 30: 30) containing 0.2% triethylamine, at pH 2.5 with UV detection at 254 nm. The reference method for NTP [33] also used an HPLC technique with a mobile phase consisting of 25 mM potassium dihydrogen phosphate buffer of pH 7: acetonitril (60:40) with UV detection at 230 nm.

Precision

Repeatability

The repeatability was evaluated through the replicate analysis of two different concentrations of each drug, either in pure drug or in dosage forms. The mean percentage recoveries based on the average of four separate determinations for pure and dosage forms are abridged in Table 5.

Intermediate precision

It was performed through replicate analysis of two different concentrations of each drug, either in pure or dosage forms on four successive days. The percentage recoveries are based on the average of four separate determinations. The results are shown in Table 5.

Robustness of the method

The robustness of the proposed method adopted was demonstrated by the consistency of the fluorescence intensity with the deliberately minor changes in the experimental parameters such as volume of acetic acid which did not greatly affect the fluorescence intensity of the mixture.

Table 7 Application of the proposed SFS & SDSFS methods to the determination of FLZ in its single dosage form

Preparation	Concentration taken (μg/ml)	Concentration found(μg/ml)		% found		Official method [41]
		SFS	SDSFS	SFS	SDSFS	
Modecate® injection (50 mg fluphenazine decanoate/2 ml)	0.5	0.498	0.499	99.60	99.92	
	1.0	1.01	1.010	100.90	100.89	
	1.5	1.505	1.49	100.33	99.97	100.63
	2.0	2.01	2.01	100.50	100.50	101.23
	2.5	2.48	2.52	99.20	100.80	101.52
	3.0	2.98	3.01	99.33	100.33	
$\bar{X}$±SD				99.98±0.69	100.40±0.41	101.13±0.45
t-test				0.64 (2.31)	0.19 (2.31)	
F- test				2.35 (5.79)	1.20 (5.79)	

Figures between parentheses are the tabulated t and F values respectively at P=0.05 [59]

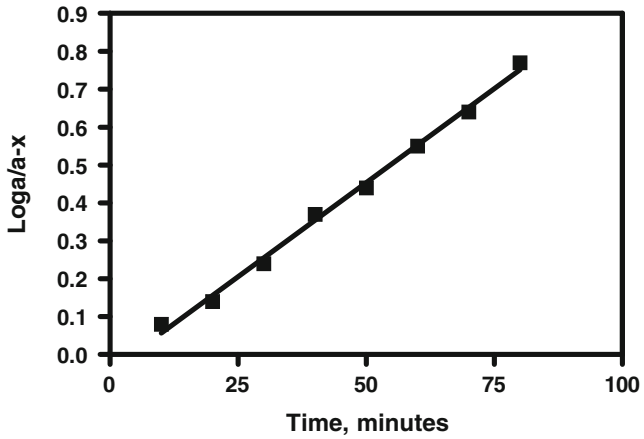

Fig. 10 Semi log plot of NTP *versus* different heating times (min) with 0.5 M sodium hydroxide at 100 °C

Pharmaceutical applications

The proposed method was further applied to the determination of the studied drugs in their co-formulated tablets.

Selectivity

Common tablet excipients such as talc, lactose, starch, gelatin, avisil and magnesium stearate did not interfere with the assay. The results are abridged in Table 6.

Accuracy

The results of the proposed method were statistically compared with those obtained using the reference method [44]. Statistical analysis of the results, using student's t-test and variance ratio (F- test), revealed no significant difference between the performance of the proposed and reference methods [44] regarding the accuracy and precision respectively (Tables 6 and 7). The reference method depends on an HPLC measurement using a mobile phase consisting of 0.02 M ammonium acetate in acetonitrile: methanol:water (50:10:40) solution and the pH was adjusted to 4.5 with acetic acid.

Stability study

Degradation of NTP was attained upon induced alkaline degradation using sodium hydroxide (0.5 M). Upon alkaline degradation of NTP, the fluorescence readings decreased gradually with time, thus indicating that the proposed method is a stability indicating one. The induced alkaline degradation of NTP followed first order kinetics (Fig. 10) with a rate constant K=0.011 min^{-1}, and $t_{½}$= 0.693/K and was found to be 64.9 min.

Complete alkaline degradation was indicated from the complete disappearance of the fluorescence spectrum of the drug. It was confirmed by performing a TLC scanning technique using a mobile phase consisting of chloroform: methanol: acetonitril (2:3:1) with UV detection, where the R_f values were found to be 0.75 and 0.43 for NTP and its alkaline degradation products respectively.

Complete alkaline degradation was attained after boiling with 2 M sodium hydroxide for 2 h. The results of statistical analysis of NTP intact drug in the presence of its degradation products is shown in Table 8. It is clear that

Table 8 Application of the proposed SFS method for the determination of NTP in the presence of its alkaline degradation product

Parameter	NTP (μg/ml)	Amount taken of degradation product (μg/ml)	Found (μg/ml)	Found %
	3.0	0.25	3.01	100.33
		0.50	2.98	99.33
		0.75	2.97	99.0
		1.0	3.02	100.67
		1.5	3.03	101.0
$\bar{X}$±SD				100.07±0.86
t-test				0.87 (2.78)
F-test				2.84 (6.94)
	7.0	1.0	7.02	100.29
		1.5	6.98	99.71
		2.0	7.04	100.57
		2.5	6.96	99.43
		3.0	6.94	99.14
$\bar{X}$±SD				99.83±0.59
t-test				0.55(2.78)
F-test				1.34(6.94)

Figures between parentheses are the tabulated t and F values respectively at P=0.05 [59]

the degradation products didn't interfere with the assay of the intact drug.

Upon exposure of FLZ and NTP to Deuterium lamp with a wavelength of 254 nm at a distance of 15 cm in a wooden cabinet for different time intervals (10 min interval up to 180 min), and then the fluorescence intensity was measured. It was found that only 15% and 23% of FLZ and NTP were decomposed respectively.

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