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Development and validation of second derivative and synchronous spectrofluorimetric methods for determination of oxytocin and ergometrine maleate in their combined formulation

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RESEARCH ARTICLE

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ABSTRACT

Simple and sensitive second derivative and synchronous spectrofluorimetric methods have been developed and validated for the quantitative determination of oxytocin and ergometrine maleate in their pure and combined dosage forms. The methods are based on the derivatization reaction of oxytocin with fluorescamine reagent, which yielded a highly fluorescent compound measured at 486 nm after excitation at 390 nm. Ergometrine was directly measured in combination with oxytocin, since it exhibits native fluorescence at 421 nm after excitation at 300 nm. Quantitation of oxytocin in presence of ergometrine was also successful at 482 and 477 nm using second derivative and synchronous spectrofluorimetry, respectively. Different experimental parameters were studied and optimized. The relative fluorescence intensity versus concentration plot was rectilinear over the range of 0.04-0.75 and 5-100 ng/mL for oxytocin and ergometrine, respectively. The methods were successfully applied for the determination of both drugs in their prepared combined ampoules. The methods were validated and compared with the reference chromatographic method; they revealed good accuracy and reproducible results. The proposed methods showed high accuracy and sensitivity, with no requirement of multiple steps or previous chemical separation.

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1. Introduction

Oxytocin (OXY) is a cyclic nonapeptide that is composed of five amino acids; cysteine, tyrosine, isoleucine, glutamine and asparagine, and the side chain contains further three amino acids; proline, leucine and glycinamide [1]. OXY causes contraction of the uterus, the effect increasing with the duration of pregnancy due to proliferation of Oxy receptors. It also stimulates the smooth muscle associated with the secretory epithelium of the lactating breast causing the ejection of milk, but having no direct effect on milk secretion. It also has a weak antidiuretic action [2].

Ergometrine (ER) is a drug that is also a smooth muscle constrictor that mostly acts on the uterus. It has more powerful action on the uterus than most other ergot alkaloids. It is used in the active management of the third stage of labor, and to prevent or treat postpartum or post portal hemorrhage; by maintaining uterine contraction and tone, blood vessels in the uterine wall are compressed, and blood flow reduced [2].

OXY and ER are sometimes found in combined ampoules (Syntometrine®), which has been shown to be more effective at preventing postpartum hemorrhage than using either alone. The dosage consists of 5 IU OXY (equivalent to 8.33 µg/mL [3]), and 500 µg ergometrine maleate in a single 1 mL ampoule. They are official also in British Pharmacopeia [4].

Very few analytical techniques appeared in the literature for determination of such mixture, all utilizing the chromatographic methods [4-6]. No spectrofluorimetric methods appeared in the literature for determination of this combination. Therefore, our aim is to introduce an easy and highly sensitive method for determination of both drugs. The method is more rapid and simpler than the reported methods with no need for tedious steps or prior separation.

Derivatization is the process by which a compound is chemically changed, producing a new one that has suitable properties to be determined by a specific analytical method. Some drugs analyzed by fluorimetry required derivatization reaction in order to produce highly fluorescent products. In addition, derivatization can improve separation between compounds and overlapping peaks [7-9].

2. Experimental

2.1. Apparatus

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All fluorescence spectra and measurements were carried out using a JASCO FP-6200 Spectrofluorometer, equipped with 150 W Xenon lamp, grating excitation and emission monochromators, and a recorder. Slit widths for both monochromators were set at 10 nm. A 1 cm compartment quartz cell was used. Spectra were evaluated using Spectra Manager FP-6200 Control Driver software, Version 1.54.03 [Build 1], JASCO Corporation. A Hanna pH-meter (Romania) equipped with a glass-calomel electrode combination was used for adjustments of pH.

2.2. Materials and chemicals

All chemicals and reagents used were of analytical reagent grade, and the solvents were of HPLC grade. Double distilled water was used throughout the study. Fluorescamine was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Solutions containing 0.025% and 1% (*w*:*v*) were freshly prepared in acetone HPLC grade, which obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Aqueous phosphate buffer solution 0.05 M, pH = 8.5 was prepared using potassium dihydrogen phosphate and the pH was adjusted by using 0.5 M sodium hydroxide (Merck, Darmstadt, Germany). Sodium chloride (NaCl) purchased from (Merck, Darmstadt, Germany). Oxytocin, pure sample (99.8%) was kindly provided by Mina Pharm, Egypt. (B.N: L00036402). Ergometrine maleate, pure sample (99.7%) was kindly provided by Teva Czech Industries (B.N: 72611000212). Pharmaceutical preparation, Syntometrine® ampoules (prepared): 5 IU OXY + 500 µg ER/1 mL.

2.3. Standard solutions

OXY standard solutions: A stock solution of concentration 833 µg/mL OXY was prepared in double distilled water. A working solution of concentration 8.33 µg/mL was obtained by further dilution of the stock solution.

ER standard solutions: A stock solution of concentration, 100 µg/mL ER, was prepared in double distilled water. A working solution of concentration 1 µg/mL was also prepared by further dilution of the previous solution.

Laboratory mixture: A stock solution containing 500 µg/mL ER and 8.33 µg/mL OXY was prepared by mixing 25.0 mg of pure ER with 0.5 mL of OXY stock solution (equivalent to 416.5 µg OXY). The solution was mixed well, dissolved in double distilled water and the volume was completed to 50 mL using the same solvent.

2.4. General analytical procedure

2.4.1. Construction of the calibration graph for ER

Different volumes were accurately transferred from ER working solution using a micropipette, into a series of 10 mL volumetric flasks and diluted to the mark using double distilled water to cover the final concentration range of 5-100 ng/mL. The resulting solutions were mixed well, a blank experiment was performed simultaneously and solutions were measured at λ_{em} 421 nm after excitation at λ_{ex} 300 nm.

2.4.2. Construction of the calibration graph for OXY

Aliquots of OXY standard working solution were accurately transferred into a series of 10 mL volumetric flasks using a micropipette to cover the final concentration range of 0.04-0.75 µg/mL. To each flask 1 mL 0.05 M phosphate buffer (pH = 8.5) was added, followed by 0.8 mL of 0.025% (*w*:*v*) fluorescamine solution [1% *w*:*v* fluorescamine was used in case of SFS only] and mixed well. The solutions were mixed and completed to the mark using double distilled water.

Direct Spectrofluorimetry: After derivatization with fluorescamine (0.025% *w*:*v*); the fluorescence of the resulting solution was measured directly at λ_{em} 486 nm after excitation at λ_{ex} 380 nm using band width 10 nm. A blank experiment was carried out simultaneously.

Second derivative (D^2) Spectrofluorimetry: After derivatization with fluorescamine (0.025% *w*:*v*); D^2 fluorescence spectra of Oxy were obtained from spectra manager software. The relative fluorescence intensity (RFI) was measured at λ_{em} 482 nm.

Synchronous fluorescence spectroscopy (SFS): After derivatization with fluorescamine (1% *w*:*v*); Synchronous fluorescence (SF) spectra of the solutions were recorded by scanning both monochromators at a constant wavelength difference $\Delta\lambda$ = 100 nm and scan rate of 500 nm/min using 10 nm excitation and emission windows. The relative intensities of SF spectra were estimated at λ_{em} 477 nm. A blank experiment was performed simultaneously.

In all experiments the RFI were plotted against the final drug concentration to obtain the calibration graph. Alternatively, the corresponding regression equation was derived.

2.4.3. Construction of the calibration graph for OXY and ER in the laboratory mixture

A laboratory mixture was prepared. Aliquots from the standard mixture solution were transferred into a series of 10 mL volumetric flasks. The procedure for determination of OXY in the mixture was similarly applied as described in Section 2.4.2. ER could be determined in the mixture, by transferring 1 mL of the mixture solution into a 250 mL volumetric flask, and completing the volume to the mark using double distilled water. The procedure was then completed to determine ER similarly as described under Section 2.4.1.

2.5. Determination of the studied drugs in their pharmaceutical preparation

A solution containing 500 µg/mL ER, 8.33 µg/mL OXY and 7 mg/mL NaCl was prepared. This ratio between ER: OXY: NaCl was carefully chosen to match the same ratio between the drugs in the dosage form Syntometrine® ampoules (5 IU OXY + 500 µg ER + 7 mg NaCl)/mL. OXY and ER were determined in the combined ampoule following the same procedure described earlier.

3. Results and discussion

OXY is a cyclic nonapeptide with only one primary amino group. It has no native fluorescence, however, derivatization wiz fluorescamine in presence of 0.05 M phosphate buffer at pH = 8.5 resulted in the formation of a highly fluorescent compound that allowed the effective quantitation of OXY at λ_{ex} = 380 nm and λ_{em} = 486 nm.

ER exhibits native fluorescence at λ_{em} 421 nm after excitation at λ_{ex} 300 nm. ER can be directly determined in combinations with OXY as the latter does not interfere with ER readings. However, analysis of OXY in such mixture is quite challenging since ER peaks clearly overlaps those of OXY resulting in fake higher Oxy fluorescence intensities. Such interference is shown in Figure 1.

In order to solve this challenging problem for determination of OXY in the mixture, we introduced two successful methods; D^2 and SFS techniques, after derivatization with fluorescamine, which are described in detail in this part to allow its quantitative determination in presence of ER. Quantitation of OXY was applicable at λ_{em} 482 nm and 477 nm using D^2 and SFS methods, respectively, as shown in Figures 2 and 3.

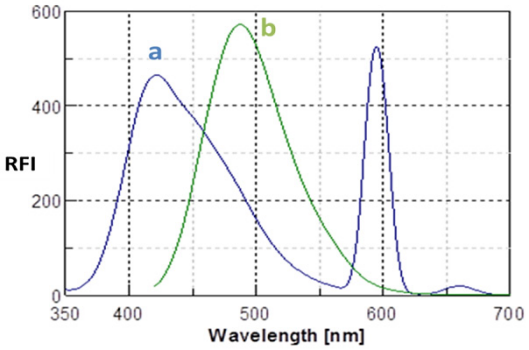

Figure 1. Spectra of (a) 40 ng/mL ER and (b) 0.42 µg/mL derivatized OXY peaks at λ_{max} 421 and 486 nm, respectively.

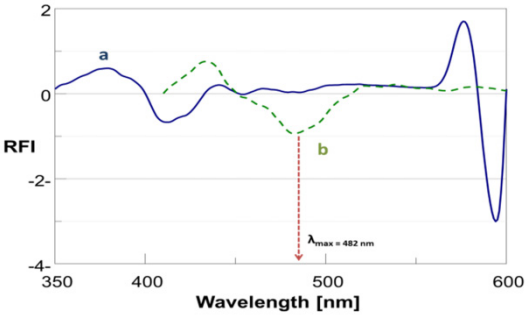

Figure 2. D^2 spectra of (a) 40 ng/mL ER and (b) 0.75 µg/mL OXY.

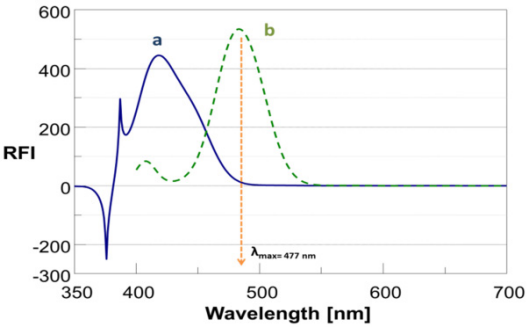

Figure 3. SF spectra of (a) 40 ng/mL ER and (b) 0.42 µg/mL OXY at Δλ = 100 nm and scan rate of 500 nm/min.

3.1. Optimization of the reaction conditions

The fluorescence characteristics of the reaction product between OXY and fluorescamine, as well as the different experimental parameters affecting its development and stability were carefully investigated and optimized. Each factor was changed individually while other factors were kept constant. These factors included: type and concentration of buffer, pH, concentration of reagent, reaction time and temperature.

3.1.1. Effect of pH

Reactions of amines with fluorescamine were found to be pH-dependent [10]. It was also found that fluorescence was developed only in alkaline medium and completely disappeared in acidic medium [10].

Therefore, the study of pH was extended over the range of 6.0-10.0 using 1 mL 0.05 M phosphate buffer and 0.8 mL 0.025% fluorescamine reagent. It was found that increasing the pH resulted in a corresponding increase in the RFI of the reaction product up to pH = 8.0, after which it remained nearly constant till pH = 9.5 and then slightly decreased again (Figure 4). Therefore, pH = 8.5 was chosen as the optimum pH for this study.

3.1.2. Effect of buffer type and concentration

The effect of buffer type was studied using the same concentration of different buffers. 0.05 M borate, phosphate and Britton-Robinson buffers were prepared and adjusted to pH = 8.5. The effect of buffer concentration was also investigated using 1 mL of 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1 M phosphate buffer, at pH = 8.5. Comparison of the obtained data revealed that 1 mL 0.05 M phosphate buffer showed the highest RFI results as shown in Figures 5 and 6, which was chosen for all further measurements.

3.1.3. Effect of fluorescamine concentration

Fluorescamine is insoluble in water. Alcoholic solutions also couldn't be used because they hinder the reagent activity. Acetonitrile and acetone gave nearly the same RFI of the reaction product using a constant volume of the drug.

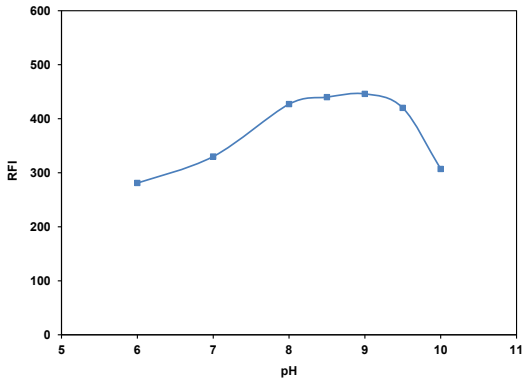

Figure 4. Effect of pH on the RFI of 0.33 µg/mL derivatized OXY product using 1 mL 0.05 M phosphate buffer and 0.8 mL fluorescamine, 0.025%.

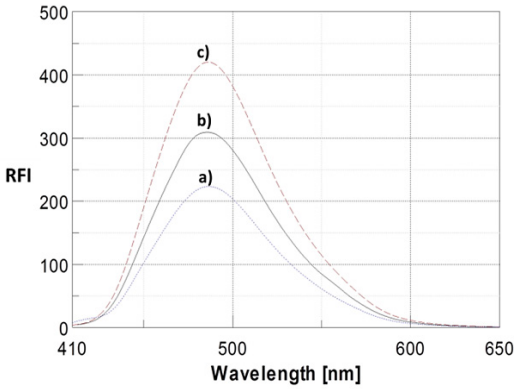

Figure 5. Effect of different types of buffers: 0.05 M (a) BR, (b) Borate, (c) Phosphate, on the RFI of 0.33 µg/mL derivatized OXY product with 0.025% fluorescamine at pH = 8.5.

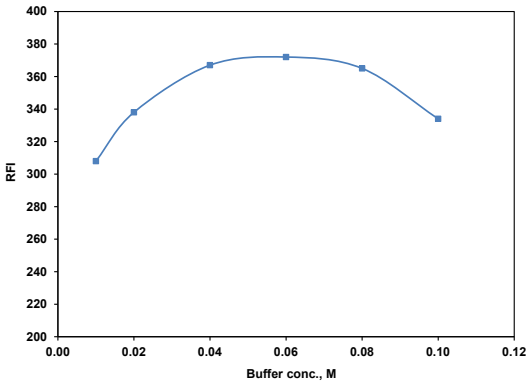

Figure 6. Effect of phosphate buffer concentration on the RFI 0.25 µg/mL derivatized OXY product with 0.025% fluorescamine at pH = 8.5.

However, acetonitrile resulted in higher readings of the blank; therefore, acetone was chosen as the most suitable solvent for fluorescamine.

The influence of the concentration of fluorescamine was studied using different volumes of 0.025% (*w*:*v*) of the reagent solution; from 0.2-1.0 mL. It was found that increasing the volume of fluorescamine resulted in a subsequent increase in RFI of the reaction product up to 0.6 mL, after which the RFI remained constant. Therefore, 0.8 mL of 0.025% fluorescamine solution was chosen as the optimal volume (Figure 7).

In case of SFS, the effect of fluorescamine reagent on ER was studied. It was found that increasing the concentration of fluorescamine reagent resulted in a quenching effect on the ER peak. This remarkable notice was in fact advantageous for our work because formulations containing OXY and ER mixtures contain relatively high concentrations of ER compared to that of OXY (60:1) which would result in a subsequent increase in ER fluorescence and peak broadening that won't allow Oxy detection.

Quenching effect of fluorescamine resulted in decrease of ER sensitivity which allowed good separation between ER and OXY. Therefore, 1% fluorescamine in acetone was used for determination of such mixture (Figure 8).

3.1.4. Effect of reaction time and temperature

Different time intervals ranging from zero time up to 2 hours were tested to ascertain the time after which the product attained its highest RFI. It was found that, the reaction product was formed immediately and remained stable for at least 2 hours with no increase in the RFI. Therefore, all measurements were performed instantaneously.

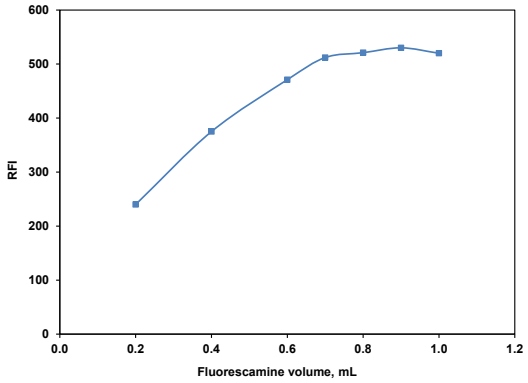

Figure 7. Effect of volume of 0.025% fluorescamine on the RFI of 0.42 µg/mL derivatized OXY product using 1 mL phosphate buffer, 0.05M, pH = 8.5.

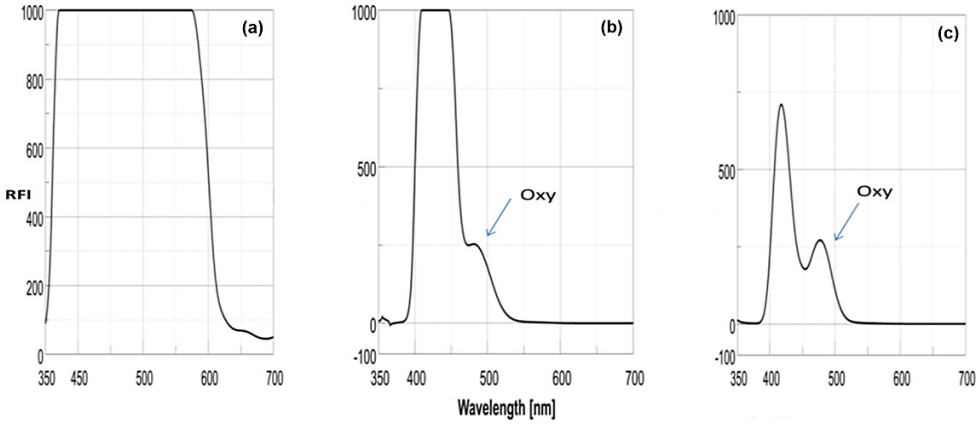

Figure 8. Effect of different fluorescamine concentrations (a) 0.025%, (b) 0.5%, (c) 1%, using a fixed concentration of OXY+ER mixture.

Increasing the reaction temperature higher than the room temperature resulted in an obvious subsequent decrease of the reaction product. This may be attributed to the instability and degradation of the reaction product at high temperatures [11]. Therefore, the reaction was carried out at room temperature.

3.1.5. Selection of the optimum Δλ (in case of SFS)

The optimum Δλ value is an essential factor for performing the SF scanning technique with regards to its resolution, sensitivity and features. It can directly influence spectral shape, band width and signal value. For this reason different Δλ ranging from 20 to 120 nm were examined. Δλ = 100 nm was chosen as the optimal for separation of Oxy and ER mixtures. At this Δλ, two distinct peaks with good regular shapes were obtained. It also eliminated the spectral interference caused by each compound in the mixture and gave the highest sensitivity.

3.2. Stoichiometry and mechanism of the reaction of OXY with fluorescamine

The stoichiometry of the reaction between OXY and fluorescamine was studied adopting the limiting logarithmic method [10]. The RFI of the product was alternatively measured in the presence of excess of either fluorescamine or OXY. A plot of log RFI *vs* log [OXY] and log [fluorescamine] gave straight lines, the values of the slopes were 0.67 and 0.58, respectively (Figure 9). Hence, it was concluded that, the molar reactivity of the reaction is 0.67/0.58 which is equal to 1.15 and so the reaction proceeds in a ratio of 1:1. Based on the observed molar ratio, and depending on the presence of one primary amino group and by analogy to previous similar reports [12-14], the reaction pathway is postulated to proceed as shown in Figure 10.

3.3. Method validation

3.3.1. Linearity and range

The calibration graph for the determination of OXY and ER by the proposed methods was constructed by plotting the RFI vs the drugs' concentrations (Figures 11-14). The calibration graphs were found to be rectilinear over the concentration ranges of 0.04-0.75 µg/mL and 5-100 ng/mL in case of OXY and ER, respectively, with regression equations:

ER (by native fluorescence)
$RFI = 9.8557 \times C + 7.6802$ ($R^2 = 0.9998$) (1)

OXY
$RFI = 1107.3 \times C + 59.537$ ($R^2 = 0.9999$) Direct (2)

$RFI = -1.2246 \times C - 0.1119$ ($R^2 = 0.9999$) D^2 (3)

$RFI = 877.77 \times C + 196.36$ ($R^2 = 0.9999$) SFS (4)

where, RFI is the relative fluorescence intensity, and C is the drugs' concentrations.

Statistical analysis of data according to Miller and Miller [15] gave high values of square correlation coefficient (r^2), small values of the standard deviation (SD), relative standard deviation (%RSD) and percentage error (%Er) as shown in Table 1. The obtained data proved the linearity of the method over the specified range of concentrations.

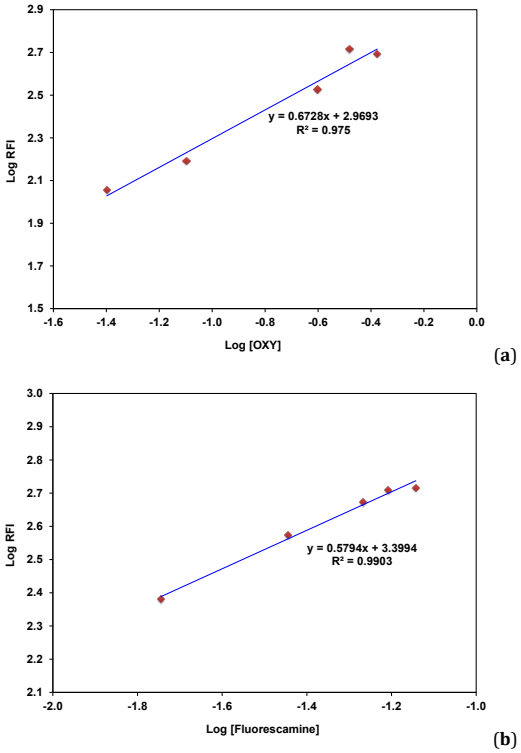

Figure 9. Stoichiometry of the reaction between OXY and fluorescamine adopting the limiting logarithmic method. (a) Log RFI *vs* log [OXY] using 0.8 mL 0.025% fluorescamine, (b) Log RFI *vs* log [Fluorescamine] using 0.75 µg/mL OXY.

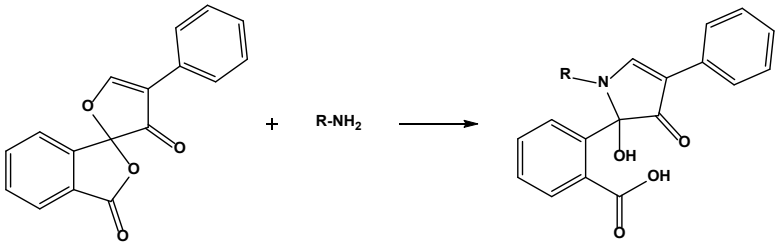

Figure 10. The proposed mechanistic pathway of the reaction between OXY and fluorescamine.

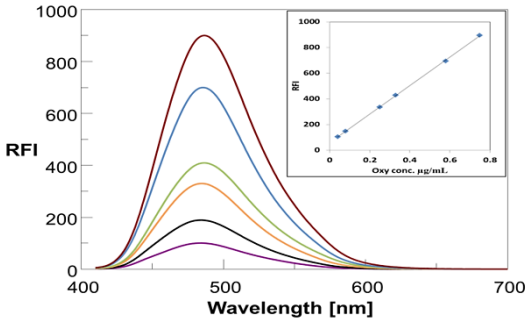

Figure 11. Spectrum and Calibration graph for determination of the derivatized OXY product at 486 nm with 0.8 mL 0.025% fluorescamine in 0.05 M phosphate buffer, pH = 8.5.

3.3.2. Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were determined according to the ICH guidelines [16]. The LOD is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The LOQ is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ results are shown in Table 1.

3.3.3. Accuracy

In order to test the validity of the proposed method, it was applied to the determination of pure samples of OXY and ER alone and in laboratory mixture over the working concentration ranges.

The results obtained were in good agreement with those obtained using the reference chromatographic method [4].

Table 1. Analytical performance data for the determination of ER and Oxy by the proposed spectrofluorimetric methods *.

Parameter	ER	OXY		
		Direct	D^2	SFS
Concentration range	5-100 ng/mL	0.04-0.75 µg/mL (0.024-0.450 IU)	0.04-0.75 µg/mL (0.024-0.450 IU)	0.04-0.75 µg/mL (0.024-0.450 IU)
LOD	1.77 ng/mL	0.01 µg/mL	0.01 µg/mL	0.01 µg/mL
LOQ	5.37 ng/mL	0.03 µg/mL	0.03 µg/mL	0.03 µg/mL
r^2	0.9998	0.9999	0.9999	0.9999
Slope (b)	9.86	1107.30	-1.22	877.77
Intercept (a)	7.68	59.54	-0.11	196.36
No. of experiments	8	6	7	6
Mean±SD	99.5±1.65	100.0±0.66	99.6±1.69	100.3±0.77
%RSD	1.66	0.66	1.70	0.77
SE	0.58	0.27	0.64	0.31
%ER	0.58	0.27	0.64	0.31

* RSD is the relative standard deviation, SE is the standard error and %ER is the percentage error.

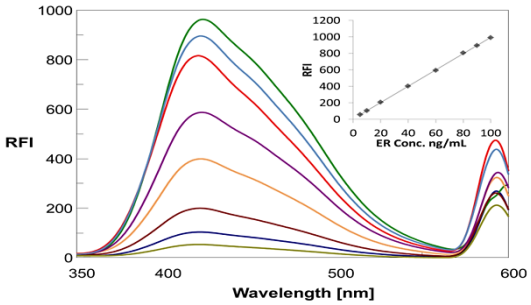

Figure 12. Native fluorescence spectrum and calibration graph of pure ER at 421 nm.

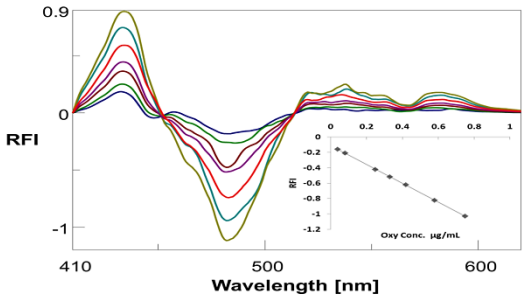

Figure 13. D^2 fluorimetric spectrum and calibration curve of derivatized OXY product at 482 nm.

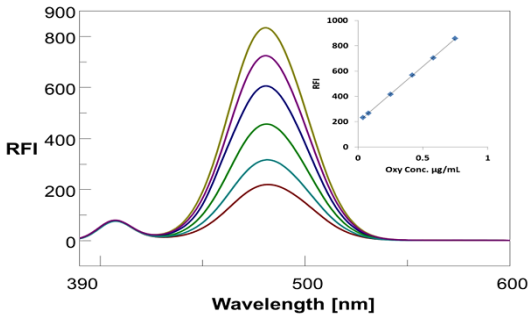

Figure 14. SFS spectrum and calibration graph of derivatized OXY product at 477 nm.

Using Student t-test and the variance ratio F-test [12] revealed no significant differences between the performance of the two methods regarding the accuracy and precision (Tables 2 and 3).

3.3.4. Precision

To evaluate the intra-day precision of the proposed methods, it was applied for the determination of three concentrations of each drug in pure form three successive times on the same day. Inter-day precision was also assessed through repeated analysis of the studied drugs in pure form using the concentrations shown in Tables 4 and 5 over a period of three successive days.

3.3.5. Robustness

The robustness of an analytical procedure is a measure of the capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [13].

Table 2. Application of the proposed and reference methods to the determination of OXY and ER in their pure form.

Standard	Parameters	Proposed method *	Official method [4] *
OXY (Direct)	% Recovery	101.6	99.9
		99.9	101.2
		99.7	100.8
	Mean±SD	100.4±1.04	100.6±0.67
	Student t-value	0.33	
	Variance ratio F-test	2.46	
OXY (D^2)	% Recovery	100.1	
		98.8	
		100.3	
	Mean±SD	99.7±0.81	
	Student t-value	1.48	
	Variance ratio F-test	1.50	
OXY (SFS)	% Recovery	98.7	
		101.1	
		100.3	
	Mean±SD	100.0±1.22	
	Student t-value	0.75	
	Variance ratio F-test	3.37	
ER	% Recovery	100.9	101.1
		100.5	100.7
		100.0	100.3
	Mean± SD	100.5±0.45	100.7±0.40
	Student t-value	0.67	
	Variance ratio F-test	1.27	

* Each result is the average of three different separate determinations; theoretical values at p = 0.05 for t- and F-tests were 2.78 and 19.00, respectively.

Table 3. Application of the proposed and reference methods to the determination of OXY and ER in their laboratory mixture.

Standard	Parameters	Proposed method *	Official method [4] *
OXY (Direct)	% Recovery	99.0	99.9
		100.7	101.2
		98.1	100.8
	Mean±SD	99.3±1.32	100.6±0.67
	Student t-value	1.6	
	Variance ratio F-test	3.93	
OXY (D^2)	% Recovery	99.2	
		97.5	
		100.2	
	Mean±SD	99.0±1.37	
	Student t-value	1.9	
	Variance ratio F-test	4.2	
OXY (SFS)	% Recovery	98.8	
		97.3	
		100.8	
	Mean±SD	99.0±1.76	
	Student t-value	1.54	
	Variance ratio F-test	6.95	
ER	% Recovery	98.3	97.9
		99.9	98.2
		100.0	100.5
	Mean±SD	99.4±0.95	98.9±1.42
	Student t-value	0.54	
	Variance ratio F-test	2.22	

* Each result is the average of three different separate determinations; theoretical values at p = 0.05 for t- and F-tests were 2.78 and 19.00, respectively.

Table 4. Precision of the proposed spectroflourimetric method for determination of ER.

Parameter		ER * (ng/mL)		
		20	40	80
Intra-day	% Recovery	101.6	102.7	97.4
		102.4	101.2	98.4
		99.6	100.5	100.0
	Mean±SD	101.2±1.44	101.5±1.12	98.6±1.31
	%RSD	1.42	1.10	1.33
	SE	0.83	0.65	0.76
	%Er	0.82	0.64	0.77
Inter-day	% Recovery	98.3	100.7	99.9
		98.7	101.9	98.9
		100.2	100.2	100.3
	Mean±SD	99.1±1.00	100.9±0.87	99.7±0.72
	%RSD	1.01	0.86	0.72
	SE	0.58	0.50	0.42
	%Er	0.59	0.50	0.42

* Each result is the average of three separate determinations.

Table 5. Precision of the proposed spectroflourimetric method for determination of OXY.

Parameter		OXY * (µg/mL)								
		Direct			D2			SFS		
		0.08	0.25	0.58	0.08	0.25	0.58	0.08	0.25	0.58
Intra-day	% Recovery	99.9	101.2	99.5	99.8	101.7	99.2	101.3	102.4	99.7
		97.8	98.8	100.3	100.1	98.8	100.5	99.9	101.6	100.5
		100.6	100.3	102.3	100.3	102.2	97.7	100.0	99.9	101.9
	Mean	99.4	100.1	100.7	100.1	100.9	99.1	100.4	101.3	100.7
	±SD	1.46	1.21	1.44	0.25	1.84	1.40	0.78	1.28	1.11
	%RSD	1.47	1.21	1.43	0.25	1.82	1.41	0.78	1.26	1.10
	SE	0.84	0.70	0.83	0.14	1.06	0.81	0.45	0.74	0.64
	%Er	0.85	0.70	0.82	0.14	1.05	0.82	0.45	0.73	0.64
Inter-day	% Recovery	97.7	99.9	101.5	100.8	99.9	102.5	100.5	99.6	97.3
		98.9	100.3	103.2	100.3	100.5	100.5	103.1	98.6	100.3
		101.2	102.4	99.1	101.1	98.6	99.8	101.9	101.9	101.1
	Mean	99.3	100.9	101.3	100.7	99.7	100.9	101.8	100.0	99.6
	±SD	1.78	1.34	2.06	0.40	0.97	1.40	1.30	1.69	2.00
	%RSD	1.79	1.33	2.03	0.40	0.97	1.39	1.28	1.69	2.01
	SE	1.03	0.77	1.19	0.23	0.56	0.81	0.75	0.98	1.16
	%Er	1.04	0.76	1.17	0.23	0.56	0.80	0.74	0.98	1.16

* Each result is the average of three separate determinations.

Table 6. Application of the proposed method for the determination of ER and OXY in their prepared combined ampoules.

Combined ampoules	Parameters	Proposed method *	Official method [4] *
OXY (Direct)	% Recovery	98.8	99.7
		97.4	97.8
		99.7	98.3
	Mean± SD	98.6±1.16	98.6±0.98
	Student t-value	0.38	
	Variance ratio F-test	1.38	
OXY (D^2)	% Recovery	97.9	
		100.2	
		99.5	
	Mean± SD	99.2±1.18	
	Student t-value	0.68	
	Variance ratio F-test	1.43	
OXY (SFS)	% Recovery	99.3	
		102.1	
		98.3	
	Mean± SD	99.9±1.97	
	Student t-value	1.02	
	Variance ratio F-test	4.00	
ER	% Recovery	100.4	101.9
		102.5	100.6
		99.7	100.2
	Mean± SD	100.9±1.46	100.9±0.89
	Student t-value	0.03	
	Variance ratio F-test	2.69	

* Each result is the average of three different separate determinations; theoretical values at p = 0.05 for t- and F-tests were 2.78 and 19.00, respectively.

The robustness of the procedure adopted in the proposed method was demonstrated by the constancy of the RFI with the minor changes in the experimental parameters, such as the volume of fluorescamine; 0.8 mL ± 0.2 mL, and change in pH 8.5 ± 0.5. These minor changes that may take place during the experimental operation did not affect the RFI of the reaction product.

3.3.6. Selectivity

The proposed methods allowed the selective determination of each drug in presence of the other without any interference proving its selectivity and ability to resolve a mixture of the two drugs.

3.3.7. Application of the proposed methods to analysis of OXY and ER in pharmaceutical dosage forms

The proposed methods were successfully applied to the determination of OXY and ER in the prepared combined ampoule (5 IU OXY + 500 µg ER + 7 mg NaCl)/mL.

The results obtained were in good agreement with those of the reference chromatographic method as shown in Table 6. The concentrations were calculated from the regression equations and consequently the percentage recoveries were obtained.

4. Conclusion

New, rapid, sensitive and valid D^2 and SFS methods were explored for the determination of OXY in combination with ER. The proposed methods showed high accuracy and precision, with no requirement of multiple steps or previous chemical separation as those associated with the cited chromatographic methods. The method could easily be used in quality control analysis of the drugs in mixtures, owing to its simplicity, low cost, high sensitivity and accuracy.

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Disclosure statement

Conflict of interests: The authors declare that they have no conflict of interest.

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Ethical approval: All ethical guidelines have been adhered.
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