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Stability indicating RP-HPLC method for the simultaneous determination of Spironolactone and Hydroflumethiazide

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ABSTRACT

A stability indicating RP-HPLC method was developed for the simultaneous determination of Spironolactone (SRL) and Hydroflumethiazide (HFM) in pharmaceutical dosage form. Inertsil ODS - C_{18} (250 mm x 4.6 mm, 5 µm) column and mobile phase of methanol: acetonitrile : phosphate buffer in the ratio of 55:40:05 v/v at a flow rate of 1.0 mL/min was used for separation of the components. The components were detected at a wavelength of 221nm using UV detector. The Spironolactone and Hydroflumethiazide were separated at retention time 4.67 and 6.74 min respectively. The developed method was validated in terms of precision, accuracy, linearity, specificity, limit of detection, limit of quantitation. The range of linearity was found to be 5-30 µg/mL for Hydroflumethiazide and 5-30 µg/mL for Spironolactone. The proposed method was applied to study the stability of the drugs under different degradation conditions such as acid, alkali, peroxide, thermal and photo light. The developed method was found to be simple, sensitive and rapid and hence, It can be adopted in any laboratory for quality control analysis.

Keywords: Spironolactone , Hydroflumethiazide, quality control, stability, validation.

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INTRODUCTION

Spironolactone:

Spironolactone[1,2] is known as 7α-Acetylthio-17α-hydroxy-3-oxopregn-4-ene-21-carboxylic acid γ-lactone sulfonamide with molecular formula $C_{24}H_{32}O_4S$ and molecular weight 416.574 g/mol. Spironolactone is official in BP, JP, EP and USP[3-6] and the molecular structure is presented in Figure 1. Spironolactone is a potassium-sparing diuretic (water pill) that prevents your body from absorbing too much salt and keeps your potassium levels from getting too low. Spironolactone is used to diagnose or treat a condition in which you have too much aldosterone in your body. Spironolactone also treats fluid retention (edema) in people with congestive heart failure, cirrhosis of the liver, or a kidney disorder called nephrotic syndrome. This medication is also used to treat or prevent hypokalemia (low potassium levels in the blood). Spironolactone is used to treat high blood pressure and heart failure. Lowering high blood pressure helps to prevent strokes, heart attacks, and kidney problems. It is also used to treat swelling (edema) caused by certain conditions (such as heart failure, liver disease) by removing excess fluid and improving symptoms such as breathing problems. This medication is also used to treat low potassium levels and conditions in which the body is making too much of a natural chemical (aldosterone). Spironolactone is also known as a "water pill" (potassium-sparing diuretic).

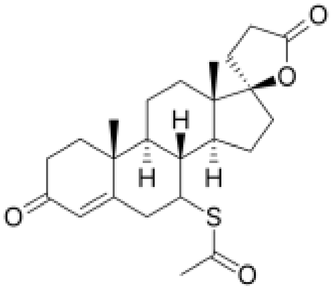

Figure 1: Molecular structure of Spironolactone

Hydroflumethiazide:

Hydroflumethiazide is chemically known as 1,1-dioxo-6-(trifluoromethyl)-3,4-dihydro-2H-1λ^6,2,4-benzothiadiazine-7-sulfonamide, The molecular formula and molecular weight of the drug are given as $C_8H_8F_3N_3O_4S_2$ and 331.292 g/mol respectively. Hydroflumethiazide is official in BP and USP [3-6] . Hydroflumethiazide is an oral thiazide which is used to treat hypertension and edema. High blood pressure adds to the workload of the heart and arteries. Hydroflumethiazide is a thiazide diuretic that inhibits water reabsorption in the nephron by inhibiting the sodium-chloride symporter (SLC12A3) in the distal convoluted tubule, which is responsible for 5% of total sodium reabsorption. Normally, the sodium-chloride symporter transports sodium and chloride from the lumen into the epithelial cell lining the distal convoluted tubule. The energy for this is provided by a sodium gradient established by sodium-potassium ATPases on the basolateral membrane. Once sodium has entered the cell, it is transported out into the basolateral interstitium via the sodium-potassium ATPase, causing an increase in the osmolarity of the interstitium, thereby establishing an osmotic gradient for water reabsorption. By blocking the sodium-chloride symporter, Hydroflumethiazide effectively reduces the osmotic gradient and water reabsorption throughout the nephron. If it continues for a long time, the heart and arteries may not function properly. This can damage the blood vessels of the brain, heart, and kidneys, resulting in a stroke, heart failure, or kidney failure. High blood pressure may also increase the risk of heart attacks. The molecular structure of Hydroflumethiazide is given in Figure 2.

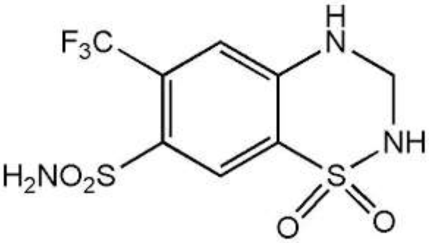

Figure 2: Molecular structure of Hydroflumethiazide

Literature review:

Different analytical methods [7-13] were reported in the literature for the determination of Spironolactone in combination with other drugs such as Hydrochlorothiazide, Chlorthalidone, Furosemide, Torsemide, Metolazone. One spectrophotometric method[14] and two HPLC methods [15,16] were reported for simultaneous quantitation of Spironolactone and Hydroflumethiazide in tablet dosage form. The determination of drugs under various stress conditions has not been carried out. Hence, the authors have attempted to develop stability indicating RP-HPLC method for the simultaneous determination of the two drugs.

MATERIALS AND METHOD

Instrumentation

Chromatographic separation was performed on a PEAK chromatographic system equipped with LCP7000 isocratic pump with variable wavelength programmable UV detector UV7000 and the output signal was monitored and integrated by PEAK chromatographic software version 1.06. Rheodyne injector with 20 µL fixed volume loop was used to inject the sample. Tec comp UV-2301 double beam UV Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Ultrasonicator was used to sonicate the mobile phase and samples. Denver electronic analytical balance (SI-234) was used for weighing the sample.

Standards and reagents

Hydroflumethiazide and Spironolactone working standard were obtained from Nicholas Piramal India Limited, Hyderabad. The formulation was taken from local market. HPLC grade methanol and water, analytical grade hydrochloric acid, sodium hydroxide, hydrogen peroxide were purchased from Merck Specialties Private Limited, Mumbai, India

Preparation of mobile phase and stock solutions

Preparation of working standard solution

Exactly 25 mg of Hydroflumethiazide and 25 mg of Spironolactone were accurately weighed, transferred into a 25 mL volumetric flask, dissolved in 15.0 mL of mobile phase, sonicated for 15 minutes and made up to the mark with water. Then 1.0 mL of the above solution was diluted to 10 mL with diluents to get concentrations of 100 µg/mL of each drug. After that 2.0 mL of the above solution was accurately transferred into a 10 mL volumetric flask, and made up to the mark with diluents to obtain the final concentration 20 µg/mL of Spironolactone and Hydroflumethiazide

Preparation of formulation solution

Average weight of ten Aldactide tablets of composition 25 mg of Spironolactone and 25 mg of Hydroflumethiazide was grinded to a fine, uniform size powder using mortar and pestle. An amount of the fine powder equivalent to one tablet (average weight) was accurately weighted and quantitatively transferred into 25 mL volumetric flask, approximately 15 mL of mobile phase was added and the solution was sonicated for 15 minutes and made up to volume the mark with mobile phase and mixed well. Then the solution was filtered through 0.45 µm nylon 6 ,6 membrane filter paper. Then above stock sample solution was diluted in series with mobile phase to obtain the final concentration of 20 µg/mL of Spironolactone and Hydroflumethiazide

Method development

The development of liquid chromatographic method was based on physic-chemical properties such as molecular weight, molecular formula, chemical structure, solubility, pK_a value and UV absorption maxima of selected drugs. The selected drugs were completely soluble in water and methanol; hence a reversed phase liquid chromatographic technique was adopted. The optimum chromatographic conditions were established by different trials by changing one of the chromatographic conditions such as column, mobile phase and its composition, flow rate of the mobile phase, injection volume, run time, column temperature and detection wavelength keeping other constant. Finally in the optimized chromatographic procedure, precisely 20 µL of the standard or sample was injected into Inertsil ODS C_{18} (4.6 x 150mm, 5.0µm) column which was kept at ambient temperature, mobile phase of methanol: acetonitrile : phosphate buffer of pH 5.5 in the ratio of 55:40:05 v/v allowed to flow through the column at a flow rate of 1.0 mL/min and the response of the detector was measured at a wavelength of 221 nm.

Method validation

The objective of validation of an analytical procedure is to demonstrate that the new method is suitable for its intended purpose. Assay procedures are intended to measure the analyte present in a given sample. In the context of this document, the assay represents a quantitative measurement of the major component(s) in the drug substance. For the drug product similar validation characteristics also apply when assaying for the active or other selected component(s).

System suitability

The system suitable parameters such as tailing factor, plate count, and resolution between two adjacent peaks were determined by injecting working standard solution of Spironolactone (SRL) and Hydroflumethiazide (HFM) in triplicate into the chromatographic system and chromatograms were obtained under the optimized chromatographic conditions. A typical HPLC chromatogram of standard was presented in Figure 3. The system suitable parameters were presented in Table 1.

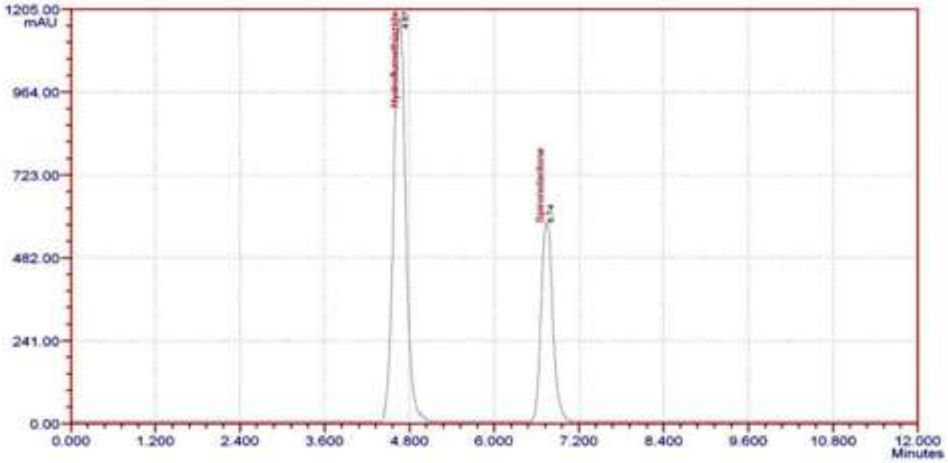

Figure 3: A typical HPLC chromatogram of HFM and SRL working standard

Table 1: System suitable parameters, retention time, peak area and peak height *

S.No.	Retention time	Peak area	Peak height	USP resolution	USP plate count	USP tailing
HFM	4.668	1384531	116736		5088	1.1
SRL	6.738	655503	57382	3.68	6935	1.16

*Mean of three values

Precision

Intraday precision

Precision is defined as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. To determine intraday precision, working standard solution of HFM and SRL was injected six times into the column; chromatograms were obtained as per the prescribed procedure. Mean and standard deviation of peak area of six replicate measurements were determined, and therefore percent of relative standard deviation (%RSD) was calculated. The results of system precision were presented in Table 2.

Inter day precision

To determine inter day precision, working standard solution of HFM and SRL was prepared as described in experimental section on different days; chromatograms were obtained as per the prescribed procedure by injecting the solution into the column. Mean and standard deviation of peak area of six replicate measurements were determined, and therefore %RSD was calculated, and the results of inter day precision were presented in Table 3

Table 2: Intraday precision of the developed method

S.No.	HFM	SRL
1	1393184	653989
2	1379766	656277
3	1381455	656274
4	1395303	657966
5	1378221	654329
6	1376994	656221
%RSD	**0.577**	**0.224**

Table 3: Inter day precision of the developed method

S.No.	HFM	SRL
1	1378384	658600
2	1387572	657651
3	1378899	650333
4	1388332	653844
5	1381630	653827
6	1379519	650703
%RSD	**0.322**	**0.524**

Linearity studies

Into a series of five 10 mL volumetric flasks, different aliquots (0.5 to 3.0 mL) of stock standard solution were accurately transferred, diluted up to the mark, injected each solution in duplicate into the column, chromatograms were recorded and measured the peak area. Linearity plots (Figure 4 and Figure 5) were drawn by taking peak area against concentration of the drugs, and calculated the slope, intercept and correlation coefficient, and were reported in Table 4.

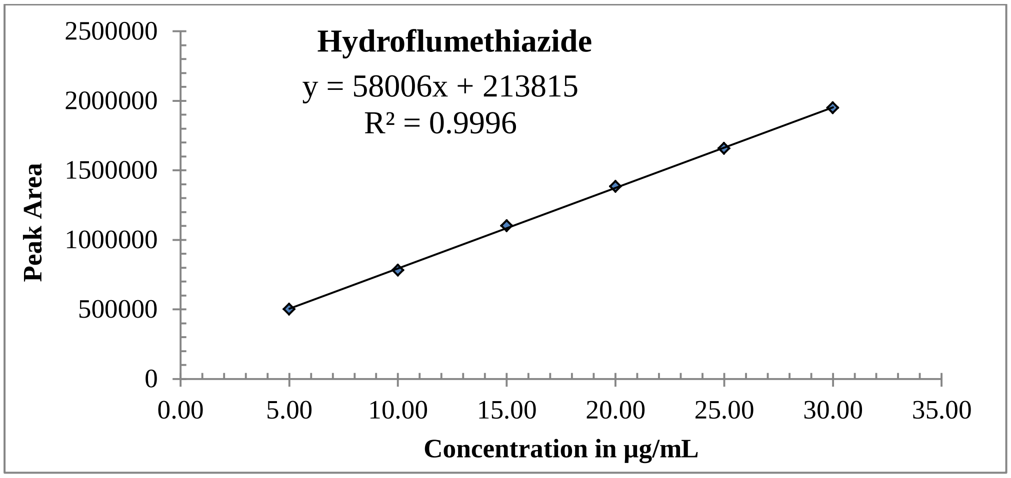

Figure 4: Linearity plot of Hydroflumethiazide

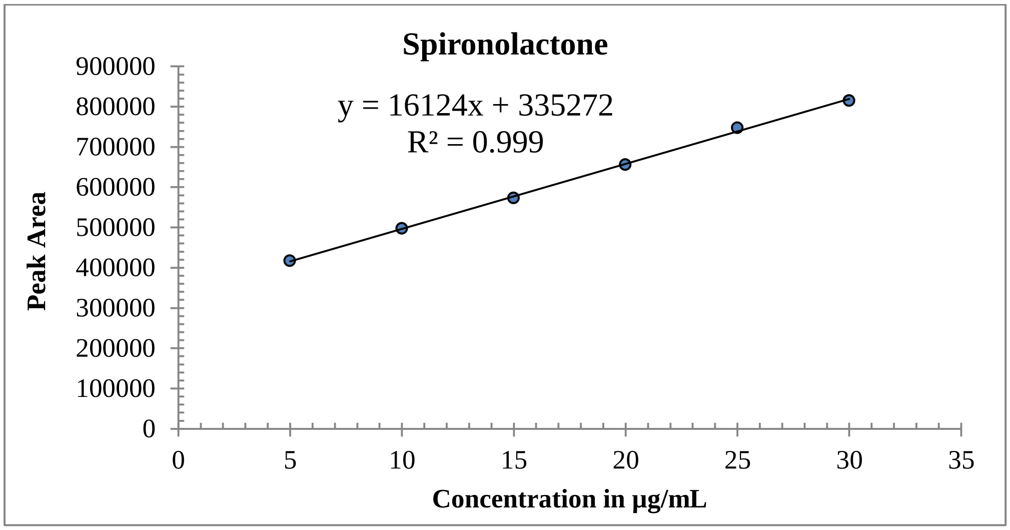

Figure 5: Linearity plot of Spironolactone

Table 4: Results of linearity studies

S.NO	Hydroflumethiazide		Spironolactone	
	Concentration in µg/mL	**Peak Area**	**Concentration in µg/mL**	**Peak Area**
1	5	501026	5	416819
2	10	781122	10	497343
3	15	1100858	15	573157
4	20	1384531	20	655503
5	25	1656989	25	746881
6	30	1948974	30	814978
	Slope	**58006**	**Slope**	**16124**
	Intercept	**21381**	**Intercept**	**33527**
	r^2	**0.999**	**r^2**	**0.999**

Sensitivity

Sensitivity of an analytical technique is defined as the instrument response to the low concentration of the analyte. The sensitivity is expressed in terms of limit of detection (LOD) and limit of quantization (LOQ), and were determined from the signal-to-noise ratio. The LOD and LOQ were calculated using the following formulae: LOD=3.0*SD /S and LOQ= 10.0*SD/S where SD=standard deviation of response (peak area) and S= average of the slope of the calibration curve. The results of sensitivity were given in Table 5

Table 5: Results of LOD and LOQ

Test	Hydroflumethiazide	Spironolactone
LOD	0.003µg/mL	0.05µg/mL
LOQ	0.01µg/mL	0.20µg/mL

Accuracy

Exactly 5/10/15 µg/mL of HFM and 5/10/15 µg/mL of SRL working standard solutions were prepared, each solution was spiked with sample solution of concentration 10 µg/mL to obtain the final concentration of 15/20/25 µg/mL, then each of the solutions were injected in triplicate into the column, chromatograms were obtained, calculated the percent of recovery. The results of recovery were presented in Table 6 and Table 7.

Table 6: Results of recovery of Hydroflumethiazide

S. No	Target	Spiked	Total	Amount found	% Recovery
1	10	5	15	14.93	99.53
2	10	5	15	15.01	100.08
3	10	5	15	15.20	101.37
1	10	10	20	20.06	100.31
2	10	10	20	20.04	100.22
3	10	10	20	20.03	100.13
1	10	15	25	24.87	99.46
2	10	15	25	25.08	100.35
3	10	15	25	25.02	100.10

Table-7: Results of recovery of Spironolactone

S. No	Target	Spiked	Total	Amount found	% Recovery
1	10	5	15	14.93	99.54
2	10	5	15	15.10	100.69
3	10	5	15	15.04	100.25
1	10	10	20	19.89	99.49
2	10	10	20	19.88	99.42
3	10	10	20	20.07	100.35
1	10	15	25	25.31	101.23
2	10	15	25	24.84	99.35
3	10	15	25	25.02	100.09

Robustness

In the study of robustness one of the parameters like flow rate, percentage of methanol in the mobile phase and pH of mobile phase were deliberately changed keeping other constant, chromatograms were recorded by injecting working standard solution of concentration level 20 µg/mL for Hydroflumethiazide and 20 µg/mL Spironolactone and system suitable parameters were evaluated. Experimental results of study of robustness were presented in Table 8.

Table 8: Results of robustness of the developed method

	Hydroflumethiazide at 20µg/mL		**Spironolactone at 20µg/mL**	
Condition	**Mean area**	**% difference**	**Mean area**	**% difference**
Standard	1384531	….	655503	….
More organic comp	1383571	0.069	658285	0.424
Less organic comp	1384622	0.007	659682	0.637
More wavelength	1387616	0.223	658454	0.450
Less wavelength	1388733	0.303	654680	0.126
More pH buffer	1386792	0.163	651756	0.572
Less pH buffer	1383366	0.084	656170	0.102

Stability studies

In order to test the stability of the drugs, sample solution of concentration 20 µg/mL of each drug was subjected to acidic, alkaline, oxidizing, thermal and photolytic degradation conditions. For acidic degradation, the sample was subjected to 0.1M HCl for 48 h and the mixture was negated. For alkaline erosion the drug was treated with 0.1 M NaOH for 48 h and the mixture was negated. For erosion under oxidizing conditions, the drug was exhibited for H_2O_2 for 48 h. For thermal degradation, the powdered drug was exhibited at 70° for 48 h. For photolytic degradation, the powdered drug was exposed to sunlight for 48 h. After completion, the solutions were liquidated and diluted with solvent mixture to furnish 20µg/mL solutions. Each solution was injected into the column; chromatograms were obtained under the optimized chromatographic conditions, measured peak area. The measured peak area was compared with peak area of standard chromatogram and percent of degradation was calculated and presented in Table 9. Ultimately, the drugs are mostly stable at the sunlight, peroxide, UV and aqueous condition and sensible in alkali and acidic conditions.

Table 9: Results of degradation of Hydroflumethiazide and Spironolactone

S.No.	**Condition**	**Number of addition peaks**	**% of Degradation**
1	Acidic	3	9.5
2	Base	3	10.2
3	Peroxide	2	11.3
4	Thermal	1	6.7
5	UV	2	9.2
7	Aqueous	0	0.51

Assay studies

Standard and sample stock solutions of concentration 100 µg/mL of HFM and 100 µg/mL of SRL were freshly prepared as per the procedure given in section preparation of solutions. Exactly 1.0 mL of standard and sample solutions were accurately transferred into two separate 10 mL volumetric flasks, diluted up to the mark with diluents, and then precisely 2.0 ml of each solution was transferred into two separate 10 mL volumetric flasks and made up to the mark; triplicate chromatograms were obtained under the optimized chromatographic conditions. A typical chromatogram of sample was presented in Figure 6. Percent of assay was determined from mean peak area of standard and sample. The results of % assay were presented in Table 10.

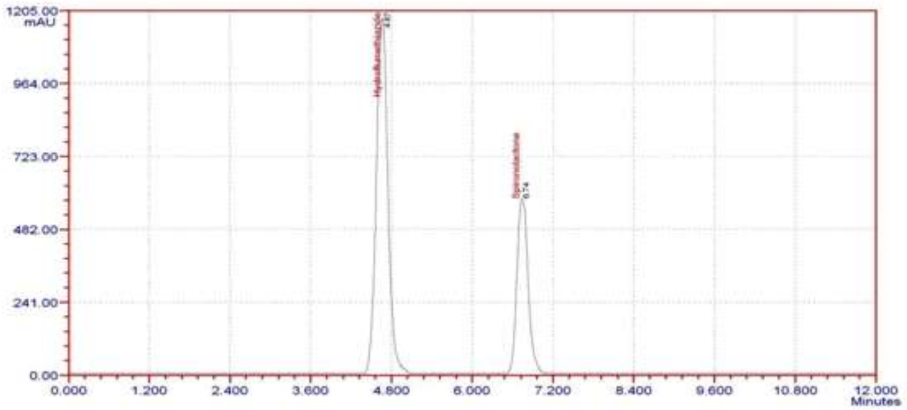

Figure 6: A typical HPLC chromatogram of HFM and SRL in sample

Table 10: Results of analysis of a marketed formulation

S.No.	Drug	Brand	Dosage	Amount Found	%Assay
1	Hydroflumethiazide	Aldactide	25 mg	24.74 mg	98.96
2	Spironolactone		25 mg	24.92 mg	99.68

RESULTS AND DISCUSSION

An isocratic mode RP-HPLC method was developed for the simultaneous determination of Spironolactone (SRL) and Hydroflumethiazide (HFM) in pharmaceutical dosage form, and to study the stability of the sample under different degradation conditions. Inertsil ODS - C_{18} (250 mm x 4.6 mm, 5 µm) column and mobile phase of methanol: acetonitrile: phosphate buffer in the ratio of 55:40:05 v/v at a flow rate of 1.0 mL/min was used for separation of the components. The components were detected at a wavelength of 221 nm using UV detector. The Spironolactone and Hydroflumethiazide were separated at retention time 4.67 and 6.74 min respectively. The developed method was validated as per the ICH guidelines. Intraday and inter day precision were determined and the %RSD values were found to be 0.577 & 0.224 and 0.322 & 0.524 respectively.

The results of precision were given in Table 2 and Table 3. Linearity study was carried out and it was found that peak area was found to be linear to the concentration of HFM and SRL in the range 5.0-30.0 µg/mL. Slope, intercept and correlation coefficient were determined, from the squared correlation coefficient; it was found that there was a good positive correlation between mean peak area and concentration. The results of linearity were presented in Table 4. The values of LOD and LOQ were found to be 0.003&0.05 and 0.01&0.20 µg/mL for HFM and SRL respectively. Study of accuracy was carried out at three different concentrations; percent of recovery was determined and found to be in between 99.42-100.69, and results of accuracy were presented in Table 6 and Table 7.

From the study of robustness, it was evident that there was no significant variation in system suitable parameter; the results of robustness were presented in Table-8. Stability of the drug sample was examined under forced degradation and found that the drug components were stable. The results of stability were presented in Table 9. Assay of HFM and SRL in tablet dosage form was determined and found to be 99.96 and 99.68 respectively. The results of assay were presented in Table 10.

CONCLUSION

The proposed method was found to be accurate, precise, sensitive and repeatable. The method was successfully applied for determination of drugs in a pharmaceutical formulation. The developed method was used to determine the stability of Hydroflumethiazide and Spironolactone under various forced degradation conditions viz. acid, base, dry heat, photolytic, neutral and UV degradation. It can be concluded that the method separates the drugs from their degradation products. Therefore it can be concluded that the proposed method may be adopted for assay, study of stability and quality control in any pharmaceutical laboratory.

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