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Original Article

# SYNCHRONOUS FLUORESCENCE SPECTROSCOPY COUPLED WITH CONTINUOUS WAVELET TRANSFORMS AND SAVITZKY-GOLAY DERIVATIZATION TECHNIQUE FOR THE SIMULTANEOUS DETERMINATION OF TADALAFIL AND DAPOXETINE HCl

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**ABSTRACT**

**Objective:** A novel combination of Tadalafil (TAD) and Dapoxetine HCl (DAP) has been recently introduced into the market for the treatment of premature ejaculation. The aim of this work is the development and validation of simple, sensitive and accurate analytical methods for the determination of TAD and DAP in their binary mixture without prior separation.

**Methods:** Synchronous fluorescence spectroscopic (SFS) methods coupled with continuous wavelet transforms (CWT) and Savitzky-Golay (SAVGOL) derivatization technique have been developed.

**Results:** Under optimum conditions, TAD and DAP were determined in the concentration ranges of 0.01–3 µg/ml and 0.01–1.2 µg/ml, respectively.

**Conclusion:** The developed methods have the requisite accuracy, selectivity, sensitivity and precision and were satisfactorily applied for the simultaneous determination of TAD and DAP in bulk powder and pharmaceutical preparations. The results obtained for the analysis of both drugs in their pure forms by the proposed methods were statistically compared to those obtained by applying a reported high performance liquid chromatographic method (HPLC) method. The statistical comparison showed that there is no significant difference between the proposed methods and the reported one with respect to accuracy and precision.

**Keywords**: Synchronous fluorescence spectroscopy, Tadalafil, Dapoxetine HCl, Continuous wavelet transforms, Savitzky-Golay technique



## INTRODUCTION

Fluorescence Spectroscopy provides a major role in analysis owing to its high sensitivity and selectivity compared to UV-spectroscopy.

Conventional fluorescence methods have limited practical applicability as most spectra of complex mixtures often cannot be resolved satisfactorily without previous separation. However, this can be overcome by using special techniques such as SFS [1].

In SFS, both the excitation and emission monochromators are scanned simultaneously. The main characteristics of SFS are narrowing of the spectral band [2], simplification of emission spectra by the choice of suitable Δ λ [3] and contraction of spectral range [4]. This result in sharp and narrow peaks compared to those of conventional spectrum.

The combination of synchronous and derivative fluorimetry enhances minor spectral features and allows more reliable identification of chemical species.

Wavelet transform (WT) was proposed for the approximate derivative calculation. It has a major advantage of enhancing the signal to noise ratio for calculations of the higher order derivatives in contrary to the derivative technique which has a major drawback in increasing the noise level in calculations of the higher order derivative [5]. WT contains two distinct parts, discrete and continuous which were developed independently in several fields [6-8].

WT methods have been successfully used for the resolution of overlapped spectra for the quantitative determination of multi-component mixtures by means of spectrophotometric methods [9-11].

An SAVGOL filter is a digital filter that can be applied to a set of digital data points for the purpose of smoothing the data, that is, to increase the signal-to-noise ratio without distorting the signal. The method is based on established mathematical procedures. It was first developed by Savitzky and Golay [12] who published tables of convolution coefficients for various polynomials and subset sizes [13]. Some errors in the tables have been corrected [14, 15]. The method has been extended for the treatment of 2-and 3-dimensional data.

Tadalafil [fig. 1], is (6R, 12aS)-6-(1,3-benzodioxol-5-yl)-2,3,6, 7,12,12a-hexahydro-2-methylpyrazino[1',2':1,6] pyrido [3,4-b] indole-1,4-dione [16]. It is a selective, long-acting $PDE_5$ inhibitor [17, 18]. Tadalafil has shown to be safe and effective in the treatment of erectile dysfunction across a variety of clinical populations [19-22].

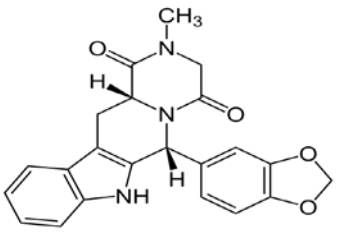


**Fig. 1: Chemical structure of Tadalafil**

Dapoxetine HCl (fig. 2), is a short-acting selective serotonin reuptake inhibitor (SSRI) developed for the treatment of premature ejaculation [23, 24]. Dapoxetine has a unique pharmacokinetic profile compared to other SSRIs as it is rapidly absorbed and eliminated after oral administration [25-28]. Dapoxetine doesn't have pharmacokinetic interactions with PDE5 inhibitors which allow its combination with several PDE5 inhibitors for the treatment of premature ejaculation [29].

A survey of the literature showed that very few methods have been reported for the simultaneous determination of TAD and DAP in bulk powder and combined dosage form. The reported methods include HPLC coupled with UV detection [30, 31] and a single spectrophotometric method [32]. Both drugs were reported to be fluorescent [33-35], thus they could be determined by fluorescence detection.

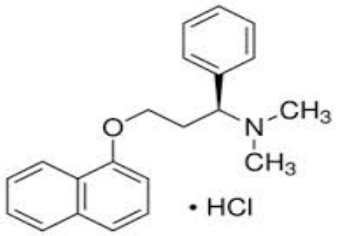


**Fig. 2: Chemical structure of Dapoxetine HCl**

The aim of this work is to develop two simple, sensitive and selective spectrofluorimetric methods based on the coupling of SFS with CWT and SAVGOL derivatization technique for the simultaneous determination of TAD and DAP in bulk powder and pharmaceutical formulation without prior separation.

## MATERIALS AND METHODS

### Chemicals and reagents

TAD and DAP standard materials were kindly supplied by El Andalus Medical company, 6th of October City, Giza-Egypt. They were found to contain 99.7±0.924 % and 101.07±0.539 %, respectively according to a reported HPLC method [30].

Super Tadarise® tablets (Batch No. ST 1514) labeled to contain 20 mg TAD and 60 mg DAP per tablet were manufactured by Sunrise Remedies Pvt. Ltd., India.

HPLC grade Methanol was Sigma–Aldrich, Germany.

### Instruments and software

Fluorescence spectra were recorded using a Shimadzu (model: RF 5301 PC, Japan) spectrofluorometer, equipped with a 150-watt Xenon lamp. Slit widths for both the excitation and emission monochromators were set at 5 nm. A 1 cm quartz cell was used. The computations were done using the Matlab® 7.0.1 software.

### Spectral characteristics

The emission spectra of 0.1 µg/ml of each of TAD and DAP in methanol were scanned over the range of 240–500 nm as shown in [fig. 3].

### Solutions and calibrations

Different aliquots of TAD and DAP standard solutions (each, 100 µg/ml) were transferred into two sets of 10-mL volumetric flasks and diluted to volume with methanol to give final concentrations equivalent to 0.01–3 µg/ml and 0.01–1.2 µg/ml, respectively. The prepared solutions were scanned for their synchronous fluorescence spectra using Δ λ of 20 nm. The spectra were transferred to Matlab® 7.0.1 for signal processing and analysis.

### Continuous wavelet transforms (CWT)

The synchronous spectra of TAD and DAP were divided by 0.1 µg/ml of DAP and TAD, respectively. The first coefficients of CWT of the ratio spectra were then calculated.

The amplitude values were then plotted versus the corresponding concentrations at wavelengths of 302 nm and 342 nm for TAD and DAP, respectively. The regression equations were computed.

### Savitzky-Golay (SAVGOL) method

The first derivative of the synchronous spectra was calculated by SAVGOL technique, and the amplitude values were then plotted versus the corresponding concentrations at wavelengths of 330 nm and 312 nm for TAD and DAP, respectively. The regression equations were then computed.

### Analysis of laboratory prepared mixtures

Five laboratory prepared mixtures of TAD and DAP were prepared by transferring suitable aliquots of TAD and DAP from their stock solutions (each, 100 µg/ml) into 10-mL volumetric flasks and were completed to volume with methanol. The synchronous fluorescence spectra of the prepared mixtures were scanned from 240–500 nm using Δ λ = 20 and transferred to Matlab® for signal processing and analysis.

### Continuous wavelet transforms (CWT)

The stored synchronous spectra of the laboratory prepared mixtures were divided by the synchronous spectra of 0.1 µg/ml of each of standard TAD and DAP then first coefficients of CWT of the obtained ratio spectra were calculated. The amplitude values of the obtained spectra were recorded at 302 nm and 342 nm for TAD and DAP, respectively. The concentrations of the drugs were calculated from the corresponding computed regression equations.

### Savitzky-Golay (SAVGOL) method

The first derivative of the stored synchronous spectra of the laboratory prepared mixtures was calculated by SAVGOL method. The amplitude values of the obtained spectra were recorded at 330 nm and 312 nm for TAD and DAP, respectively. The concentrations of both drugs were calculated from the corresponding computed regression equations.

### Application to pharmaceutical formulation

Ten tablets of Super-Tadarise® [labeled to contain 20 mg TAD and 60 mg DAP] were accurately weighed and finely powdered. An accurate weight of powder equivalent to 10 mg TAD and 30 mg DAP was transferred into a 100-mL volumetric flask, sonicated with 50 mL methanol for 15 min and diluted to volume with the same solvent. The solution (100 µg/ml TAD and 300 µg/ml DAP) was sonicated again for 10 min and filtered. An aliquot (0.01 mL) was transferred to a 10-mL volumetric flask and completed to volume with methanol to give final concentration equivalent to 0.1 µg/ml TAD and 0.3 µg/ml DAP.

The proposed methods were applied for the analysis of the studied drugs in their pharmaceutical formulation using the procedures mentioned under the analysis of laboratory prepared mixtures for each method and the concentrations of the cited drugs were calculated from the corresponding regression equations.

## RESULTS AND DISCUSSION

By reviewing the literature, it was found that no spectrofluorimetric methods have been developed for the simultaneous determination of TAD and DAP in their binary mixture without prior separation which adds novelty to the proposed methods. Moreover, the developed methods offer higher sensitivity than the reported spectrophotometric methods [32].

In this work, two spectrofluorimetric methods coupled with CWT and SAVGOL derivatization technique were developed for the simultaneous determination of TAD and DAP in their bulk powders and pharmaceutical dosage forms.

The developed methods were simple, sensitive and selective with good precision and accuracy. They were successfully applied for the determination of TAD and DAP in their bulk powders and pharmaceutical formulations.

TAD was found to exhibit native fluorescence at λem of 331 nm, after excitation at 227 nm, and DAP also showed native fluorescence at λem of 339 nm after excitation at 227 nm. The emission spectra of both drugs show severe overlap, [fig. 3].

Synchronous fluorescence spectra using Δ λ 0f 20 nm were recorded for both drugs, [fig. 4]. The synchronous spectra of TAD and DAP resulted in a better resolution and more featured spectra than the conventional ones, but the spectra of the two drugs are still overlapped which hinders their direct determination. Different experimental and instrumental parameters were found to affect the shape and intensity of the synchronous spectra. These parameters were studied and-and optimized using 0.1 µg/ml TAD and DAP. They include the diluting solvent, Δ λ, response time, excitation and emission bandwidths and the instrument sensitivity.

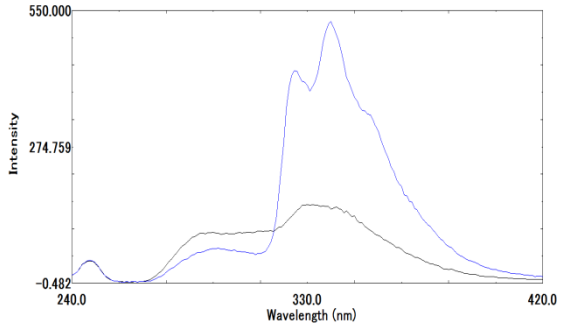


**Fig. 3: Emission spectra of 0.1 µg/ml of each of Tadalafil and Dapoxetine HCl in methanol**

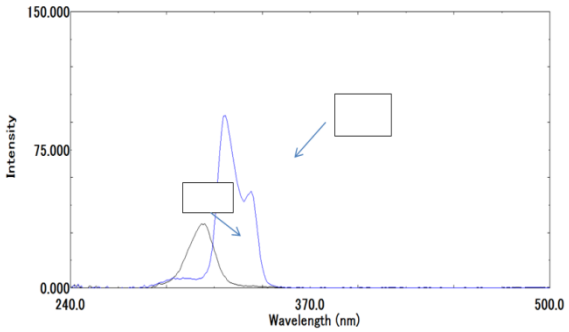


**Fig. 4: Synchronous fluorescence spectra of 0.1 µg/ml of each of Tadalafil and Dapoxetine HCl**

Dilutions with different organic and inorganic solvents were tried including methanol, ethanol, acetonitrile, water, 0.1 N HCl and 0.1 M sulfuric acid.

Organic solvents showed higher fluorescence intensities than inorganic solvents. Methanol was selected for dilution as it showed higher fluorescence intensity and better linearity compared to other organic solvents.

Different excitation bandwidths were tried (5, 10 and 20 nm) while keeping the emission bandwidth constant and vice versa. Both the excitation and emission bandwidths were fixed at 5 nm, where they provide reasonable intensities.

The choice of the optimum Δ λ value is a critical factor for performing SFS. It can directly affect the spectral shape, bandwidth, and signal value. Different Δ λ values were examined (20–100 nm). Δ λ of 20 nm was chosen as it resulted in the narrower spectral band, better resolution and higher intensity of spectral features.

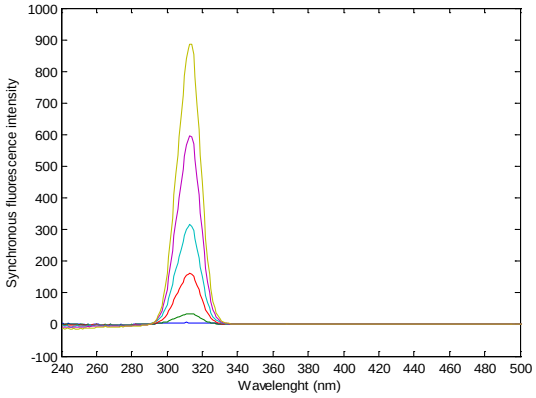


**Fig. 5: Synchronous fluorescence spectra of Tadalafil (0.03 µg/ml–3 µg/ml) in methanol**

The instrument was adjusted at auto response time. High sensitivity was chosen to increase the fluorescence intensity.

Aliquots of TAD and DAP standard solutions were diluted with methanol to give final concentrations of 0.01–3 µg/ml and 0.01–1.2 µg/ml of TAD and DAP, respectively. They were then scanned for their synchronous fluorescence spectra using Δ λ of 20 nm, [fig. 5 and 6].

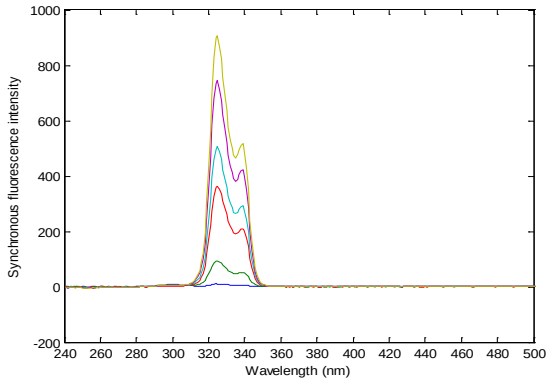


**Fig. 6: Synchronous fluorescence spectra of Dapoxetine HCl (0.01 µg/ml–1.2 µg/ml) in methanol**

**Continuous wavelet transforms (CWT)**

The synchronous spectra of TAD and DAP were divided by 0.1 µg/ml of each of DAP and TAD, respectively, and the ratio spectra were obtained, as shown in [fig. 7 and 8].

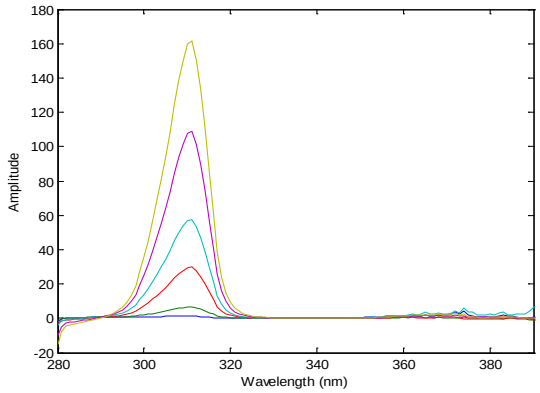


**Fig. 7: Ratio spectra of Tadalafil (0.01–3 µg/ml) in methanol using standard spectrum of 0.1 µg/ml of Dapoxetine HCl as a divisor**

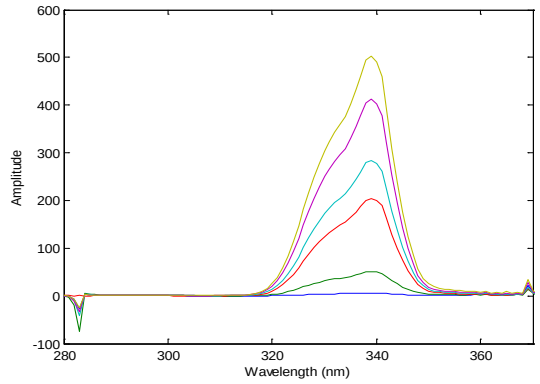


**Fig. 8: Ratio spectra of Dapoxetine HCl (0.01–1.2 µg/ml) in methanol using standard spectrum of 0.1 µg/ml of Tadalafil as a divisor**

First coefficients of CWT of ratio spectra were calculated using a scale of 25, [fig. 9 and 10]. TAD showed two peak maxima at 302 nm and 317 nm, also DAP showed two peak maxima at 328 nm and 345 nm. TAD and DAP were determined at 302 nm and 328 nm, respectively, owing to the better correlation coefficients obtained on calibration.

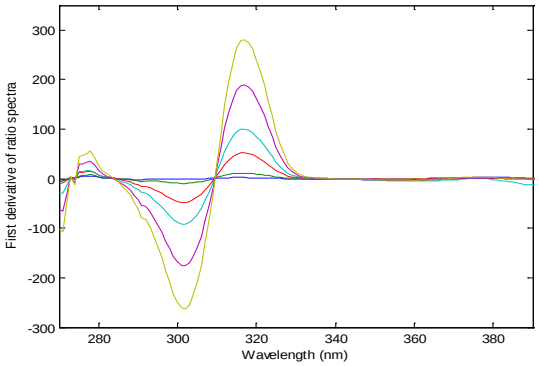


**Fig. 9: First coefficients of CWT of ratio spectra of Tadalafil (0.03–3 µg/ml) in methanol using standard spectrum of 0.1 µg/ml of Dapoxetine HCl as a divisor**

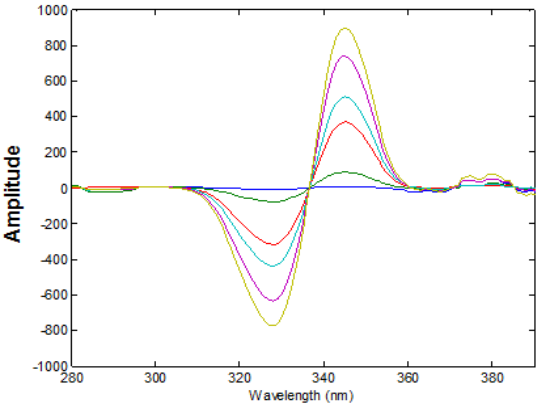


**Fig. 10: First coefficients of CWT of ratio spectra of Dapoxetine HCl (0.01–1.2 µg/ml) in methanol using standard spectrum of 0.1 µg/ml of Tadalafil as a divisor**

The concentrations of TAD and DAP in the mixtures could be obtained from the corresponding regression equations (obtained by plotting the amplitude values at 302 nm and 328 nm for TAD and DAP, respectively against the corresponding concentrations).

**Savitzky-Golay (SAVGOL) method**

First derivative of the synchronous fluorescence spectra of TAD and DAP were calculated by SAVGOL technique using a width of 50, [fig. **11]**. TAD was determined at 330 nm and DAP were determined at 312 nm which corresponds to the zero crossing points of DAP and TAD, respectively.

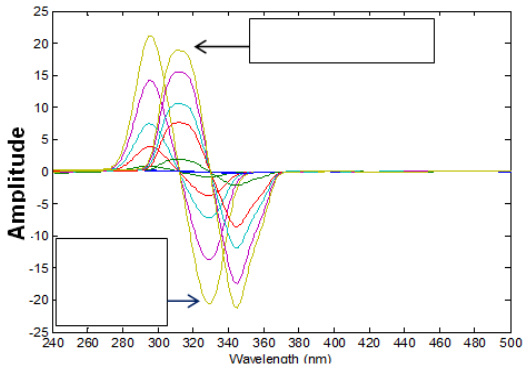


**Fig. 11: First derivative spectra of Tadalafil (0.01–3 µg/ml) and Dapoxetine HCl (0.01–1.2 µg/ml) in methanol calculated by SAVGOL function**

The concentrations of TAD and DAP in the mixtures could be obtained from the corresponding regression equations (obtained by plotting the amplitude values at 330 nm and 312 nm for TAD and DAP, respectively against the corresponding concentrations).

**Method validation**

Validation was performed according to ICH guidelines [36].

**Linearity and range**

The linearity of the proposed methods was assessed by analyzing six concentrations of TAD and DAP ranging from 0.01–3 µg/ml and 0.01–1.2 µg/ml, respectively. The assay was performed according to the previously mentioned experimental conditions, and the linear equations are summarized in [table 1].

**Table 1: Regression and validation parameters for the determination of Tadalafil and DapoxetineHCl in their pure forms by the proposed methods**

| Parameter | Tadalafil | | Dapoxetine HCl | |
|---|---|---|---|---|
| | CWT at 302 nm | SAVGOL at 330 nm | CWT at 328 nm | SAVGOL at 312 nm |
| Range (µg/ml) | 0.01–3 | 0.01–3 | 0.01–1.2 | 0.01–1.2 |
| Slope | 87.192 | 6.7809 | 632.78 | 15.7624 |
| Intercept | 2.0458 | 0.2098 | 5.2213 | -0.2330 |
| *SE of the slope | 0.8089 | 0.0600 | 8.077 | 0.2017 |
| SE of the intercept | 1.2470 | 0.0925 | 5.890 | 0.1609 |
| Correlation coefficient (r) | 0.9998 | 0.9998 | 0.9997 | 0.9997 |
| *LOD (µg/ml) | 0.024 | 0.0102 | 0.008 | 0.009 |
| *LOQ (µg/ml) | 0.073 | 0.0310 | 0.023 | 0.027 |
| **Accuracy (mean±SD) | 98.98±1.826 | 100.34±1.699 | 99.90±1.047 | 99.80±1.154 |
| **Specificity (mean±SD) | 100.68±1.898 | 99.78±1.670 | 99.29±1.551 | 100.68±1.413 |
| Precision (RSD%) | | | | |
| Repeatability | 1.176 | 1.062 | 1.489 | 1.277 |
| Intermediate precision | 1.680 | 0.874 | 0.529 | 0.489 |

*SE (Standard error)., **Average of five determinations., *LOD(limit of detection)., *LOQ(limit of quantitation).

**Accuracy**

The accuracy of the results was checked by applying the proposed methods for determination of different individual samples of pure TAD and DAP. The concentrations were obtained from the corresponding regression equations. The percentage recoveries, mean recoveries, and relative standard deviations were calculated and showed good accuracy for the proposed methods. [table 1].

**Selectivity**

Selectivity of the proposed methods was assessed by the analysis of different laboratory prepared mixtures of TAD and DAP within the linearity range. Satisfactory results were obtained as shown in [table 2].

**Precision**

**Repeatability and intermediate precision**

Freshly prepared solutions of concentrations 0.7, 2 and 3 µg/ml of TAD and 0.5, 0.7 and 0.9 µg/ml of DAP were assayed in triplicates within the same day and inter-daily on three successive days by the proposed methods. The relative standard deviations were then calculated, [table 1].

**Application to pharmaceutical preparation**

The proposed methods were applied for the determination of TAD and DAP in their combined pharmaceutical formulation, Super Tadarise® tablets. The accuracy of the method was further assessed by applying the standard addition technique, and the results are shown in [tables 3 and 4].

The results obtained for the analysis of both drugs in their pure forms by the proposed methods were statistically compared to those obtained by applying a reported HPLC method [30]. The values of the calculated t and F values are less than the tabulated ones which reveal that there is no significant difference between the proposed methods and the reported one with respect to accuracy and precision, [table 5].

**Table 2**: **Results of determination of Tadalafil and Dapoxetine HCl in laboratory prepared mixtures by the proposed methods**

| **Tadalafil** | | | | | | **Dapoxetine HCl** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **CWT** | | | **Savgol** | | | **CWT** | | | **SAVGOL** | | |
| **Taken (µg/ml)** | ***Found (µg/ml)** | **Recovery %** | **Taken (µg/ml)** | ***Found (µg/ml)** | **Recovery %** | **Taken (µg/ml)** | ***Found (µg/ml)** | **Recovery %** | **Taken (µg/ml)** | ***Found (µg/ml)** | **Recovery %** |
| 0.200 | 0.205 | 102.50 | 0.200 | 0.201 | 100.50 | 0.600 | 0.581 | 96.83 | 0.600 | 0.592 | 98.67 |
| 0.700 | 0.689 | 98.43 | 0.700 | 0.681 | 97.29 | 1.200 | 1.191 | 99.25 | 0.700 | 0.709 | 101.29 |
| 0.500 | 0.513 | 102.60 | 0.500 | 0.508 | 101.60 | 0.700 | 0.702 | 100.29 | 0.900 | 0.917 | 101.89 |
| 1.000 | 1.007 | 100.70 | 1.000 | 1.005 | 100.50 | 0.900 | 0.908 | 100.89 | 0.500 | 0.509 | 101.80 |
| 2.000 | 1.983 | 99.15 | 2.000 | 1.980 | 99.00 | 0.500 | 0.496 | 99.20 | 1.200 | 1.197 | 99.75 |
| Mean | | 100.68 | Mean | | 99.78 | Mean | | 99.29 | Mean | | 100.68 |
| RSD% | | 1.885 | RSD% | | 1.674 | RSD% | | 1.562 | RSD% | | 1.404 |

*Average of three determinations

**Table 3**: **Results obtained by applying the proposed CWT method for the determination of Tadalafil and DapoxetineHCl in Super-Tadarise® tablets and application of standard addition technique**

| **Tadalafil** | | | | | | **Dapoxetine HCl** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Super-Tadarise® tablets (Batch No. ST1514)** | | | **Standard addition** | | | **Super-Tadarise® tablets (Batch No. ST1514)** | | | **Standard addition** | | |
| **Taken (µg/ml)** | ***Found (µg/ml)** | **Recovery%** | **Added (µg/ml)** | ***Found (µg/ml)** | **Recovery%** | **Taken (µg/ml)** | ***Found (µg/ml)** | **Recovery%** | **Added (µg/ml)** | ***Found (µg/ml)** | **Recovery%** |
| 0.100 | 0.100 | 100.00 | 0.080 | 0.079 | 98.75 | 0.300 | 0.303 | 101.00 | 0.100 | 0.098 | 98.00 |
| | 0.099 | 99.00 | 0.100 | 0.101 | 101.00 | | 0.305 | 101.67 | 0.300 | 0.299 | 99.67 |
| | 0.098 | 98.00 | 0.12 | 0.117 | 97.50 | | 0.304 | 101.33 | 0.500 | 0.499 | 99.80 |
| Mean | | 99.00 | Mean | | 99.08 | Mean | | 101.33 | Mean | | 99.16 |
| RSD% | | 1.010 | RSD% | | 1.790 | RSD% | | 0.331 | RSD% | | 1.012 |

*Average of three determinations

**Table 4: Results obtained by applying the proposed SAVGOL method for the determination of Tadalafil and Dapoxetine HCl in Super-Tadarise® tablets and application of standard addition technique**

| **Tadalafil** | | | | | | **Dapoxetine HCl** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Super-Tadarise® tablets (Batch No. ST1514)** | | | **Standard addition** | | | **Super-Tadarise® tablets (Batch No. ST1514)** | | | **Standard addition** | | |
| **Taken (µg/ml)** | ***Found (µg/ml)** | **Recovery%** | **Added (µg/ml)** | ***Found (µg/ml)** | **Recovery%** | **Taken (µg/ml)** | ***Found (µg/ml)** | **Recovery%** | **Added (µg/ml)** | ***Found (µg/ml)** | **Recovery%** |
| 0.100 | 0.101 | 101.00 | 0.080 | 0.082 | 102.50 | 0.300 | 0.305 | 101.67 | 0.100 | 0.102 | 102.00 |
| | 0.099 | 99.00 | 0.100 | 0.103 | 103.00 | | 0.305 | 101.67 | 0.300 | 0.300 | 100.00 |
| | 0.096 | 96.00 | 0.12 | 0.122 | 101.67 | | 0.307 | 102.33 | 0.500 | 0.495 | 99.00 |
| Mean | | 98.67 | Mean | | 102.39 | Mean | | 101.89 | Mean | | 100.33 |
| RSD% | | 2.551 | RSD% | | 0.656 | RSD% | | 0.374 | RSD% | | 1.522 |

*Average of three determinations.

**CONCLUSION**

Synchronous fluorescence spectroscopy is more advantageous than conventional spectrofluorimetry due to narrowing of the spectral band and simplification of emission spectra which results in sharper and narrower peaks. The developed spectrofluorimetric methods offer high sensitivity and selectivity for the simultaneous determination of TAD and DAP in their binary mixture. Both CWT and SAVGOL techniques have the advantage of combining smoothing and differentiation in a single operation. They enhance the signal to noise ratio without deterioration of the signal compared to a derivative calculation by numerical differentiation. The proposed methods are simple precise and don't need any sophisticated apparatus. They could be applied in quality control laboratories for the routine analysis of the studied drugs either in their bulk powders or in pharmaceutical dosage forms without preliminary separation.

**Table 5**: **Statistical comparison of the results obtained by the proposed methods and the reported HPLC method for the determination of Tadalafil and DapoxetineHCl in their pure forms**

| Parameter | Tadalafil | | | Dapoxetine HCl | | |
|---|---|---|---|---|---|---|
| | CWT method | Savgol method | **Reported method | CWT method | SAVGOL method | **Reported method |
| Mean | 98.98 | 100.34 | 99.70 | 99.89 | 99.80 | 99.56 |
| SD | 1.854 | 1.699 | 0.921 | 1.048 | 1.153 | 0.917 |
| n | 5 | 5 | 5 | 5 | 5 | 5 |
| Variance | 3.438 | 2.887 | 0.848 | 1.098 | 1.330 | 0.842 |
| t-test* | 1.223(2.306)* | 0.740(2.447)* | | 0.547(2.306)* | 0.373(2.306)* | |
| F* | 1.100(6.388)* | 3.406(6.388)* | | 1.287(6.388)* | 1.580(6.388)* | |

*The fig. in parenthesis are the corresponding theoretical values at p=0.05., **HPLC method (C-18, using methanol: acetonitrile: triethylamine in the ratio of 95: 5: 0.5 v/v/v at a flow rate of 1 mL/min and detection at 290 nm).

**CONFLICT OF INTERESTS**

Declared none

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