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DEVELOPMENT AND VALIDATION OF SPECTROPHOTOMETRIC METHODS FOR SIMULTANEOUS ESTIMATION OF FUROSEMIDE AND SPIRONOLACTONE BY VIERORDT'S METHOD IN BULK AND COMBINED TABLET DOSAGE FORM

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Abstract: Anew simple, convenient and suitable spectrophotometric method for simultaneous determination of Furosemide and Spironolactone in combined dosage form has been developed and validated. Simultaneous equation method (Vierordt's method) was used for determination of Furosemide and Spironolactone in combined dosage form. For spectrophotometric method development double distilled water and ethanol were used as a solvent in the ratio of (20:80). The proposed method was quantitatively evaluated in terms of linearity, precision, accuracy, lower limit of detection (LOD) and quantification (LOQ), recovery and robustness. All the parameters were found to be within the acceptance limit. λmax of Furosemide and Spironolactone was found to be 275 and 237 nm respectively. Beer's law was obeyed over the concentration ranges of 2-10 μg mL^{-1} for both Furosemide and Spironolactone respectively. The % assay for commercial formulation was found to be 99.60%±0.0500 for Furosemide and 100.26%±1.17 for Spironolactone by the proposed methods. The overall recovery was observed to be 100.38±0.09% for Furosemide and 100.49±0.4197% for Spironolactone by

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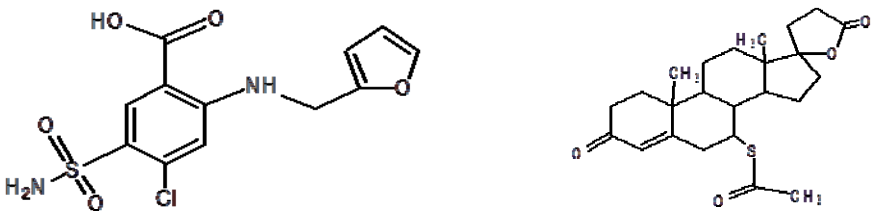
simultaneous equation method (Vierordt's method). LOD and LOQ were 0.76 and 2.32 µg mL^{-1} for Furosemide, 1.99 and 6.04 µg mL^{-1} for Spironolactone. A new simple, convenient, precise, rapid, accurate and economical and reliable spectrophotometric method was developed and validated for the analysis of Furosemide and Spironolactone in bulk drug and their formulations.

***Keywords*:** Furosemide; Spironolactone; Vierordt's Method; Validation

Introduction

Furosemide (FUR), is chemically 5-(aminosulfonyl)-4-chloro-2-[(2 furanylmethyl) amino] benzoic acid, a loop diuretic that is used in the treatment of congestive heart failure and edema (**Figure 1a**). FUR acts on thick ascending limb of the loop of Henle, which leads to loss of sodium, potassium, and chloride that are dispatched in the urine.[1]Itresults in a decrease in sodium and chloride reabsorption, while increasing the excretion of potassium in the distal renal tubule. The diuretic effect of orally administered FUR appears within 30 minutes to 1 hour and is maximal in the first or second hour.[2]



(a) (b)

Figure 1. Structure of (a) Furosemideand (b) Spironolactone.

Spironolactone (SPI) is chemically known as 17-hydroxy-7α-mercapto-3-oxo-17α-pregn-4-ene-21-carboxylic acid γ-lactones acetate shown in (Figure 1b).[3] SPI is most commonly used anti-diuretic agent in clinical practices. It acts on the intracellular aldosterone receptors in the distal tubule cells, which ultimately increases the excretion of water and sodium and cause a decrease in the excretion of potassium.[4,5]

Literature survey revealed reports on analytical methods such as UV-VIS for the determination of FUR[6-9] and SPI [4, 10-13] either single or other drug(s) in different formulations. Few UV/VIS[5, 14-19] methods have also been reported for the simultaneous estimation of SPI and FUR.However, as single drug candidate in biological fluid(s) very few methods have been reported for simultaneous estimation of FUR and SPI in combination with other drugs from biological samples. Moreover, the reported methods were not much cost-effective in terms of solvent consumption. Thus, it was felt essential to develop a procedure which will serve as a reliable, accurate UV method for the simultaneous estimation of FUR and SPI. The present investigation was therefore undertaken with an intention of establishing a simple, rapid, accurate, economic, precise and robust UV method for the simultaneous estimation of FUR and SPI in bulk drug and formulations.

Results and Discussion

Overlain spectra of the FUR and SPI depicted occurrence of two peaks at 275 nm and 237 nm, shown in Figure 2.

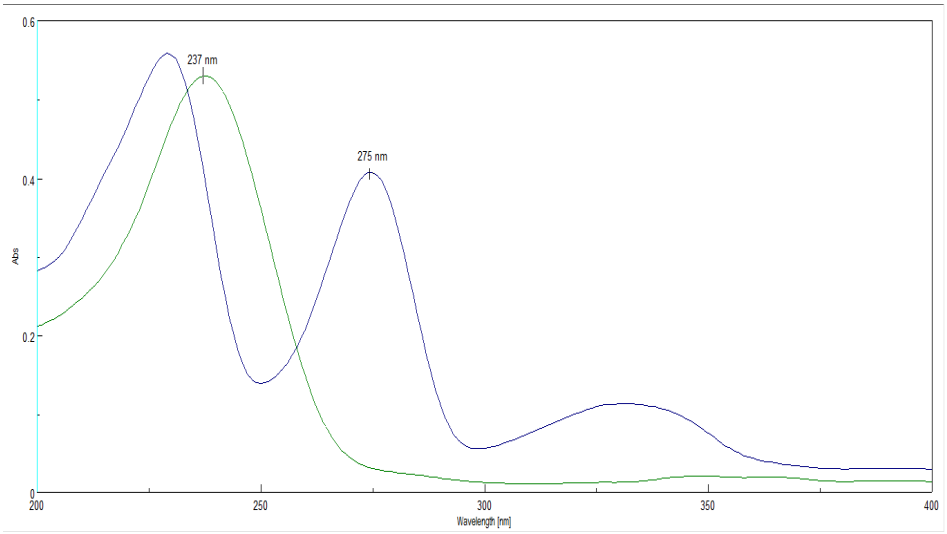


Figure 2. Overlap spectra of Furosemide & Spironolactone indouble distilled water and ethanol (20:80).

To optimize the UV parameters, several conditions were tried to achieve a good absorption and peak shape for FUR and SPI. Several solvents of different compositions were tried to provide sufficient selectivity towards the drugs. To optimize the UV/VIS parameters, several conditions were tried to achieve a good absorbance and a peak shape for SPI and FUR. Several solvents were tried for better absorbance and proper peak shape namely water, phosphate buffer, methanol, acetonitrile etc. Methanol and water (80:20) demonstrated good peak shape and absorbance than others. Moreover, methanol and water (80:20) as organic components resulted in better sensitivity.

Method validation

The developed method was validated according to International Conference on Harmonization guidelines for validation of analytical procedures in order to determine linearity, precision, LOD, LOQ and accuracy for the analyte.[20]

Linearity

The linearity of the calibration curve was evaluated by linear regression analysis. The calibration curves were obtained with concentrations of the standard solutions of 2-10 μg mL^{-1} for SPI and FUR respectively shown in Table 1 and Figure 3. For acceptance, a correlation coefficient of 0.99 or better was required.

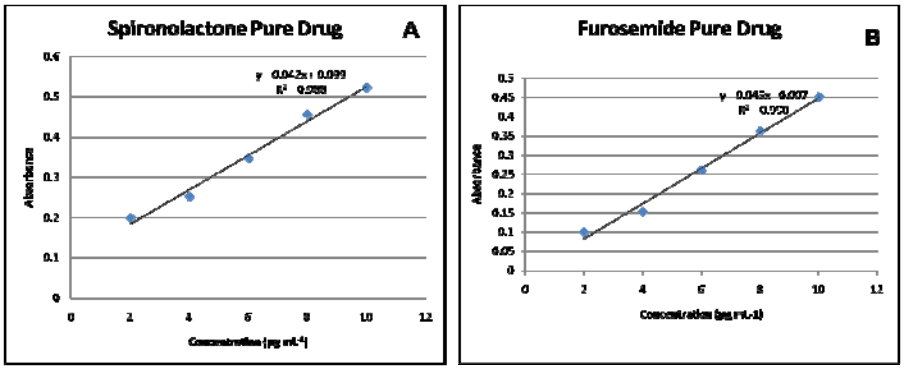


Figure 3. Calibration curve of Furosemide at 275 nm and Spironolactone at 237 nm.

Table 1. Linearity data for FUR and SPI.

Concentration (μg mL^{-1})	Absorbance of FUR at 275 nm*	Absorbance of SPI at 237 nm*
2	0.09	0.18
4	0.17	0.25
6	0.26	0.34
8	0.36	0.45
10	0.44	0.52
Slope	0.04	0.04
Intercept	0.09	-0.01
Correlation Coefficient (R^2)	0.98	0.99
LOD (μg mL^{-1})	0.76	1.99
LOQ (μg mL^{-1})	2.32	6.04

Precision

Repeatability measurements were carried out by analyzing six different solutions containing concentration 2, 6 and 10 μg mL^{-1} of SPI and FUR, % RSD was calculated. Precision were carried out by performing inter day and intraday variation. In inter day variation the sample was analyzed on three consecutive days.[21, 22] For determination of intraday variation the absorbance were measured three times in a day. Inter and intraday precision was determined using 2, 6 and 10 μg mL^{-1} concentration.

Intraday Precision

The intraday precisions were determined for solutions (2, 6 and 10 μg mL^{-1}) and were analyzed three times for the consecutive days (i.e. morning, afternoon, evening). Mean, standard deviation and % RSD was calculated and the results are shown in Table 2. Acceptance criteria: % RSD should not be more than 2.0.

Table 2. Results of intraday precision for FUR and SPI (n = 3).

Theoretical Concentration (µg mL^{-1})	FUR			SPI		
	Mean % Measured concentration	Mean Standard Deviation	Mean% RSD	Mean % Measured concentration	Mean % Standard Deviation	Mean % RSD
2	100.26	0.13	0.13	98.93	0.13	0.13
6	101.90	0.10	0.10	101.26	1.28	1.27
10	100.16	0.08	0.08	99.60	0.16	0.16
Overall mean % recovery	100.77			99.93		
Overall SD	0.11			0.53		
Overall % RSD	0.10			0.52		

*Each value is a mean of three observations.

Interday Precision

The interday precisions were determined for solution (2, 6 and 10 µg mL^{-1}) and were analyzed for the three times on different day. Percentage relative standard deviation (% RSD) was calculated and the results are shown in Table 3.

Acceptance criteria: % RSD should not be more than 2.0.

Table 3. Results of interday precision for FUR and SPI (n = 3).

Theoretical Concentration (μg mL^{-1})	FUR			SPI		
	Mean % Measured concentration	Mean Standard Deviation	Mean% RSD	Mean % Measured concentration	Mean % Standard Deviation	Mean % RSD
2	99.72	0.32	0.32	98.94	0.28	0.28
6	100.09	0.06	0.06	100.86	0.52	0.51
10	99.68	0.15	0.15	98.65	0.07	0.08
Overall mean % recovery	99.83			99.48		
Overall SD	0.17			0.29		
Overall % RSD	0.18			0.29		

*Each value is a mean of three observations.

Accuracy

The degree of accuracy of UV method and recovery studies were performed in triplicate by the standard addition method at 50%, 100% and 150% levels. The pre-quantified 10 μg mL^{-1} sample solution of SPI and FUR were spiked with an extra 50%, 100%, and 150 % of the standards SPI and FUR. Absorbances were measured at 237 and 275 nm and the concentration of drug was determined. These mixtures were analyzed by the developed method. The percentage recovery of the samples, % RSD and the percentage were calculated at each concentration level and results are shown in Table 4 and Table 5.

Table 4. Results of recovery studies (n = 3).

Level of Recovery in %	% Recovery		Mean		SD		% RSD	
	FUR	SPI	FUR	SPI	FUR	SPI	FUR	SPI
Level – 1 (0%)	99.20 99.30 99.20	102.20 102.10 102.30	99.23	102.20	0.05	0.10	0.06	0.09
Level – 2 (50%)	100.26 100.13 100.40	99.06 98.93 98.80	100.26	98.93	0.13	0.13	0.12	0.13
Level – 3 (100%)	101.90 101.80 102.00	99.80 101.80 102.20	101.97	101.26	0.10	1.28	0.09	1.26
Level – 4 (150%)	100.24 100.08 100.16	99.60 99.44 99.76	100.16	99.60	0.80	0.16	0.08	0.16
Overall mean % recovery			FUR SPI			100.38% 100.49%		
Overall SD			FUR SPI			0.09 0.41		
Overall % RSD			FUR SPI SPI			0.09 0.41 0.41		

*Each value is a mean of three observations

Table 5. Assay of marketed formulation.

Sr. No	Drug Label claim (mg)	Amount found (mg)	% Label claim	Over all mean	Over all SD
1	FUR (20mg)	19.92	99.60%	99.60%	0.05
	FUR (20mg)	19.93	99.65%		
	FUR (20mg)	19.91	99.55%		
2	SPI(50mg)	49.90	99.80%	100.26%	1.17
	SPI(50mg)	49.70	99.40%		
	SPI(50mg)	50.80	101.60%		

*Each value is a mean of three observations.

Limit of detection and Limit of quantification

Limit of detection (LOD) is the lowest amount of an analyte that can be detected but not necessarily as an exact value. Limit of quantification (LOQ) is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy [23]. Limit of detection (LOD) and limit of quantification (LQD) of UV method were calculated by using the values of slopes and intercepts of the calibration curves for FUR and SPI. The results of LOD and LOQ are shown in Table 2.

LOD and LOQ were based on the standard deviation of the response and the slope of the corresponding calibration curve using the equations.[23- 24] The following formulas were used for determination of LOD and LOQ.[25]

$$\textbf{LOD} = 3.3 \times \sigma/s, \qquad \textbf{LOQ} = 10 \times \sigma/s$$

Where, σ =Standard deviation of y intercept of regression lines (six independent measurements of a sample with very low concentrations), s =Slope of calibration Curve.

Figure of merits

Most of the reported methods were determined SPI and FUR by using UV/VIS simultaneous method. However Parimoo et al. and Naveed et al. have reported UV spectroscopic method for the single drug (FUR). The developed methods were not validated as per guidelines by Millership and Gahandule et al.Hence, the used method provided high recovery efficiencies for all the studied drugs. In addition, the best recoveries were obtained using methanol and water (80:20) increased method sensitivity. The developed method is less time consuming and cost effective method with high reproducibility than other. Although the extraction procedures were carried out in simple step, it is still easy to manipulate in a short time. Table 6 represents performances of the developed method in comparison with the

reported methods for analysis of SPI and FUR. These results pointed for the usefulness of the developed method in analysis of SPI and FUR in UV/VIS studies.

Table 6. Comparative Study of UV/VIS Method for FUR and SPI.

Method		Drug	Linearity µg mL^{-1}	LOD µg mL^{-1}	Solvent Used	% Recoveries	λ_{max} Used	Ref.
UV-Method	Absorbance maxima Method	FUR	5-25	-	Methanol	100.46	277 nm.	7
	Area Under the Curve Method	FUR	5-25	-		100.86	258.40 nm – 293.80 nm	
UV-Method	-	FUR	6.25-100	-	Water	103.45	276 nm	8
UV-Method	-	FUR		-	Ethanol	102.10	278nm	14
		SPI	-			101.40		
UV-Method		FUR	-	-	Methanol	100.50	276nm 238nm	15
		SPI				101.12		
UV-Method	First Order Derivative Method	FUR	2-10	0.82	Methanol	100.88– 101.46	350 nm 250.80 nm	16
		SPI	5-25	0.75		98.25– 100.00		
	Absorbance Ratio (Q – Absorbance) Method	FUR	2-10	0.02		99.24– 102.00	276 nm 261.21 nm	
		SPI	5-25	0.93		98.80-100.55		

Table 6. Continued.

UV-Method	Second Order Derivative Method	FUR	0.8-4.0	0.09	methanol in water(70:30)	99.40	277nm 238nm	17
		SPI	2–10	0.03		99.67		
	Area Under Curve Method	FUR	0.8-4.0	0.10		99.79	267.0 nm to 287.0 nm	
		SPI	2–10	0.17		100.12	228.0 nm to 248.0 nm	
UV-Method	Principal component regression Method	FUR	2.0-12.0	-	Methanol	101.36	272nm 235nm	18
		SPI	5.0-30.0			89.94		
UV-Method	Vierordt's method	FUR	2-10	0.76	double distilled water and ethanol (20:80)	99.23±0.057-101.97±0.10	275nm 237nm	This Study
		SPI	2-10	1.99		98.93±0.13-102.02±0.10		

Experimental

Chemicals and reagents

Spectroscopic grade of Methanol and Deionised water were filtered through 0.45 µm filter paper. Tablet used for analysis was Lasilactone manufactured by Sanofi India limited Ankleshwar-393002 containing equivalent weight of Furosemide and Spironolactone 20 and 50 mg respectively. API of Furosemide was kindly supplied as a gift sample by Yarrow Chemical Productions Mumbai (INDIA) 421201. Spironolactone was gifted by Aarti Pharma and Aarti Plastic Bhandup (West) Mumbai 400078.

Instrumentation

An UV-Visible double beam spectrophotometer JASCO V 630 with 1 cm matched quartz cells was used. All weighing operations were done on electronic balance (Model Shimadzu AUW-220D), Ultrasonicator model 5.0L150H was used.

Preparation of standard stock solution

Stock solution was prepared containing 10 mg of FUR and SPI pure drug in 100 mL volumetric flask. Then the volume were adjusted up to 100 mL with the ratio of double distilled water and ethanol (20:80), respectively to get a final concentration of 100 μg mL^{-1} for FUR and SPI.

Preparation of working solution

Suitable aliquots of 100 μg mL^{-1} solutions were diluted up to the mark with double distilled water and ethanol (20:80) to get the concentration range of 2, 4, 6, 8 and 10 μg smL^{-1} for FUR and SPI. The absorbances were recorded at 275 and 237 nm for FUR and SPI, respectively.

Preparation of working sample solutions of formulation

20 tablets of FUR and SPI combination were purchased from medical store Kasegaon - 415404 (INDIA). The tablet comprised of 50 mg of spironolactone and 20 mg of Furosemide. 20 tablets were weighed separately by removing the tablet coating and crushed uniformly with the help of mortar and pestle. Calculated an average weight of sample powder equivalent to 10 and 4 mg of SPI and FUR were transferred into 100 mL volumetric flask. Then 10 mL solvent containing double distilled water and ethanol (20:80) was added. The solution was sonicated for 10 min and volume was adjusted up to 100 mL with double distilled water and ethanol

(20:80) to get a concentration of SPI and FUR as 100 μg mL^{-1} and 40 μg mL^{-1} respectively. Then the solution was filtered through the Whatman's filter paper. Then 1 mL of the above solution was pipetted out and transferred in a 10 mL volumetric flask and diluted up to 10 mL using double distilled water and ethanol (20:80) to get final concentrations of 10 μg mL^{-1} for SPI and 4 μg mL^{-1} for FUR. The absorbance was measured for FUR and SPI at 275 nm and 237 nm respectively and simultaneous equation method used for determination of concentrations of both the drugs.

Selection of analytical wavelength

From the standard solution of FUR (100 μg mL^{-1}) and SPI (100 μg mL^{-1}), 10 μg mL^{-1} solution of FUR and SPI was prepared. The scanning for solution of FUR and SPI were carried out in the range of 200-400 nm against using double distilled water and ethanol (20:80) as a blank. The maximum absorption (λ_{max}) of FUR and SPI (Figure 2) was found at 275 nm and 237 nm respectively. Absorbances and absorptivity for a series of standard solutions were recorded at selected wavelengths. The two wavelengths selected should be such that at each wavelength the difference in the absorptivity between the two components should be as large as possible.

Determination

The standard solution of FUR was scanned in the range of 200-400 nm and the λ_{max} was found to be 275 nm against double distilled water and ethanol (20:80) (Figure 2).

Similarly, the standard solution of SPI was scanned in the range of 200-400 nm and the λ_{max} was found to be 237 nm against double distilled water and ethanol (20:80) (Figure 2). The absorbances of both the drugs were recorded at 275 nm and 237 nm and molar Absorptivity (Є) for both the drugs are shown in Table.7.

Table 7. Absorptivity data for FUR and SPI.

Sr. No	Conc. (µg mL^{-1})	FUR				Conc. (µg mL^{-1})	SPI			
		Abs at 275nm*	Abs at 237nm*	ax1	ax2		Abs at 275nm*	Abs at 237nm*	ay1	ay2
1	2	0.09	0.09	0.04	0.05	2	0.01	0.18	0.01	0.09
2	4	0.17	0.12	0.04	0.03	4	0.02	0.25	0.01	0.06
3	6	0.26	0.23	0.04	0.03	6	0.03	0.34	0.01	0.06
4	8	0.36	0.35	0.04	0.04	8	0.04	0.44	0.01	0.06
5	10	0.45	0.46	0.04	0.04	10	0.04	0.52	0.01	0.05

*Each value is a mean of three observations, ax1 = Absorptivity value of FUR at 275 nm, ax2 = Absorptivity value of FUR at 237 nm, ay1 = Absorptivity value of SPI at 275 nm, ay2 = Absorptivity value of SPI at 237 nm.

Simultaneous equation method by vierordt's method

In a multicomponent system consisting of two components uses the absorbance's ratio at a two selected wavelength, λ_{1max} is wavelength of one of the two components and λ_{2max} is wavelength of another remaining component. From the overlay spectra of two drugs, it is evident that FUR shown at 275 nm (A1) and the SPI shown at 237 nm (A2). From this exact amount of each drug was determined using simultaneous equation as mentioned as.

$$Cx = \frac{A2ay1 - A1ay2}{ax2ay1 - ax1ay2} \quad (1)$$

$$Cy = \frac{A1ay2 - A2ay1}{ax2ay1 - ax1ay2} \quad (2)$$

Where,

Cx = Conc^{n} of FUR (µg mL^{-1}), **Cy** = Conc^{n} of SPI (µg mL^{-1}), **ax1** = Absorptivity value of FUR at 275 nm, **ax2** = Absorptivity value of FUR at 237 nm, **ay1** = Absorptivity value of SPI at 275 nm, **ay2** = Absorptivity value of SPI at 237 nm, **A1** = Absorbance of sample at 275nm, **A2** = Absorbance of sample at 237nm**.**

Conclusion

This validated methodis new, rapid, accurate, precise, sensitive, and reproducible and can be employed for routine analysis for simultaneous estimation of Furosemide and Spironolactone in combined dosage form.

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Abbreviation

UV/VIS: Ultraviolet visible; ICH: International Conference on Harmonization; LOQ: Limit of quantitation; LOD: Limit of detection; RSD: Relative standard deviation; SPI: Spironolactone; FUR: Furosemide; SD: Standard deviation.

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