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Method development and validation of spectrophotometric and RP-HPLC methods for simultaneous estimation of spironolactone and furosemide in bulk and combined tablet dosage forms

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Abstract: The intent of the present work was to develop a simple, sensitive, accurate, precise, rapid and economical UV- spectrophotometric and reverse phase high pressure liquid chromatographic method for the simultaneous estimation of Spironolactone and Furosemide in bulk and combined tablet dosage forms. UV-Spectrophotometry was carried out by simultaneous equation method using 0.02 M potassium dihydrogen phosphate buffer pH 3.5: Acetonitrile (50:50) v/v as a solvent. The linearity range was 2-14 μg mL^{-1} for Spironolactone and Furosemide with a correlation coefficient > 0.99. The chromatographic separation was achieved on 250 mm × 4.6 mm, hypersil BDS C18 column with particle size 5 μm, by using an isocratic mixture of 0.02 M potassium dihydrogen phosphate buffer pH 3.5: Acetonitrile: tert butyl methyl ether (49:50:1) v/v/v as a solvent at a flow rate of 1 mL min^{-1} and UV detection was carried out at 254 nm. The retention time were observed to be 3.666 and 6.661 minutes for Furosemide and Spironolactone respectively. The two developed methods were validated according to the ICH guidelines for accuracy, precision, linearity, LOD, LOQ and were found to be within the limits. It can be concluded that these two methods could be successfully used for the simultaneous estimation of Spironolactone and Furosemide in bulk and combined tablet dosage forms.

Key words: UV-spectroscopic, RP-HPLC, spironolactone, furosemide

1. Introduction

Furosemide (FUR), is chemically 5 - (aminosulfonyl) - 4-chloro – 2 - [(2 furanylmethyl) amino] benzoic acid, a loop diuretic that has been used in the treatment of congestive heart failure and edema (*Fig.* 1(a)). FUR acts on thick ascending limb of the loop of Henle leading to a loss of sodium, potassium and chloride that are dispatched in the urine.[1] This results 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

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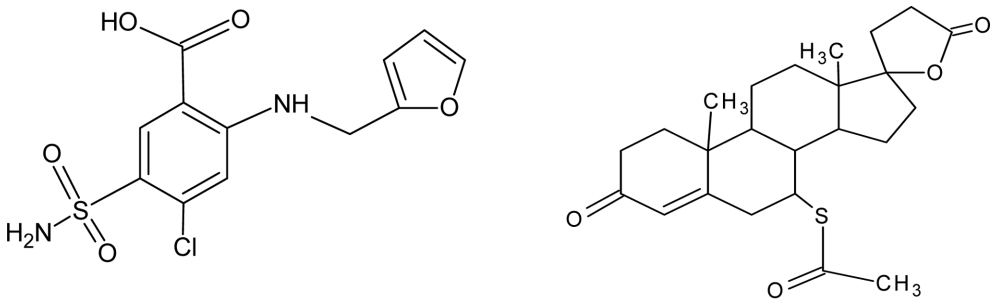

(a) (b)

Fig. 1. (a) Structure of Frusemide, (b) Structure of Spironolactone.

FUR appears within 30 minutes to 1 hour and is maximal in the first or second hour.[2]

Spironolactone (SPL) is chemically known as 17 – hydroxyl - 7α-mercapto – 3 – oxo - 17α-pregn – 4 – ene – 21-carboxylic acid γ-lactones acetate shown in (*Fig*. 1(b)).[3] SPL is most commonly used antidiuretic agent in the field of clinical practices. Its site of action is intracellular aldosterone receptors in the distal tubule cells. It increases the excretion of water and sodium and decreases the excretion of potassium.[4,5] FUR and SPL containing formulations are available in the market, which are widely used to treats oedema (fluid overload) associated with heart, liver, kidney or lung disease. Moreover, both drugs appears to be the preferred approach in achieving rapid natriuresis and maintaining normokalemia.[6] High doses of furosemide and spironolactone, or concomitant use of these diuretics, seem to be an important cause of hyponatremia in heart failure patients, particularly in combination with advanced age, diabetes and alcohol consumption.[7] We have selected the said combination for HPLC method development due to its high demand in global market. Literature survey revealed that analytical methods like UV,[5,8-15] RP-HPLC[8,16-20] have been used for the estimation of these drugs individually or in combination with other drugs. But very few methods UV.[21-24] RP-HPLC[9,13,25-27] are available for the simultaneous estimation of these drugs. In addition, it has been observed that the retention times for the said drugs are high, which also makes the method costlier due to requirement of excess solvent and time. Patel and Solanki[9] have reported that the retention time of Furosemide and Spironolactone was 3.81 min and 7.28 min, having a very big difference in resolution of the drugs. Bhojani *et al*.[22] have reported different linearity range for the FUR and SPL, which was the drawback of the method. Vadloori *et al*.[28] have reported good resolution between the two drugs but according to linearity range it proved to be a less sensitive method. Also some of the methods have used costlier mobile phase, whose pH adjusted below 2.5, which may possibly cause damage to the column. Therefore, it becomes prudent to develop newer analytical method(s) for routine analysis of these drugs, which helps to ensure the identity, purity, potency and performance of the drug in the dosage forms. The developed methods were validated according to International Conference on Harmonization guidelines for validation of analytical procedures.[29]

2. Materials and Methods

2.1. Chemicals and reagents

Spironolactone was gifted by Aarti Pharma and Aarti Plastic Bhandup (West) Mumbai-(INDIA) 400078. Furosemide was kindly supplied as a gift sample by Yarrow Chemical Productions Mumbai (INDIA) 421201. HPLC grade Acetonitrile and Methanol was purchased from Loba Chemicals private ltd. Analytical grade Potassium dihydrogen phosphate, o-phosphoric acid were of Research Lab, Mumbai, Filter paper was purchased from Research lab Islampur

(INDIA) 415409. All the chemicals for the analysis were freshly prepared, analyzed and used.

2.2. Instrumentation

UV-Visible double beam spectrophotometer JASCO V-530 made in Japan with 1 cm matched pair quartz cell and spectral bandwidth of 1 nm was used. HPLC system (Dionex) with variable wavelength detector (vwd) (model summit) was used. Chromeleon 6.8 SR11 as data processing software was used. Analysis was performed on 250 mm × 4.6 mm, hypersil BDS C_{18} column with internal particle size 5 μm. All weighing operations were performed by using an electronic balance (Model Shimadzu AUW-220D). Ultrasonicator model 5.0L150H was used.

2.3. Chromatographic condition

The mobile phase containing potassium dihydrogen phosphate buffer maintained at pH 3.5 using o-phosphoric acid and acetonitrile: tert butyl methyl ether in the ratio of 49:50:1 v/v/v was selected as the optimum composition of the mobile phase, as this solvent system ideally resolved the components. C_{18} (4.6 × 250 mm) Hypersil BDS was used as a stationary phase for the selected method. The flow rate was set to 1.0 mL min^{-1} and UV detection was carried out at 254 nm. The mobile phase and the sample were degassed by sonication for 10 min and filtered through 0.4 μm membrane filter paper. All the determinations were performed at a constant column temperature (25 °C).

2.4. Preparation of standard solutions

2.4.1. For UV method

10 mg of SPL and FRM were accurately weighed and transferred to a 10 mL volumetric flask separately. 7 mL of Phosphate buffer maintained at a pH of 3.5: Acetonitrile (50:50) v/v mobile phase was added to the both flasks and sonicated for 10 minutes. The final volume was adjusted thereafter to obtain 1000 μg mL^{-1} concentration of SPL and FRM. The stock solutions were subsequently prepared in 10 mL volumetric flasks to get the final concentration of 100 μg mL^{-1} of SPL and FRM.

2.4.2. For HPLC

100 mg API of SPL and FRM as a standard was weighed and transferred to 100 mL labelled volumetric flask. 70 mL of Phosphate Buffer pH 3.5: Acetonitrile: tert butyl methyl ether (49:50:1 v/v/v) mobile phase was added and subjected to sonication for 10 minutes. The final volume was adjusted to obtain concentration of 1000 μg mL^{-1} for SPL and FRM. The stock solutions were subsequently prepared in 10 mL volumetric flasks to get the final concentration of 100 μg mL^{-1} of SPL and FRM.

2.5. Preparation of working solution

2.5.1. For UV method

Suitable aliquots of standard stock solutions were diluted up to the mark with Phosphate Buffer (pH 3.5): Acetonitrile (50:50) to yield a concentration range of 2-14 μg mL^{-1} for SPL and FRM.

2.5.2. For HPLC Method

Suitable aliquots of standard stock solutions were diluted up to the mark with Phosphate Buffer (pH 3.5): Acetonitrile: tert butyl methyl ether (49:50:1 v/v/v) to yield a concentration range of 2-14 μg mL^{-1} for SPL and FRM.

2.6. Sample solution preparation

20 tablets of Fruselac (Lupin Ltd INDIA) were weighed and then crushed in a mortar using a pestle to obtain a fine powder. 50 mg and 20 mg equivalent weight of SPL and FRM respectively were accurately weighed and transferred to a 100 mL volumetric flask. 70 mL of mobile phase was added and the solution was sonicated for 15 minutes. The volume was adjusted up to the mark by addition of mobile phase. It was then filtered through Whatmann filter paper. The final volume was made up to the mark with the same solution to obtain sample stock solution of SPL (500 μg mL^{-1}) and FRM (200 μg mL^{-1}). Further, solution was filtered using 0.45 μm membrane filter.

2.7. Optimized analytical methods

2.7.1. UV Method

SPL and FRM (10 μg mL^{-1}) were scanned separately

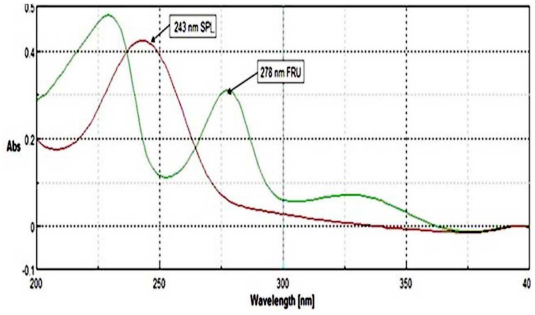

Fig. 2. Overlays of spironolactone and frusemide.

in a wavelength range of 200-400 nm against Phosphate Buffer (pH 3.5): Acetonitrile (50:50 V/V) as blank. The wavelength was selected for SPL and FRM at 243 and 278 nm respectively. The overlay spectrum of SPL and FRM is shown in *Fig.* 2.

2.7.2. For HPLC method

Finally the HPLC analysis was performed by using Hypersil BDS C_{18} column (4.6 × 250 mm) used as stationary phase. SPL and FRM were eluted with a flow rate 1.0 mL min^{-1} using mobile Phase Phosphate Buffer: Acetonitrile: tert butyl methyl ether in a proportion of 49:50:1 v/v/v, respectively. The detection wavelength was set at 254 nm and the sample concentration was used to be 10 μg mL^{-1} and 25 μg mL^{-1} for FRM and SPL respectively. The representative chromatogram is shown in *Fig.* 3.

2.8. Method validation

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

2.8.1. Specificity

It is the ability to assess unequivocally the analyte in the presence of impurities. For its determination, 12 μg mL^{-1} of blank, standard and sample solutions were injected separately and chromatograms were recorded under the optimized condition.

2.8.2. System suitability

1) For HPLC

System suitability tests are an important part of method development and are used to ensure adequate performance of the chromatographic system according to USP 24/NF 19 to confirm the reproducibility of the equipment adequate for the analysis.[30-31]

2.8.3. Linearity

1) For UV

The linearity of this method was evaluated by linear regression analysis and calculated by least square method and the drug showed linearity at the concentration of 2, 4, 6, 8, 10, 12 and 14 μg mL^{-1} for SPL and FRM.[32,33]

2) For HPLC

Linearity was demonstrated by analyzing six different concentrations of active compound. Peak areas were recorded for all peaks and calibration curve was constructed by plotting peak area versus concentration of SPL and FRM. Linearity experiment was performed six times to check the detector's response to be linear in function with various concentrations of drugs.[34]

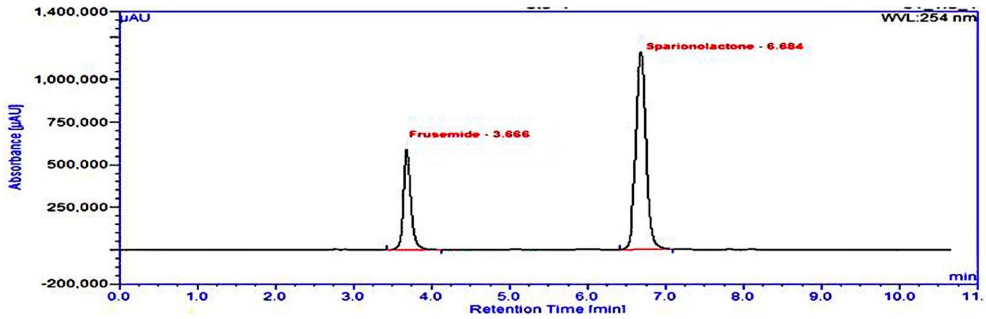

Fig. 3. Chromatogram showing well resolved peaks of SPL and FRM.

The linearity was studied using six concentrations at 2, 4, 6, 8, 10, 12 and 14 mg mL^{-1} of SPL and FRM. Several approaches are given in ICH guidelines to determine the detection (LOD) and quantification (LOQ) limits. In this study, LOD and LOQ were based on the standard deviation of the response and the slope of the corresponding calibration curve using the equations.[35]

2.8.4. Accuracy (% Recovery)

It is the measure of closeness between the actual value and the analytical value that is calculated by applying the test procedure for a number of times. Recovery was done at three different levels viz. 80 %, 100 % and 120 %, within the Beer's limit for both the drugs.[34] Previously analyzed tablet sample of concentration 10 μg mL^{-1} was spiked with known concentrations of the pure samples and then reanalyzed using the proposed methods.

2.8.5. Precision

Precision of the analytical procedure expresses the closeness of agreement (Degree of scatter) between series of measurement obtained from multiple sampling of the same homogeneous sample under prescribed condition.[35] Repeatability measurements were carried out by analyzing different solutions containing SPL and FRM and % RSD was determined. Precision was carried out by performing inter day and intraday variation.

2.8.6. Robustness

The study of robustness was carried out to evaluate the influence of small but deliberate variations in the chromatogram conditions on the determinations of both drugs. Robustness study was performed by changing the wavelength of both the drugs, changing the flow rate of both drugs, changing the composition of mobile phase and changing the temperature of the column.

2.9. Analysis of the marketed formulation

The chromatograms of the drug samples extracted did not show any change in the retention time and thus, correlated that there was no interaction between the drug and other excipients present in the marketed formulations. % RSD value indicated the suitability of the method for the routine analysis of SPL and FRM in the marketed formulation.

3. Results

The suitability of the method was validated by injecting 10 μg mL^{-1} of working standard solution into the system under the optimized chromatographic conditions prior to the analysis. System suitability parameters are shown in *Table* 1, which exhibited acceptable results of Peak area, Theoretical Plates, Tailing factor and Retention time of the SPL and FRM**.** Peak area, theoretical plates, tailing factor and retention time of SPL exhibited 208854, 10214, 0.92 and 6.66 respectively. In case of FRM Peak area, theoretical plates, tailing factor and retention time was observed to be 165471, 12451, 0.62 and 3.66 respectively.

The specificity of the method was validated by recording chromatograms of blank and sample under optimized analytical conditions and compared them with standard solution. It was observed that no additional peaks were found in sample solution, SPL and FRM peaks are completely separated in HPLC chromatogram even in the presence of other excipients (*Fig.* 4). The linearity parameter was employed for the UV/VIS and HPLC developed method. The response of the drug for the UV/VIS method was found to be linear in the investigated concentration ranges of 2-14 μg mL^{-1} for SPL and FRM. The response of the drug for the HPLC method was

Table 1. System suitability parameters of HPLC method

Parameter	Observations* (Mean±%RSD)	
	SPL	FRM
Concentration (μg mL^{-1})	10.00	10.00
Peak Area	208854	165471
Theoretical Plates	10214±1.2541	12451±1.0214
Tailing factor	0.92±1.5241	0.62±1.6254
Retention time	6.66±0.4215	3.66±0.9214

*Average of six readings

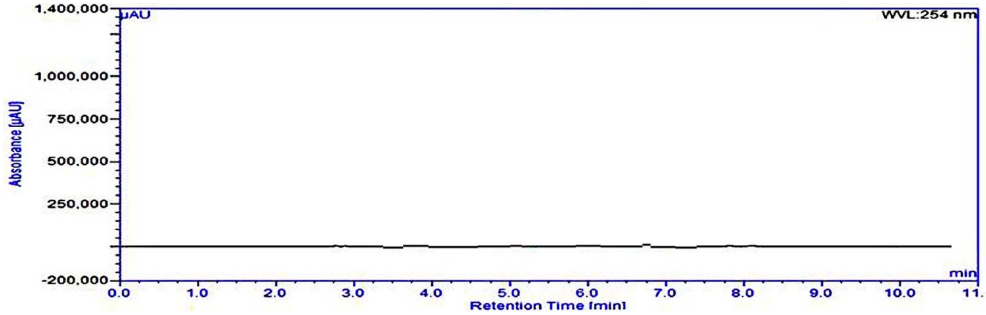

Fig. 4. A typical Chromatogram showing the specificity of SPL and FRM.

Table 2. Linearity values of SPL and FRM

Method	Parameters	SPL	FRM
UV	Regression Equation	y = 0.016x + 0.044	y = 0.027x + 0.044
	Linearity (µg mL^{-1})	2-14 µg mL^{-1}	2-12 µg mL^{-1}
	Correlation coefficient	0.9998	0.9991
HPLC	Regression Equation	y = 1025.2x + 2541.1	y = 254.2x – 1021.1
	Linearity (µg mL^{-1})	2-14 µg mL^{-1}	2-12 µg mL^{-1}
	Correlation coefficient	0.9997	0.9998

Table 3. Recovery values of SPL and FRM

UV method						
Drug	Recovery			% RSD		
	80 %	100 %	120 %	80 %	100 %	120 %
SPL	99.97	99.96	100.00	0.51	0.46	0.53
FRM	99.99	99.96	99.94	0.28	0.30	0.42
HPLC method						
Drug	Recovery			% RSD		
	80 %	100 %	120 %	80 %	100 %	120 %
SPL	100.25	99.85	100.31	1.254	1.254	0.254
FRM	100.78	98.65	100.21	1.021	0.954	0.854

Table 4. Precision values of SPL and FRM

Method	Drug	Concentration (µg mL^{-1})	Intraday (% RSD)	Interday (% RSD)
UV	SPL	2	0.44	0.45
		8	0.80	0.52
		12	0.74	0.68
	FRM	2	0.22	0.36
		8	0.26	0.45
		12	0.34	0.54
HPLC	SPL	2	0.25	0.21
		8	0.15	0.65
		12	0.85	0.57
	FRM	2	0.12	0.25
		8	0.19	0.78
		12	0.21	0.84

found to be linear in the investigated concentration ranges of 2-14 µg mL^{-1} for SPL and FRM. UV/VIS method was found to be linear; with correlation of coefficient at 0.9998 and 0.9991 for SPL and FRM, respectively (*Table* 2). Also, the HPLC method for SPL and FRM has shown the correlation coefficient at 0.9997 and 0.9998, respectively. Recovery studies were performed at three different levels viz. 80 %, 100 % and 120 %. Percentage recoveries for SPL and FRM are shown in *Table* 3 and chromatograms of these drugs for 80 %, 100 %, and 120 % are shown in *Fig.* 5. Obtained results of UV and HPLC developed method were found to be within the limits, in the range of 99.65-100.78 %, which indicated the accuracy of the UV and HPLC developed method. The precision of the UV and HPLC method was calculated in the term of % RSD as shown in *Table* 4.

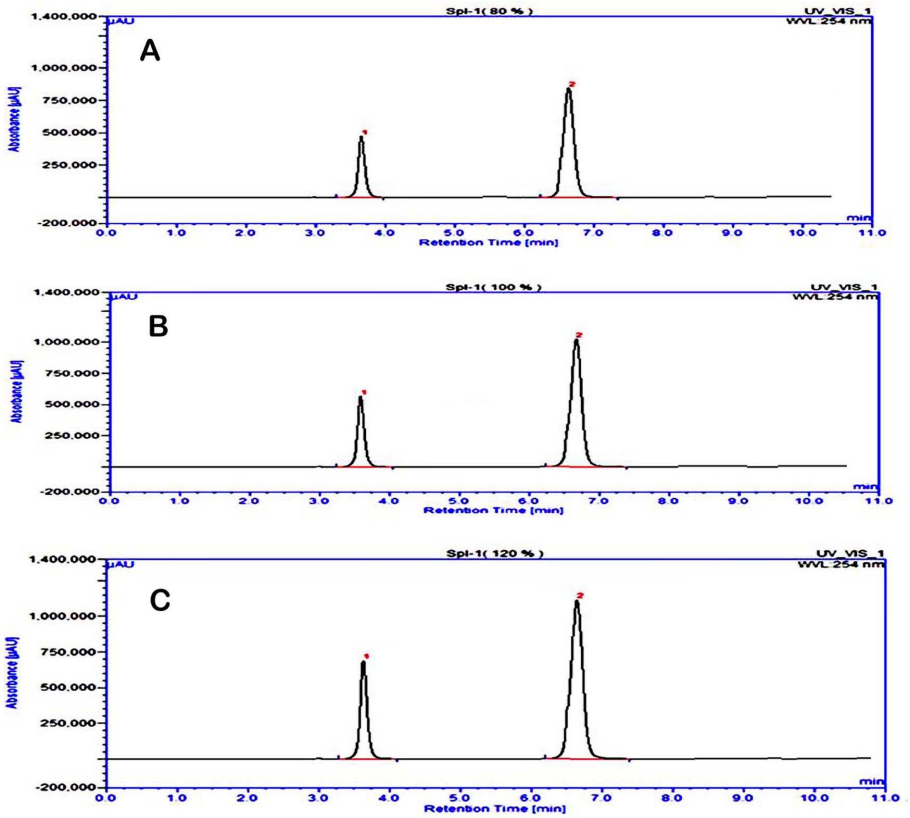

Fig. 5. Chromatogram of Spironolactone and Frusemide at A) 80 %, B) 100 % and C) 120 %

Table 5. LOD and LOQ of SPL and FRM

Method	Drug	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
UV	SPL	0.144	0.438
	FRM	0.122	0.371
HPLC	SPL	0.652	1.891
	FRM	0.921	1.352

In Inter day variation the sample was analyzed on three consecutive days for the both method. The results of interday and intraday precision of SPL and FRM were below 2 %, which indicated that both the developed methods are precise (*Table* 4). The LOD and LOQ were calculated using values of slopes and intercepts of the calibration curves for both the drugs as shown in *Table* 5. In the UV method, the LODs for SPL and FRM were found to be 0.144 and 0.122 μg mL^{-1}, while the LOQs for SPL and FRM were 0.652 and 0.921 μg mL^{-1}, respectively. In the HPLC method, the LODs for SPL and FRM were found to be 0.607 and 0.801 μg mL^{-1}, while the LOQs for SPL and FRM were 1.891 and 1.352 μg mL^{-1}, respectively. For robustness studies, conditions like flow rate and wavelength were changed and the method was performed in HPLC system. The results of robustness after changing the flow rate, wavelength and changing mobile phase composition are shown in *Table* 6 and respective chromatograms are depicted in *Fig.* 6, 7 and 8. The chromatograms of the drug samples extracted did not show any change in the retention time, which proved that the developed method is robust. The drug content was found to be 49.95 and 49.85 with a % RSD of 0.39 and 0.89 for SPL in UV and HPLC method, respectively. The drug content was found to be 19.99 and 19.98 with a % RSD of 0.31 and 0.53 for FRM in UV and HPLC method,

Table 6. Robustness parameters of SPL and FRM

Sr.No.	Parameter Flow Rate (mL min^{-1})	SPL	FRM
		Rt (Min)	Rt (Min)
1	0.8	6.678	3.654
2	1	6.665	3.687
3	1.2	6.691	3.612
Sr.No.	Parameter Wavelength (nm)	SPL	FRM
		Rt (Min)	Rt (Min)
1	252	6.667	3.667
2	254	6.648	3.641
3	256	6.685	3.632
Sr.No.	Parameter Composition of Mobile Phase (0.02 M potassium dihydrogen phosphate buffer (pH 3.5): acetonitrile: tert butyl methyl ether)	SPL	FRM
		Rt (Min)	Rt (Min)
1	47:53:1	6.671	3.670
2	49:50:1	6.661	3.663
3	51:48:1	6.651	3.642
Sr.No.	Parameter Temperature (°C)	SPL	FRM
		Rt (Min)	Rt (Min)
1	23	6.661	3.665
2	25	6.659	3.657
3	27	6.666	3.669

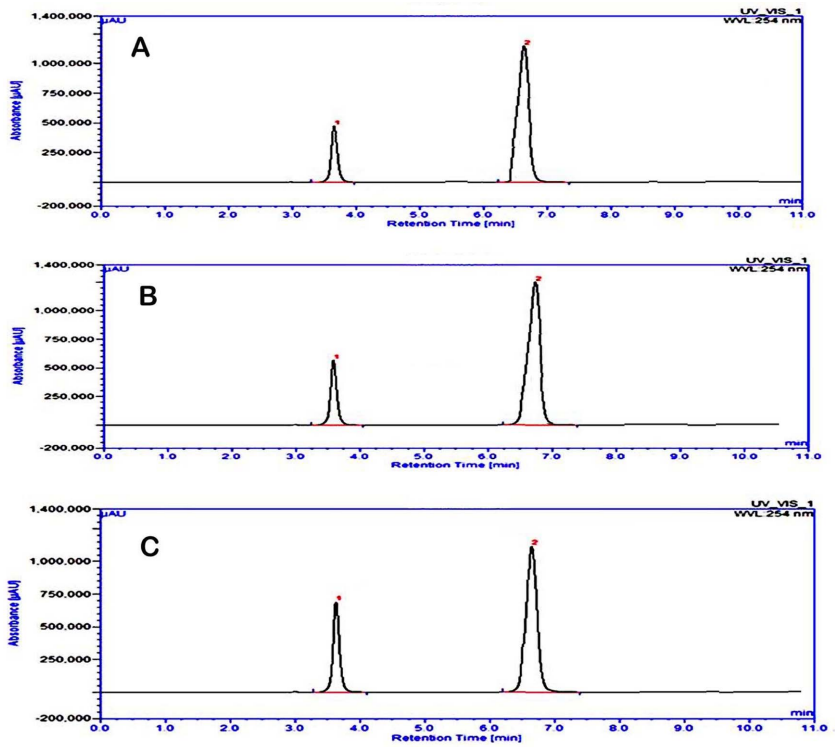

Fig. 6. Chromatogram of spironolactone and frusemide at flow rate A) 0.8 mL min^{-1}, B) 1 mL min^{-1} and C) 1.2 mL min^{-1}

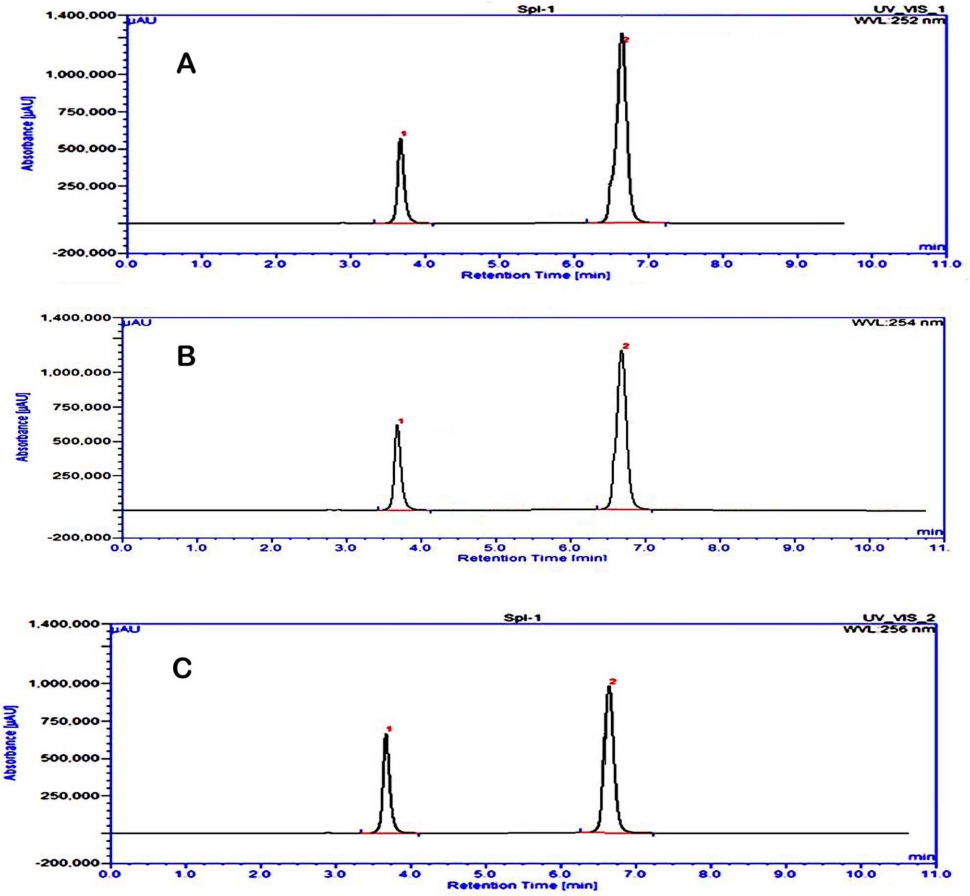

Fig. 7. Chromatogram of Spironolactone and Frusemide at A) 252 nm, B) 254 nm and C) 256 nm

respectively as shown in *Table* 7. The % RSD value indicated the suitability of the UV and HPLC method for the routine analysis of SPL and FRM in the marketed formulation.

4. Discussion

The present study aimed at development of an UV and HPLC method for the estimation of SPL and FRM. For optimizing good peak shape and absorbance for simultaneous estimation of SPL and FRM, several conditions were tried in the UV method. For the selectivity towards the SPL and FRM, we had tried several solvents with different compositions. Phosphate buffer maintained at pH at 3.5: Acetonitrile (50:50 v/v) component resulted in a better sensitivity. The methods discussed in the present work provide a convenient, sensitive, precise and accurate way for the simultaneously analysis of SPL and FRM from bulk and tablet dosage form by UV Spectrophotometry method. The wavelength was selected for SPL at 243 nm and FRM at 278 nm.

To optimize the RP-HPLC parameters, several chromatographic conditions were tried to get a good peak shape, low retention time with good resolution in SPL and FRM. Different compositions of mobile phase containing acetonitrile, methanol, water and buffer were tried to provide sufficient selectivity toward the SPL and FRM. Water and acetonitrile resulted in better sensitivity for the determination of SPL and FRM. As compared to water, phosphate buffer contributed high sensitivity and selectivity to

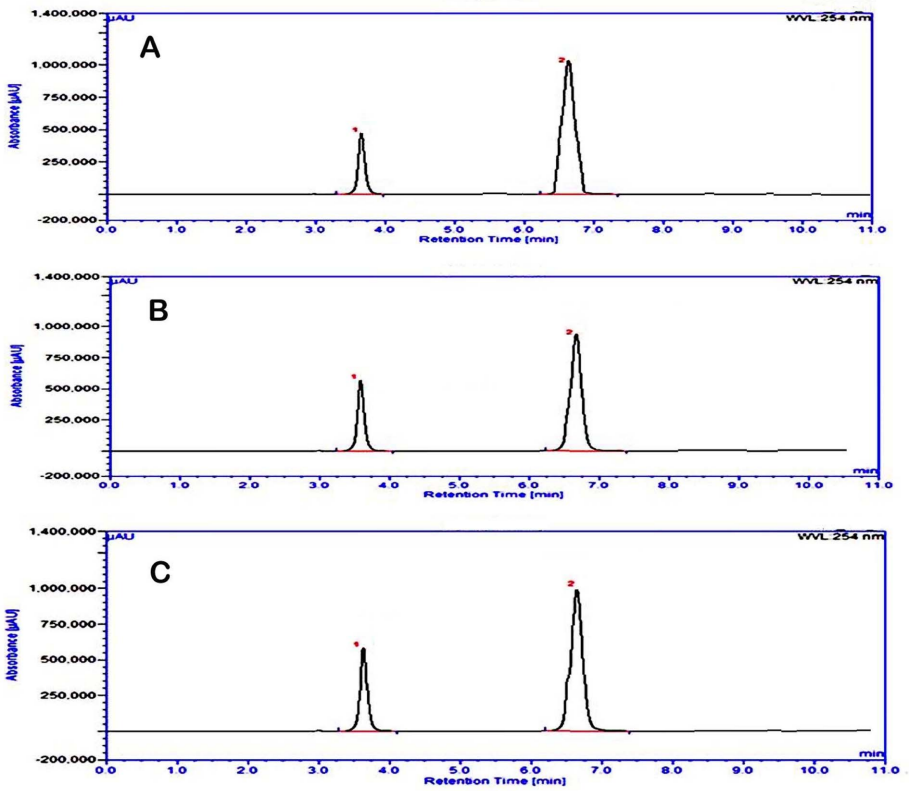

Fig. 8. Chromatogram of Spironolactone and Frusemide at A) 47:53:1, B) 49:50:1 and C) 51:48:1

Table 7. Assay data of the marketed formulation

Method	Drug	Amount labelled	Amount found*	% Assay	% RSD
UV	SPL	50 mg	49.95	99.83	0.39
	FRM	20 mg	19.99	99.95	0.31
HPLC	SPL	50 mg	49.85	99.70	0.89
	FRM	20 mg	19.98	99.90	0.53

analyze the drugs.[30,34,36] Thereafter, SPL peak showed tailing effect, therefore the pH of the buffer was adjusted at 3.5 using diluted ortho-phosphoric acid. The optimized mobile phase consisted of 0.02 M potassium dihydrogen phosphate buffer (pH 3.5): acetonitrile: tert butyl methyl ether (49:50:1 v/v/v). The column effluence was monitored at 254 nm. Injection volume was optimized to 10 µL. The column temperature was maintained at 25 ºC (ambient). Intersil C_{18} column (250 mm × 4.6 mm, particle size 5 µm) in isocratic mode at a flow rate of 1 mL min^{-1} was used. The retention time of approximately 3.661 ± 0.9214 and 6.66 ± 0.4215 min was consistently observed for SPL and FRM, respectively, throughout all the analytical runs. Changing of flow rate affected the resolution of SPL and FRM. Therefore, 1 mL min^{-1} was observed to be the ideal condition for the separation of SPL and FRM.

5. Figure of Merits

Patel *et al.*[13] and Bhojani *et al.*[21] have developed method using pure water as a mobile phase composition, however, the retention time of the drugs are

Table 8. Comparison of the developed HPLC method and the reported methods for analysis of SPL and FRM

Author Name(s)	Method	Linearity	LOD	Retention Time	% Recovery	Mobile Phase	Detection at λ_{max}	Reference Number
Patel H, Solanki S.	RP-HPLC	FRM-2-10 μg mL^{-1} and SPL-5-25 μg mL^{-1}	FRM-0.0025 μg mL^{-1} and SPL-0.00099 μg mL^{-1}	FRM - 3.73, SPL - 7.21 (Extra solvent peak was appeared)	FRM-99.25 %-101.45 % and SPL-99.1 %-100.26 %	Acetonitrile : Water (not mentioned proportion of mobile phase)	237 nm	11
Patel H, Solanki S.	UV	FRM-2-10 μg mL^{-1} and SPL-5-25 μg mL^{-1}	FRM-0.825 μg mL^{-1} and SPL-0.875 μg mL^{-1}	N/A	FRM-98.25-100.00 % and SPL-100.88 %-101.46 %	--	276 nm	12
Bhojani M, Dadhania K, Faldu S.	RP-HPLC	FRM-10-60 μg mL^{-1} and SPL-50-125 μg mL^{-1}	FRM-1.24 μg mL^{-1} and SPL-0.46 μg mL^{-1}	FRM-3.64, SPL-6.69	FRM-99.06-99.33 % and SPL -98.68 %-99. 16 %	Methanol:Water (70:30 v/v), pH 3.20±0.05	236 nm	19
Israt SS, Uddin MN, Jahan RA, Karim MM	UV Method	FRM-2-12 μg mL^{-1} and SPL-5-30 μg mL^{-1}	--	--	FRM-92.72 % and SPL-87.52 % -99.16 %	--	FRM-272 and SPL -235	20
Ram VR, Dave PN, Joshi HS	RP-HPLC	FRM-40-160 μg mL^{-1} and SPL-40-160 μg mL^{-1}	Not performed	FRM-around 2.8 min SPL-Around 7.2 min	FRM-98.05 and 100.17 % SPL-and 99.07 and 100.58 %	acetonitrile - 0.01M ammonium acetate buffer, pH 3.9 (50: 50, v/v).	254 nm	23
Chavan RR, Bhinge SD, Bhutkar MA, Randive DS	UV-HPLC	FRM-2 to 14 μg mL^{-1} SPL-2 to 14 μg mL^{-1}	SPL 0.144 and FRM-0.122 μg mL^{-1}	FRM-3.684 min and SPL-6.684 min	SPL-99.85 to 100.31 % FRM-99.65 to 100.78 %	0.02 M potassium dihydrogen phosphate buffer pH 3.5: Acetonitrile: tert butyl methyl ether (49:50:1) v/v/v	254 nm	Proposed Method

not stable in pure water. Therefore, most of the methods have used the buffer in the mobile phase to stabilize it and achieve reproducible retention time.[37] Moreover, Patel's method has observed an extra peak for the pharmacotherapeutic agent. Also they have not mentioned the details of the mobile phase ratio. Ram *et al.*,[24] has used limited validation parameter (excluding LOD, LOQ etc), which is an essential part of the method development process. Further, the linearity range was too high (FRM – 40-160 μg mL^{-1} and SPL - 40-160 μg mL^{-1}) than our method (2 to 14 μg mL^{-1} for FRM and SPL). The details are depicted in *Table* 8. Ram *et al*. has used short column i.e., 15 cm in length (SGE 150 3 4.6 mm SS Wakosil II 5C_8RS 5-mm column) as compared to our present method (C_{18} (4.6 × 250 mm) Hypersil BDS), which will change the retention time of the pharmacotherapeutic agent(s). Interestingly the retention time was not changed due to the phosphate buffer introduced in the mobile phase. Also we have obtained the sharp shape and lower tailing effect of the observed peaks than the method suggested by Ram *et al.* Method reproducibility at short times is of major concern in the tablet formulation. The validation results proved that the usefulness of the developed aqueous method in analysis of FRM and SPL by using different size of column along with routinely used buffers.

6. Conclusions

The two developed methods based on the spectrophotometry and RP-HPLC, were validated as per the ICH guidelines. The standard deviation and % RSD calculated for the developed methods indicated a high degree of precision. The results of recovery studies revealed a high degree of accuracy of the developed methods. From the experimental data, it can be concluded that the developed and validated methods are rapid, accurate, precise, sensitive and reproducible and can be employed for routine analysis for the

simultaneous estimation of Spironolactone and Furosemide in combined dosage form.

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Conflict of Interest

Authors have no conflicts of interest to declare.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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