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Article

Smart Spectrophotometric Methods for Concurrent Determination of Furosemide and Spironolactone Mixture in Their Pharmaceutical Dosage Forms

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Simple, precise, accurate and specific spectrophotometric methods are progressed and validated for concurrent analysis of Furosemide (FUR) and Spironolactone (SPR) in their combined dosage form depend on spectral analysis procedures. Furosemide (FUR) in the binary mixture could be analyzed at its $\lambda_{max\ 274}$ nm using its recovered zero order absorption spectrum using constant multiplication method (CM). Spironolactone (SPR) in the mixture could be analyzed at its λ_{max} 238 nm by ratio subtraction method (RS). Concurrent determination for FUR and SPR in their mixture could be applied by amplitude modulation method (AM), absorbance subtraction method (AS) and ratio difference (RD). Linearity ranges of FUR and SPR were (2.0µg /mL -22.0 µg /mL) and (3.0µg /mL -30.0 µg /mL), respectively. Specificity of the proposed spectrophotometric methods was examined by analyzing the prepared mixtures in laboratory and was applied successfully for pharmaceutical dosage form analysis which have the cited drugs without additives contribution. The proposed spectrophotometric methods were also validated as per as the guidelines of ICH. Statistical comparison was performed between the obtained results with those from the official methods of the cited drugs, using one-way ANOVA, F-test and student t-test. The results are exhibiting insignificant difference concerning precision and accuracy.

Keywords: Absorbance subtraction method (AS). Amplitude modulation method (AM). Ratio subtraction method (RS). Constant multiplication method (CM). Ratio difference method (RD).

INTRODUCTION

FUR [4-chloro-2-(furan-2-ylmethylamino)-5-sulfa moylbenzoic acid] as in Figure 1.a is a potent diuretic acting by inhibiting electrolytes' active reabsorption in kidney and increasing chloride ions, calcium, potassium and sodium excretion(Reynolds, 1982).

SPR [7α-acetylthio-3-oxo-17α-pregn-4-ene-21, 17-carbolactone] as in Figure 1b is a potent diuretic acting by inhibiting aldosterone's effect, leading to an increase in water and sodium excretion and a decrease in potassium excretion(Reynolds, 1982).

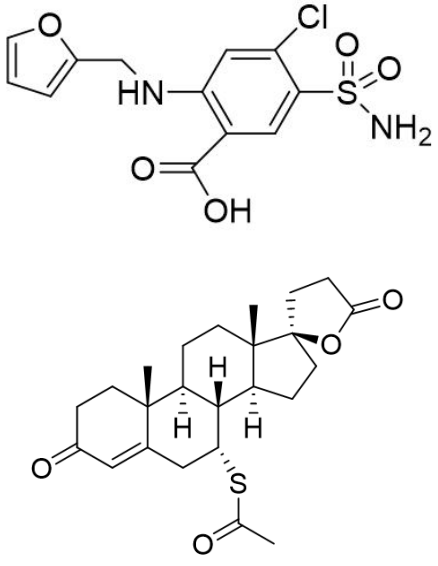

FIGURE1 – Structural formulae for (a) FUR, (b) SPR.

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Combinations of FUR and SPR are frequently prescribed to treat congestive heart failure, renal diseases and hypertension.

Various analytical methods were established for the analysis of each of these of them, either alone or in combination with other drugs. Various methods were established for analysis of FUR, including polarographic and electrochemical methods(Semaan *et al.,* 2008, Santini *et al.,* 2009, Malode *et al.,* 2012; Kor, Zarei, 2016; Medeiros *et al.,* 2016), spectrophotometric methods(Bosch *et al.,* 2013; Sawant Ramesh *et al.,* 2015; Emam *et al.,* 2018) and chromatographic methods. These were to include also HPLC (Bosch *et al*., 2013)and HPTLC methods(Kher *et al.,* 2013).

Various methods were established for analysis of SPR, including polarographic and electrochemical methods (Al-Ghamdi, Al-Ghamdi, Al-Omar, 2008; El-Shahawi *et al.,* 2013; Smajdor, Piech, Paczosa-Bator, 2018), spectrophotometric methods (Dinç, Üstündağ, 2003; Tekerek, Şukuroglu, Okan, 2008; Hegazy *et al*., 2010; Golher, Kapse, Singh, 2011; Patel, Solanki, 2012, Kundu *et al.,* 2017; Emam *et al.,* 2018) and various chromatographic methods. These included HPLC (Vaidya, Khanolkar, Gadre, 2002; Baranowska, Markowski, Baranowski, 2009; Ma *et al.,* 2010; Vlase *et al.,* 2011; Ram, Dave, Joshi, 2012; Walash *et al.,* 2013; Woo *et al.,* 2013; Lee *et al.,* 2015; Rajalakshmi *et al.,* 2018), HPTLC (Sharma *et al.,* 2010; Kher *et al.,* 2013).

Various methods were established for simultaneous analysis of FUR and SPR, including spectrophotometric methods (Millership, Parker, Donnelly, 2005; Israt *et al.,* 2016; Chavan *et al.,* 2018; Emam *et al.,* 2018) and chromatographic methods (Maulik, Ketan, Shital, 2012; Patel, Solanki, 2012; Ram, Dave, Joshi, 2012; Walash *et al.,* 2012; Ram, Ram, Joshi, 2015; Naguib *et al.,* 2018).

Creating simple spectrophotometric methods which were able to resolve the spectral overlapping of FUR and SP is the main aim of this work. The proposed spectrophotometric methods could be easily used for accurate and precise concurrent determination of FUR and SPR binary mixture applying simple manipulation steps. The proposed methods could be utilized in estimation of their market formula named Lasilactone® tablet. A comparative study was performed between the attained results to check the efficiency of the proposed methods in FUR and SPR determination.

MATERIALS AND METHODS

Experimental

Devices and operating system

The analysis was performed using a Shimadzu UV-1800 spectrophotometer attached to ACER computer. Quartz cells-1.0 cm were used for recording the absorption spectra of both reference and test solutions at the range of 200 nm - 400 nm.

Materials

- *Authentic materials*

FUR and SPR were generously provided by Mina Pharm Company, Cairo, Egypt. Their purity detected as 99.90 ± 0.61 and 99.87 ± 0.60 for FUR and SPR, respectively, as stated in the official BP method(Pharmacopoeia,2007) and EP method(Pharmacopoeia,2002)for FUR and SPR, conjointly.

- *Dosage form*

Lasilactone® 20/50 tablets were used with batch number 6EG021. Every tablet is claimed to have 20 mg and 50 mg of FUR and SPR, conjointly. Lasilactone® 20/100 tablets were also used. Its number of batch is 6EG015. Every tablet is claimed to have 20 mg of FUR and 100 mg of SPR. Both dosage forms were produced by Sanofi Aventis Company for Pharmaceuticals, Cairo, Egypt, and were obtained from the local market.

- *Chemicals and solvents*

Methyl Alcohl was kindly obtained from El Nasr Chemicals & Pharmaceutical Company, Cairo, Egypt.

Standard Solutions

- *Standard stock solutions*

Preparation of standard stock solutions of FUR and SPR (1.0 mg/mL, each), were carried out by dissolving

accurately weighed 100.0 mg of each drug in methanol into two separate 100-mL volumetric flasks. The volume was adjusted till the mark with the same solvent and stored in the refrigerator.

- *Standard working Solutions*

Preparation of stock solutions of FUR and SPR (50.0 µg/mL, each) was carried out by transferring 5-mL of stock standard solution of each drug (1000.0 µg/mL, each), into two 100-mL volumetric flasks, separately then the volume was adjusted till the mark by using the same solvent and kept in the refrigerator.

Procedure

Spectrophotometric scanning of FUR and SPR

Solutions of FUR (12.0 µg/mL) and SPR (30.0 µg/mL) were prepared using their standard working solutions (50.0 µg/mL of each), and scanned in a wavelength region 200-400 nm against methanol as blank, then their spectra of zero order absorption were obtained.

Construction of calibration graphs

Aliquots equivalent to (20.0- 220.0 µg) FUR and (30.0-300.0 µg) SPR had been transmitted from their standard working solutions of FUR and SPR (50.0 µg/mL, each) to two separate sets of volumetric flasks (10-mL), the volume was then adjusted till the mark using methanol. Scanning of the spectra of absorption of previously performed standard solutions was conducted in the region from 200-400 nm and recorded onto the computer. The calibration graphs were done applying an average of three experiments.

- *Calibration graphs based on absorbance of zero order absorption spectra for FUR and SPR*

Construction of calibration graphs of FUR was carried out by plotting the absorbance at 274 nm versus the correlative FUR concentrations and the equation of regression was computed. The absorbance factor of different concentrations of pure FUR at 254.8 nm and 340 nm [$A_{254.8}$ / A_{340}] was calculated, and the average was taken while construction of calibration graphs of SPR was carried out by plotting the absorbance at 238 nm and 254.8 nm (isoabsorbtive point) versus the correlative concentrations of SPR. The equation of regression was then accurately computed.

- *Calibration graphs based on amplitude of ratio spectra for FUR and SP FUR: The use of SPR (25 µg /mL) as a divisor*

Previously stored spectra of zero order absorption of FUR were divided by the spectrum of zero order absorption of SPR (25 µg/mL) to obtain ratio spectra, and the amplitudes at 260 nm and 263 nm were recorded. The amplitude difference between the selected wavelengths was calculated. Construction of calibration graph was conducted by plotting the amplitudes difference against the correlative FUR concentrations, then equations of regression were accurately computed.

SPR: Amplitude of ratio spectra by using of FUR (18 µg /mL) as a divisor

Previously stored spectra of zero order absorption of SPR were divided by the spectrum of absorption of FUR (18.0 µg/mL) to obtain ratio spectra, and the amplitudes at 238 nm and at 249 nm were recorded, then the amplitude difference between the two selected wavelengths was calculated. Construction of calibration graph was conducted by plotting the amplitudes difference against the SPR concentrations, then equations of regression were accurately computed.

SPR: Amplitude of ratio spectra by using of normalized FUR as a divisor

Previously stored spectra of zero order absorption of SPR (3.0-22.0 µg/mL) were divided by the spectrum of absorption of normalized FUR to obtain ratio spectra, and the amplitude values at 254.8 nm were recorded against the correlative SPR concentrations. Equations of regression were then accurately computed.

Analysis of prepared mixtures in laboratory

Aliquots were accurately transferred from standard working solutions (50.0 µg/mL) of FUR and SPR. Laboratory prepared mixtures with various ratios of the mentioned drugs were prepared and the volume was adjusted till the mark by methanol. Scanning of spectra of previously prepared mixtures was carried out at wavelength region (200-400 nm) and recorded into the computer.

- *Manipulation of zero order absorption spectra of the mixtures*
  (AS) for FUR and SPR

The spectrum of zero order absorption of each laboratory prepared mixtures were measured at 254.8 nm and 340 nm. The absorbance of FUR at 254.8 nm (λ_{iso}) in the mixtures could be obtained by using previously calculated absorbance factor of pure concentrations of FUR. This obtained absorbance was subtracted from the absorbance at 254.8 nm (λ_{iso}), which was recorded previously to acquire the absorbance related to SPR. Both FUR and SPR concentrations could be calculated with accuracy using unified equation of regression at 254.8 nm.

- *Manipulation of the ratio spectra of the mixtures using FUR (18.0 µg/mL) as a divisor*

Division of the scanned spectrum of each lab prepared mixture in laboratory by the spectrum of zero order absorption of FUR (18.0 µg/mL) was performed and the ratio spectrum was obtained.

(RS) coupled with (CM) for SPR and FUR, conjointly

Measuring the constant of each of the mixture was performed in the plateau region from 285 – 374 nm, where the FUR spectrum extended farther than SPR spectrum. The spectrum of zero order absorption of SPR was obtained by the subtraction of the previously measured constant value of each mixture from the correlative ratio spectrum of the mixture, followed by multiplication by the spectrum of the divisor FUR (18.0 µg/mL), while the spectrum of zero order absorption of FUR was obtainedby multiplying the value of the constant by the divisor's spectrum (FUR 18.0 µg/mL). Both FUR and SPR concentrations were calculated using their correlative regression equations at 238 nm and 274 nm, conjointly.

(RD) for SPR

Using the obtained ratio spectra of each mixture, the amplitude difference between 238 nm and 249 nm was recorded. SPR concentration for each of previously laboratory prepared mixture was attained using relative equation of regression.

- *Manipulation of the ratio spectra of previously prepared mixtures using SPR (25.0 µg/mL).*

(RD) for FUR

Using the obtained ratio spectra of each mixture, the amplitude difference between 260 nm and 270 nm was recorded. FUR concentration in each of laboratory prepared mixture was attained using relative equation of regression.

1. *Manipulation of the ratio spectra of previously prepared mixtures using normalized spectra as a divisor.*
   (AM) for FUR and SPR

Division of the scanned spectrum of each of the previously prepared mixture by the absorption spectrum of normalized FUR was performed. Amplitudes were recorded in the plateau region at wavelength region 285-374 nm (the constant of each mixture) was recorded and subtracted from correlative recorded amplitude at 254.8 nm (the isosbestic point) of each mixture in order to obtain amplitude related to SPR. FUR and SPR concentrations were calculated, using the correlative unified equation of regression, as well as the previously recorded constant value for FUR and the previously calculated amplitude value of SPR.

Assessment of pharmaceutical dosage form

Ten tablets of Lasilactone® tablets of each concentration (20/50), (20/100) of (FUR/SPR) were accurately weighed and the average was computed, then they were grinded to obtain fine powder. An accurate amount equal to one tablet containing 20 mg FUR and 50 mg of SPR for dosage form (20/50) & 20 mg FUR and 100 mg of SPR for dosage form (20/100) were separately transmitted to two separate 100-mL beakers, followed by the addition of 50-mL methanol, then sonication of the solution was performed using ultrasonic bath for ten minutes. The solution was filtered using filter paper (Whatman No.10 filter paper with pore size = 11 µm) into 100 mL volumetric flasks. Using methanol, the volume was adjusted till the mark. Appropriate dilution was performed to obtain final concentration claimed to have 3.0 µg /mL FUR, 7.5 µg/mL in dosage form (20/50) and 2.5 µg /mL FUR, 12.5 µg/mL SPR in dosage form (20/100). The proposed methods were applied for estimation of the studied drugs through the procedures previously cited under analysis of prepared mixtures in laboratory for every method. Using relative equations of regression, FUR and SPR concentrations were calculated.

RESULTS AND DISCUSSION

FUR and SPR were scanned in a wavelength range 200-400 nm and maxima for FUR (274 nm) and SPR (238 nm) in spectra of zero order absorption was measured as revealed in Figure 2. The two cited drugs have shown same absorptivity, acting as one compund at the isoabsorptive point. Experimentally, this was confirmed by recording the spectra of zero order absorption of FUR and SPR (10.0 µg/mL) of each, conjointly, in methanol and in their binary mixture of 5.0 µg/mL of each as revealed in Figure 3.

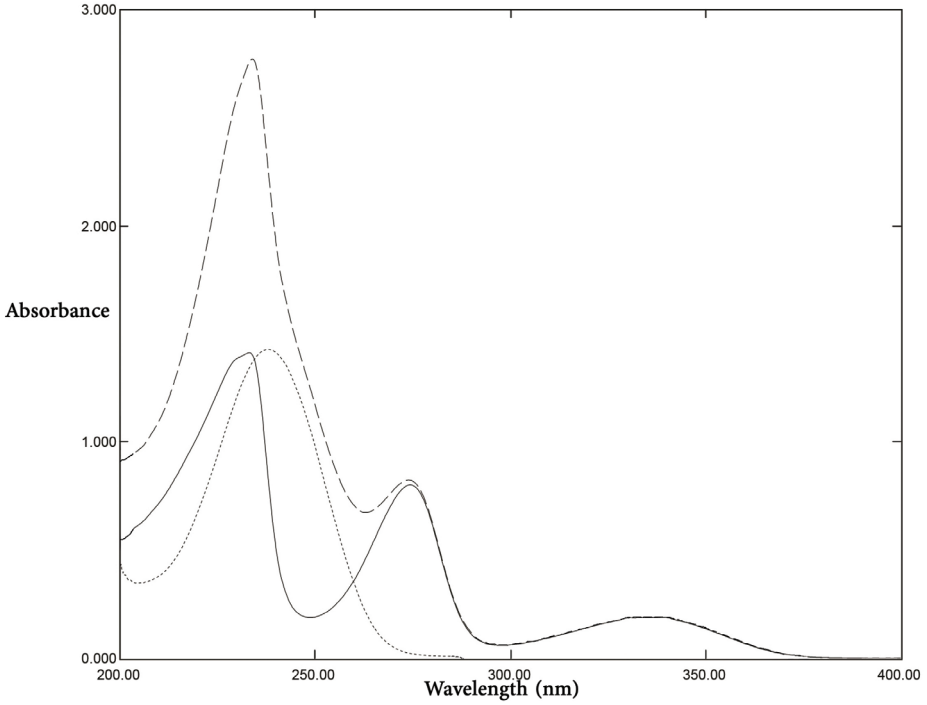

FIGURE 2 – Figure 2: Zero order absorption spectra of FUR (———) 12.0µg/mL, SPR (-------) 30.0 µg/mL and their binary mixture (– – – – –) as in pharmaceutical dosage form ratio.

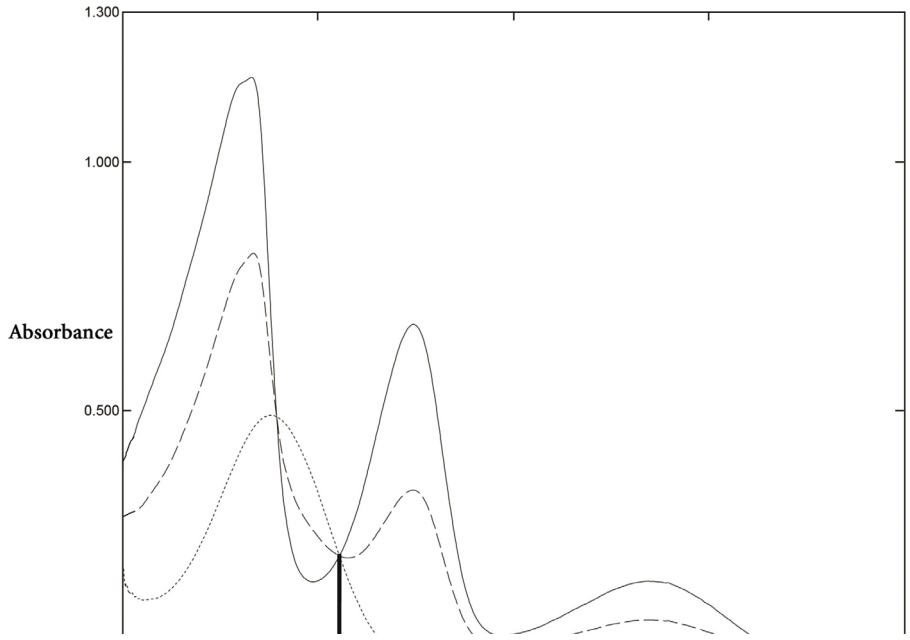

FIGURE 3 – Zero order Absorption spectra of FUR (———) 10.0µg/mL, SPR (-------) 10.0 µg/mL and their binary mixture 5µg/mL of each (– – – – –) which showing the isoabsorptive point.

The aim of this work is to create simple, accurate and sensitive methods for concurrent of FUR and SPR determination concurrently, either in their authentic form or their combined pharmaceutical formulation with acceptable precision, besides statistically comparing the proposed methods' ability for the determination of the two cited drugs.

Univerate techniques have many advantages over other chemometric and chromatographic techniques for being able to offer simple, low cost, needing few preparation steps and availability of the instrument in research labs, where as regarding chemometric method, it is complex and involves high level mathematics that require a statistical program to analyze the data and these statistical programs can be expensive for an individual to obtain and regarding the chromatographic method, it is high cost teqnique and need sophisticated instrument with well trained person.

A comparative review has been carried out between the proposed methods, ratio difference (RD), ratio subtraction (RS), constant multiplication (CM), absorbance subtraction (AS) and amplitude modulation (AM) and reported methods for this binary mixture, simultaneous equation and derivative ratio method DD(Millership, Parker, Donnelly, 2005; Chavan *et al.,* 2018) indicating satisfactory results. RS and CM methods have a privilege over other univariate spectrophotometric reported methods for resolving the overlapping spectra that it regains D^0 absorption spectrum of each component in the mixture which acts as a spectral profile of each cited drug and uses its maxima for the determination and calculation of concentration. In addition, the recovered D^0 absorption spectrum can be regarded as a suitable parameter which is useful in drug identity and purity. AS and AM methods are superior to the RS, CM and reported methods since one regression equation at iso-point can be used in estimation of both drugs in the binary mixtures.

The main drawback of the reported method (simultaneous equation) is tedious mathematical calculation and for reported method (DD) is requiring derivatization steps that increase noise, that could affect the accuracy and precision of he result. Furthermore, the choice of divisor is critical to maximize sensitivity and minimize noise

Different types of solvents were tried, including methanol and acetonitrile. Methanol showed satisfactory results for the two drugs FUR and SPR regarding selectivity, precision and the ability to determine

each drug with one preparation as it was successful in dissolving the mentioned amounts of drugs.

The divisor concentration which is to be selected should compromise between negligable noise and maximum sensitivity. Various concentrations of FUR and SPR were tested and it was found that the concentration of FUR' (18.0 µg/mL) and the concentration of SPR' (25.0µg/mL) were the best concerning sensitivity, repeatability and average recovery percent when utilized as divisor. Selection of the divisor affects the results of manipulating ratio spectra techniques as in amplitude modulation method. So, to eliminate the divisor, the normalized spectrum of FUR or SPR (the sum of the zero order absorption spectra of different concentrations within linearity range divided by their concentrations and represents absorptivity curve) was utilized versus all the measured wavelengths.

Using the absorbance of the scanned spectra of zero order absorption

AS for FUR and SPR

This method (Lotfy, Hegazy, 2013; Saleh *et al.,* 2013) depends on analysis of elements where their spectra of zero order absorption have isosbestic point which is known as the isoabsorptive point.Those elements at this point showing equal absorptivities. For FUR and SPR determination, their isoabsorptive point at 254.8 nm was used as revealed in Figure 3. Calculation of the absorbance correlating to FUR or SPR, separately at isoabsorptive point 254.8 nm, was performed by using the previously calculated absorbance factor [$abs_{254.8}$ / abs_{340}] which is calculated by taking the average of the absorbance ratio of various concentrations of pure FUR at 254.8 nm (λ_{iso}) to that at 340 nm which exhibits no interference of SPR. Then, subtraction was performed, and FUR absorbance was obtained.

Absorbance of FUR in the mixture at $\lambda_{254.8}$
= [$abs_{254.8nm}$ / abs_{340nm}] × $abs_{340nm\ (Mix)}$

Absorbance of SPR in the mixture at $\lambda_{254.8}$ =
abs $\lambda_{254.8nm\ (Mix)}$ - [$abs_{254.8nm}$ / abs_{340nm}] × $abs_{340nm\ (Mix)}$

Where, abs $\lambda_{254.8nm\ (Mix)}$ or $abs_{340nm\ (Mix)}$ is FUR and SPR absorbance at 254.8 nm or 340 nm and abs $_{254.8}$ / abs $_{340}$ is the calculated absorbance factor of pure FUR at 254.8 nm to 340 nm and it was found to be 1.3051.

The previously calculated values of absorbance correlating to FUR and SPR was utilized to derermine their concentration through using the unified equations of regression at λ_{iso} 254.8 nm.

A linear correlation between the absorbance values of SPR at 254.8 nm (λ_{iso}) against the correlating concentrations (3.0 µg/mL - 22.0 µg/mL) was used, and the equation of regression was computed as revealed in Table I.

TABLE I - Validation parameters of the proposed spectrophotometric methods for determination of FUR and SPR in pure form

Drug name	**FUR**		**SPR**			
Methods	**D^0 (274 nm)**	**RD**	**D^0 (238 nm)**	**RD**	**AM at (254.8 nm)**	**AS**
Range[a] (µg/mL)	2.00- 22.00		3.00-30.00		3.00-22.00	
Regressions Parameters						
Slope	0.0666	0.1217	0.0470	0.0804	0.9996	0.0218
intercept	0.0064	0.0603	0.0219	0.0418	0.0393	0.0002
Correlation coefficient :(r)	1.0000	0.9999	0.9999	0.9999	1.0000	0.9999

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TABLE I - Validation parameters of the proposed spectrophotometric methods for determination of FUR and SPR in pure form

Drug name	FUR		SPR			
Methods	**D^0 (274 nm)**	**RD**	**D^0 (238 nm)**	**RD**	**AM at (254.8 nm)**	**AS**
Accuracy (Mean±SD)	99.75±0.44	99.26± 1.05	100.07± 0.28	99.66± 1.00	99.31±1.08	100.04±0.60
Precision						
Repeatability[b]	99.84±0.463	100.41± 0.644	100.25 ±0.697	100.21±0.980	99.97±0.068	100.24±0.890
Inter-day precision[c]	100.24± 0.697	100.01± 1.044	100.16 ± 1.030	99.38±1.199	100.00±0.138	99.63±0.917
Robustness (%RSD[d])	0.756	0.983	0.956	0.791	0.882	0.855

[a] Six and Seven calibration points in (µg/mL) for FUR and SPR respectively, average of three experiments.
[b] Intra-day precision (n=9), average of 3 different concentrations (6.00, 14.00 and 18.00 µg/mL) for FUR and (10.00, 20.00 and 25.00 µg/mL) for SPR for D^0 and RD methods while(7.00, 13.00 and 19.00 µg/mL)for AM and AS methods, repeated 3 times each within the same day.
[c]Inter-day precision (n=9), average of 3 different concentrations (6.00, 14.00 and 18.00 µg/mL) for FUR and (10.00, 20.00 and 25.00 µg/mL) for SPR for SPR for D^0 and RD methods while(7.00, 13.00 and 19.00 µg/mL)for AM and AS methods, repeated 3 times each on 3 successive days.
[d] Average of changing of the working wavelength (±0.1 nm).

Using the amplitudes of the ratio spectra of the spectra of zero order absorption

AM for FUR and SPR

This method (Lotfy *et al.,* 2014; Saleh *et al.,* 2014) depending on presence of an isoabsorptive point in the spectra of zero order absorption of two cited components which subsequentaly will be remained as an isosbestic point at the same wavelength of their ratio spectra after division by normalized spectrum of the extended drug.

Division of binary mixture (FUR+SPR) spectrum showing the isoabsorptive point by the normalized spectrum of FUR as a divisor was performed, and the ratio spectrum was obtained as revealed in Figure 4. In each mixture, the amplitude value of the constant was measured at the plateau region at (285 - 374 nm), which is representing FUR amplitude constant value throughout the whole spectrum. At λ_{iso} (254.8 nm), the amplitude of the ratio spectra represents the sum of FUR and SPR amplitudes, then the recorded amplitude at 254.8 nm was subtracted from the previously measured constant at (285 - 374 nm). The correlating recorded amplitude of SPR was acquired, which is equal to SPR recorded concentration in the mixture ($C_{Recorded}$ of SPR). Nevertheless, the recorded amplitude of constant value will be directly equal to the FUR recorded concentration in the mixture ($C_{Recorded}$ of FUR). To eliminate any error which may be occurred due to signal to noise ratio, FUR or SPR actual concentration could be determined by using their correlative unified equation of regression equation at λ_{iso} (254.8 nm).

$C_{Recorded} = 0.9941\ C_{Actual} + 0.1256.$

$C_{Recorded}$ is the ratio spectrum's recorded amplitude at 254.8 nm, and C_{Actual} is the correlating FUR or SPR concentration.

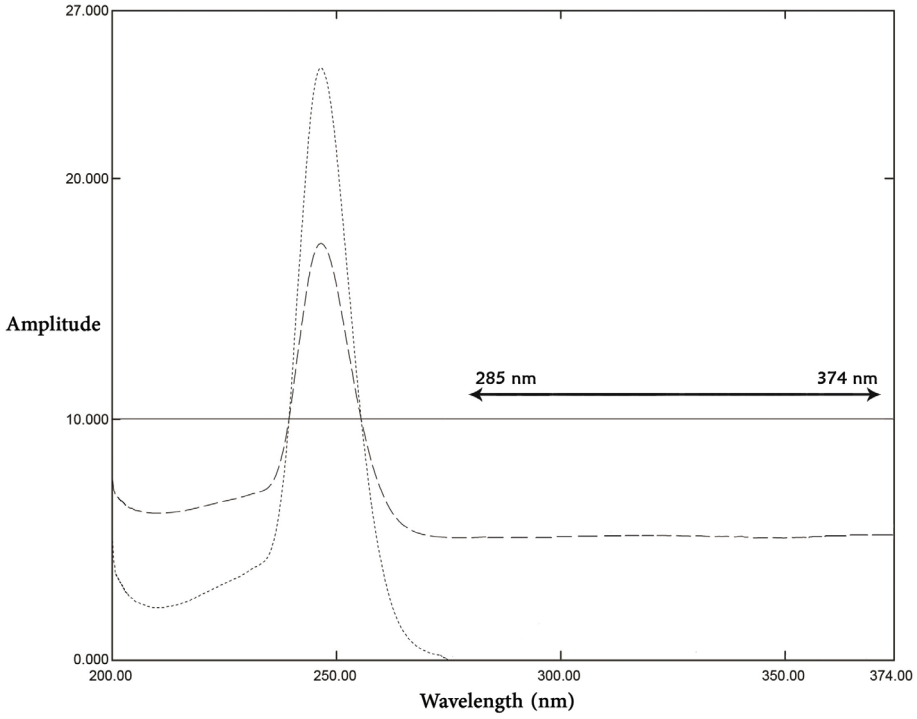

FIGURE 4 – Ratio spectra of FUR (———) 10.0µg/mL, SPR (-------) 10.0g/mL and their binary mixture 5.00µg/mL of each (– – – – –) using normalized spectrum of FUR as a divisor.

A linear relationship was obtained between peak amplitudes of the ratio spectra of SPR at λ_{iso} 254.8 nm against the corresponding concentrations (3.0 - 22.0µg/ mL). The equation of regression was computed as revealed in Table I.

(RD) for FUR and SPR

In this method, the difference in amplitude between the two selected wavelengths on the mixture's ratio spectra is in direct relationship with the desired element's concentration; it doesn't depend on one of the interfering elements (Lotfy, Hagazy, 2012).

In case of determining the concentration of FUR: The scanned absorption spectrum of the mixture is to be obtained first, and then divided by SP absorption spectrum as a divisor. Accordingly, the ratio spectrum, (which could be represented as $\frac{FUR}{SPR'}$ + constant) is obtained.

In case of determining the concentration of SPR: The scanned absorption spectrum of the mixture is to be obtained first, and then it is divided by FUR absorption spectrum as a divisor. Accordingly, the ratio spectrum, (which could be represented as $\left(\frac{FUR}{SPR'}\right)$ + constant) is obtained. Selection of two wavelength for ratio spectra of FUR and SPR and subtraction these two amplitudes $\left(\frac{FUR}{SPR'}\right)1 - \left(\frac{FUR}{SPR'}\right)2$ in case of determination of FUR and $\left(\frac{SPR}{FUR'}\right)1 - \left(\frac{SPR}{FUR'}\right)2$ in case of determination of SPR was performed, so the constant will be omitted and the contribution of the divisor element will be omitted as revealed in Figure 5 and 6, while the concentration of the other element will be directly related to the previously calculated difference. FUR and SPR concentrations in each mixture were determined through its relative equation of regression showing linear relation between the amplitude difference at (DP260-270nm) in case of determination of FUR, while at (DP238-249nm) in case of determination of SPR against their correlative concentrations of FUR (2.0 - 22.0 µg/ mL) and SPR (3.0 - 30.0 µg/ mL), and the equations of regression were computed as revealed in Table I.

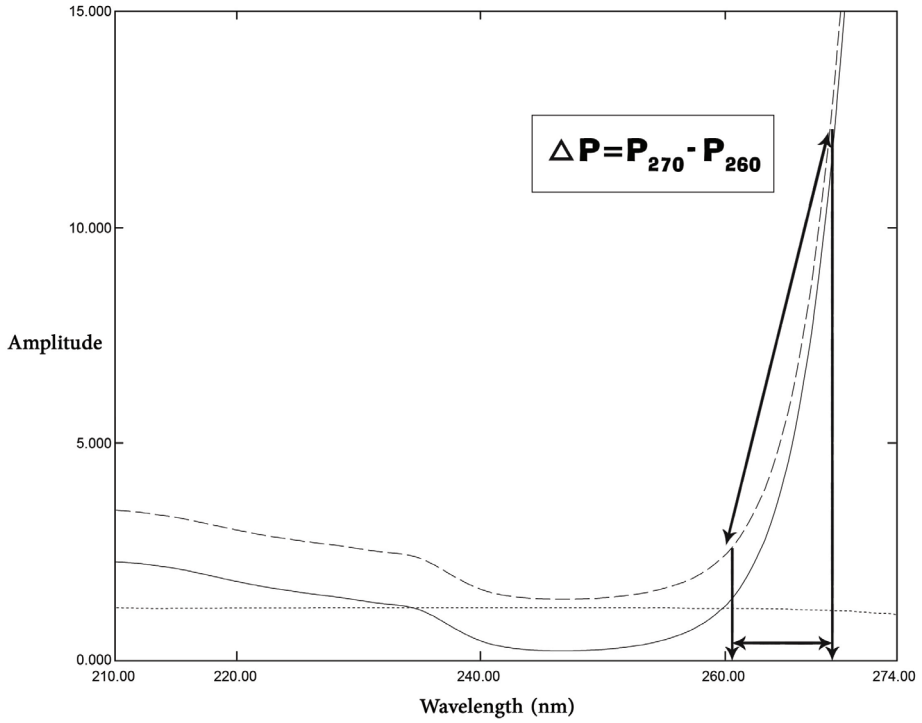

FIGURE 5 – The amplitude difference at 270 nm and 260 nm (ΔP 270-260 nm) of ratio spectra FUR (———) 12.0 µg/mL, SPR (-------) 30.0 µg/mL and their binary mixture (– – – – –) using SPR (25.0 µg/mL) as a divisor.

Appropriate selection of the two wavelengths and the divisor is very important. The divisor which will be selected should make compromising between negligible noise and maximum sensitivity, meanwhile, the prerequisite of the two selected wavelengths is that the drug of interest has highest amplitudes difference at the contribution region with the interfering substances, so the selected wavelengths were 260 and 270.0 nm for determination of FUR and 238 nm and 249nm for determination of SPR in each mixture, using SPR divisor (18.0 µg/mL) and FUR divisor (25.0 µg/mL) for determination of FUR and SPR, conjointly.

Using the amplitudes of their ratio spectra

RS) coupled with (CM)

The ratio spectra of each mixture could be obtained using FUR (18.0 µg/mL) as a divisor, the constant (FUR/FUR') at 285-374 nm was measured. Subtracting this constant from the mixture's ratio spectra and the ratio spectra of (SPR/FUR') in every mixture was obtained, then multiplying the result by the divisor FUR (18.0 µg/mL) to obtain the spectrum of zero order absorption of SPR in every mixture as revealed in Figure 7. a, b, c. While obtaining the spectrum of zero order absorption of FUR was achieved by multiplying by the previously recorded constant value of every mixture by the divisor FUR (18.0 µg/mL) as revealed in Figure 7.c.

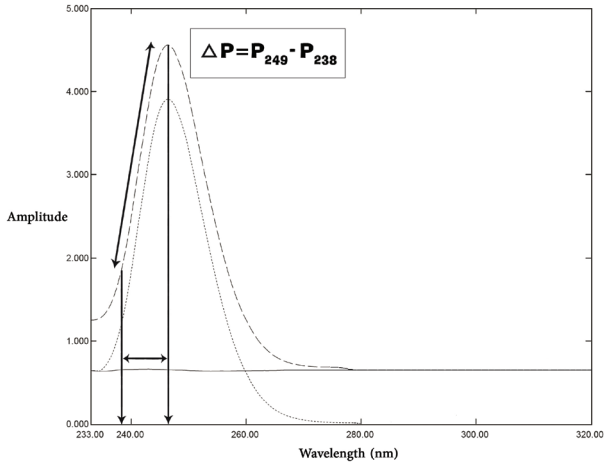

FIGURE 6 – The amplitude difference at 249 nm and 238 nm (ΔP 249-238 nm) of ratio spectra FUR (———) 12.0 µg/mL, SPR (-------) 30.0 µg/mL and their binary mixture (– – – – –) using FUR (18.0 µg/mL) as a divisor.

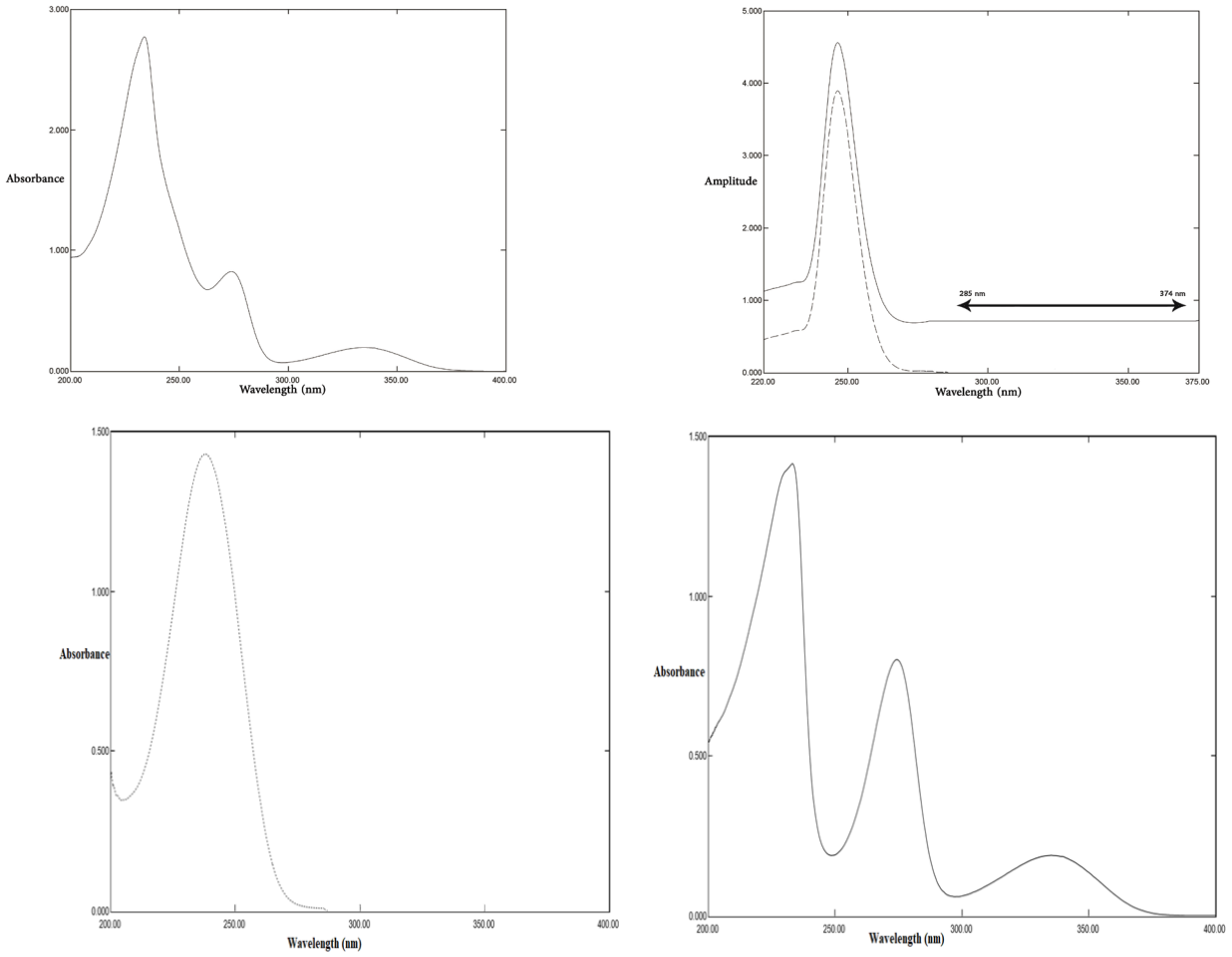

FIGURE 7 – (a) Zero order Absorption spectra of of binary mixture of FUR 12.0 µg/mL, SPR 30.0 µg/mL, (b) Ratio spectrum of the binary mixture of FUR and SPR (———) using FUR (18.0 µg/mL) as a divisor and (-------) the obtained ratio spectrum of binary mixture after subtraction of the constant., (c) The obtained zero order absorption spectrum of SPR after multiplication of resolved ratio spectrum obtained after subtraction of the constant by the spectrum of the divisor (– – – – –) and also the obtained zero order absorption spectrum of FUR after multiplication of constant by the spectrum of the divisor using FUR (18.0 µg/mL) (———).

A linear correlation was obtained between the absorbance values of FUR and SPR at its maxima 274 nm and 238 nm, conjointly against the correlating concentrations (2.0 - 22.0 µg/mL) for FUR and (3.0 – 30 µg/mL) for SPR and the equations of regression were computed as revealed in Table I.

Method validation

Validation was performed relative to the guidelines of ICH (1997) as revealed in Table I**.**

Linearity

The linearity of the proposed methods was assessed through analyzing different concentrations of FUR and SPR ranging from 2.0–22.0 µg/ml and 3.0–30.0 µg/mL, conjointly. Replication of each concentration was conducted three times. The analysis was performed as per as previously mentioned experimental conditions. Demonstration of linear equations was conducted in Table I.

Accuracy

Accuracy was investigated by applying the proposed methods for determination of various samples of FUR and SPR and and the standard addition technique was performed where various well-known concentrations of pure standard FUR and SPR were added to the pharmaceutical formulation before proceeding in previously mentioned methods. Obtaining the concentrations of both PSE and LOR was performed using the relative equations of regression. Notable good accuracy of the proposed spectrophotometric methods was attained. This was shown by the obtained percentages of recovery presented in Table I.

Range

The range of the calibration was made through using the practical range necessary according to loyalty to Beer's law and the concentration of both FUR and SPR which exist in their combined dosage form to afford linear, precise and accurate results as revealed in Table I.

Selectivity

Selectivity of the proposed methods was attained by the analysis of various laboratory prepared mixtures of FUR and SPR within the linearity range. Acceptable results were revealed in Table II.

TABLE II - Determination of FUR and SPR in the laboratory-prepared mixtures by the proposed spectrophotometric methods

%Recovery[a]±SD									
FUR: SPR		FUR				SPR			
Ratio	Concentrations (µg/mL)	CM	RD	AM	AS	RS	RD	AM	AS
(1:2.5)*	3:7.5	99.02	100.00	99.79	99.44	99.93	99.76	101.47	99.91
(1:5)*	3:15	100.00	100.17	99.86	100.10	100.00	99.71	100.19	99.98
(1:3)	4:12	100.57	100.00	100.02	99.43	100.13	99.93	100.59	100.16
(2:3.6)	5:9	100.47	100.28	99.82	99.85	99.86	100.49	100.35	100.02
(1: 6)	3:18	99.42	99.91	98.96	100.10	100.39	100.23	99.46	99.66
Mean±SD		99.90 ±0.67	100.07±0.15	99.69±0.42	99.78 ±0.33	100.06±0.21	100.02 ±0.33	100.41 ±0.73	99.95 ±0.18

[a] Average of three determinations.
*Ratio of the cited drugs as in the dosage form.

Precision

- *Repeatability and Intermediate Precision*

They could be determined through using three concentrations of FUR and SPR conjointly, then they were examined intra-daily and inter-daily three times on three different days using the proposed spectrophotometric methods. The correlating standard deviations which is correlative to every concentration were computed in Table I.

Robustness

The suggested methods' robustness was determined in order to evaluate the impact of small but intentional variations of the applied conditions on the proposed drugs' determination. When small changes in the working wavelength (±0.1 nm) were applied and minor change in the absorbance was noticed as shown in table I.

Application of the proposed analytical methods in assessment of Lasilactone®Tablet

The proposed spectrophotometric methods were used for the determination of concentration of both FUR and SPR in their combined dosage form, Lasilactone tablet® and the results are revealed in Table III. The validity of the proposed procedures is further evaluated by applying the technique of standard addition showing no interference from excipients. Good percentage recoveries were shown for all the proposed methods and this allows their usage for regular analysis of FUR and SPR in their combined formulation. The obtained results were revealed in Table III.

TABLE III - Determination of FUR and SPR in the pharmaceutical dosage form Lasilactone® (20/ 50) and (20/100) by proposed spectrophotometric methods and application of standard addition technique

	FUR				SPR			
	CM	**RD**	**AM**	**AS**	**RS**	**RD**	**AM**	**AS**
Pharmaceutical dosage form[a] **(found%±SD):**	99.70±0.36	99.78±0.42	100.52±0.37	98.94±0.32	100.44±0.34	98.58±0.42	99.51±0.37	100.30±0.32
D.F (1:2.5)								
D.F (1:5)	98.97±0.31	100.51±0.39	99.21±0.38	99.86±0.31	100.43±0.49	99.45±0.39	100.00±0.41	101.07±0.41
Standard Addition[b] **(Recovery%±SD):**	100.19±0.17	100.09±0.16	99.95±0.16	99.37±0.63	100.00±0.49	99.90±0.72	100.02±0.49	100.14±0.57
D.F (1:2.5)[c]								
D.F (1:5)[d]	100.12±0.21	100.28±0.13	99.68±0.67	99.76±0.24	100.13±0.27	99.95±0.36	99.88±0.36	99.79±0.19

[a]Average of three experiments.
[b]Average of three experiments.
[c] D.F (1:2.5) claimed amount (3 µg/ml of FUR and 7.5 µg/ml of SPR) was used and 3, 6, 9 µg/ml of pure FUR and 4,8,12 µg/ml of pure SPR were added.
[d] D.F (1:5) claimed amount (2.5 µg/ml of FUR and 12.5 µg/ml of SPR) was used and 2.5, 4, 8 µg/ml of pure FUR and 4,6, 8 µg/ml of pure SPR were added.

Statistical analysis

The comparison of the statistical data which obtained either by the proposed analytical methods or those obtained by the official BP method (Pharmacopoeia,2007) and EP method (Pharmacopoeia,2002) for FUR and SPR, respectively, showed insignificant differences as revealed in Table IV. To relate the ability of the proposed methods for the determination of FUR and SPR, the results obtained by applying the proposed methods were

subjected to statistical analysis through one-way ANOVA test for pure and the obtained values were less than the theoretical ones indicating that there was no significant difference between the proposed and the official methods with respect to accuracy and precision as revealed in Table V.

TABLE IV - Statistical comparison of the results obtained by the proposed spectrophotometric methods and those obtained by the official ones for the determination of FUR and SPR in their pure powdered form

Parameters	FUR			SPR				
	D^0 (274 nm)	RD ($\Delta P_{270-260nm}$)	Official method (Pharmacopoeia, 2007)[a]	D^0 (238 nm)	RD ($\Delta P_{249-238nm}$)	AM at (254.8 nm)	AS	Official method (Pharmacopoeia, 2002)
Mean	99.93	100.23	99.90	99.84	99.80	99.92	100.09	99.87
SD	0.54	0.88	0.61	0.78	0.98	0.32	1.05	0.60
n	6	6	6	7	7	7	7	6
Variance	0.2916	0.7744	0.3721	0.6084	0.9604	0.1024	1.1025	0.3600
Student's t-test	0.090 (2.228)	0.755 (2.228)		0.077 (2.201)	0.273 (2.201)	0.192 (2.201)	0.452 (2.201)	
F test	1.28 (5.05)	2.08 (5.05)		1.69 (4.95)	2.67 (4.95)	3.52 (4.39)	3.06 (4.95)	

[a] BP method for FUR is a potentiometric titration method, while EP method for SPR is spectrophotometric method.
[b] The figures in parenthesis are the corresponding theoretical values at P = 0.05(MR, LJ 1999).

TABLE V - ANOVA (single factor) for comparison of the results of the proposed spectrophotometric methods and those of the official methods for determination of FUR and SPR in pure powdered form

Source of variation	Sum of squares	DF	Mean Square	F value	P value	F crit
FUR						
Between exp.	1.208779	2	0.402926	1.005032	0.411089	3.098391
Within exp.	8.018183	15	0.400909			
Total	9.226963	17				
SP						
Between exp.	0.359199	4	0.0898	0.140694	0.965665	2.701399
Within exp.	18.50957	29	0.638261			
Total	18.86877	33				

-At the 0.05 level.
-The population means are not significantly different

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Advantages and limitations of the proposed methods

The main advantage of AM is that it is not affected by the divisor concentration since the normalized divisor is used. In addition, this method is also advantageous over AS method due to its ability to modulate directly the obtained amplitude at the ratio spectrum to the concentration of every drug through using the normalized divisor, and it could be applied without having to carry out calculation of absorbance factor. On the other hand, AS method has a privilege that it is applied on the zero-order absorption mode with no need of one of the interfering components as a divisor. The limitation for AS method and AM method is the presence of isoabsorptive point for the spectra of the studied drugs, thus the common concentrations in their linearity range of both drugs should be used. The most remarkable characters of the ratio difference method are simplicity with minimum manipulation steps and optimum accuracy and reproducibility. The RD method has no limitations and it is characterized by the ability of analysing the overlapped spectra without any necessary to preliminary steps. The main advantage of RS and CM is restoring the original spectra of proposed drug which allow their analysis using their maxima with maximum precision and accuracy. Meanwhile both methods are limited for analyzing binary mixtures with overlapped spectra where one of them showing extension over the other one. All the applied methods do not need any sophisticated devices or software.

CONCLUSIONS

Different spectrophotometric methods were applied for concurrent analysis of FUR and SPR in their pure powdered form, prepared mixtures in laboratory and pharmaceutical formulation, using different manipulating pathways for calculation of the values of absorbance for either spectra of zero order absorption or ratio spectra.

The validation of the proposed methods was performed using the guidelines of ICH and satisfactory results were obtained. Those methods could also be conducted in laboratories of the quality control for regular FUR and SPR analysis. The results were subjected to the statistical comparison against to each other and to the official methods of pure studied drugs. Insignificant difference was attained.

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CONFLICT OF INTEREST STATEMENT

The authors affirm that there is no conflict of interests concerning this manuscript.

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