"DEVELOPMENT AND VALIDATION OF RP - HPLC METHOD FOR ESTIMATION OF DAPAGLIFLOZIN AND SPIRONOLACTONE IN SYNTHETIC MIXTURE"

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ABSTRACT

This research article outlines the development and validation of a dependable reverse-phase high-performance liquid chromatography (RP-HPLC) method intended for the simultaneous analysis of Dapagliflozin and Spironolactone in a synthetic mixture. The method was optimized to achieve effective separation and quantification of both compounds by utilizing an appropriate stationary phase and mobile phase composition. Key parameters, including retention time, resolution, and peak symmetry, were thoroughly evaluated to ensure the method's reliability. Validation was performed following ICH guidelines, addressing factors such as specificity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). The results demonstrated strong linearity within a specified concentration range, as well as acceptable LOD and LOQ values. Overall, the developed RP-HPLC method proved to be efficient, reproducible, and suitable for routine quality control analyses.

KEY WORDS: Dapagliflozin, Spironolactone, Diabetes, Hypertension, RP - HPLC method, pharmaceutical analysis.

INTRODUCTION

Dapagliflozin, a sodium-glucose cotransporter 2 (SGLT2) inhibitor, is extensively utilized in treating type 2 diabetes mellitus (T2DM) because it effectively reduces blood glucose levels by facilitating the excretion of glucose through urine [1].

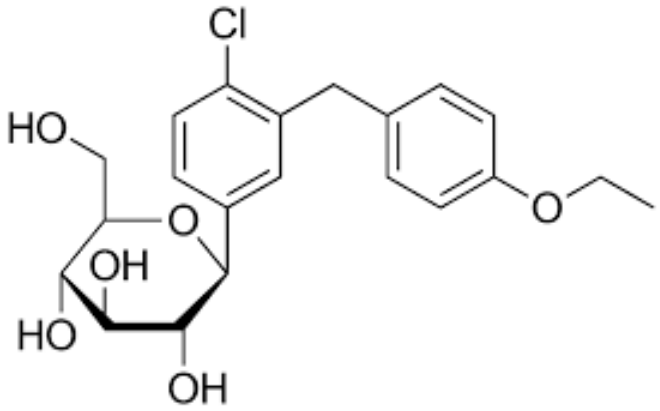
Moreover, it has been acknowledged for its cardiovascular advantages and protective effects on the kidneys, making it an important therapeutic choice for patients with concurrent health issues [2].

In contrast, Spironolactone is a potassium-sparing diuretic that primarily functions as an aldosterone antagonist. It is frequently prescribed for managing conditions such as high blood pressure, heart failure, and fluid retention related to liver cirrhosis [3] .The use of these two medications together can offer enhanced benefits, especially for individuals with both diabetes and heart failure, as they target various underlying physiological factors associated with these diseases. The concurrent measurement of Dapagliflozin and Spironolactone in a synthetic mixture is crucial for pharmacokinetic research and quality assurance in pharmaceutical products. Conventional analytical techniques like spectrophotometry and chromatography often fall short in sensitivity, specificity, and the capability to analyze multiple components at once. As a result, there is an increasing demand for reliable analytical methods that can effectively separate and quantify these substances in complex mixtures.

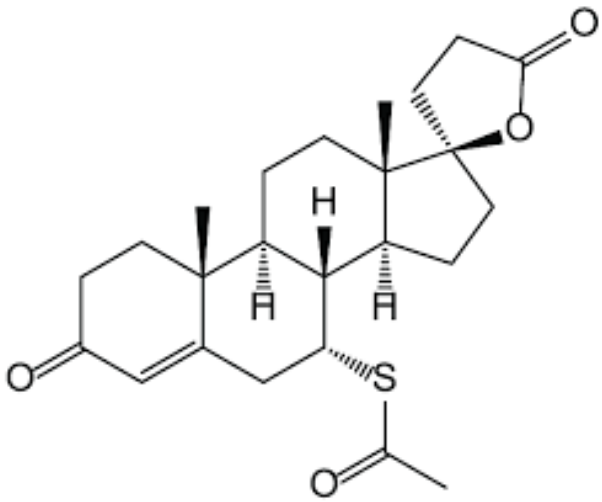
Reverse-phase high-performance liquid chromatography (RP-HPLC) has become a favoured technique because of its excellent resolution, rapid analysis, and consistent results. This method effectively separates compounds based on their hydrophobic characteristics, making it ideal for the analysis of various pharmaceuticals [4]. Establishing and validating an RP-HPLC method for the simultaneous quantification of Dapagliflozin and Spironolactone could greatly improve the efficiency of quality control procedures in pharmaceutical laboratories.

Validating analytical methods is essential to ensure compliance with regulatory standards and to guarantee dependable results. The International Council for Harmonisation (ICH) guidelines specify that validation should cover various parameters, such as specificity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) [5] . A thoroughly validated RP-HPLC method not only ensures the quality of pharmaceutical products but also aids in clinical studies focused on the pharmacokinetics and pharmacodynamics of these medications.

- **Dapagliflozin Structure**



- **Spironolactone structure**



This review article focuses on the creation and validation of a reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous determination of Dapagliflozin and Spironolactone in a synthetic mixture. We will detail the methodology used during the development phase, which includes the choice of stationary and mobile phases, optimization of chromatographic conditions, and validation criteria. Additionally, we will emphasize the importance of this analytical technique in maintaining the quality and effectiveness of pharmaceutical formulations that contain these crucial therapeutic compounds.

1. IDENTIFICATION STUDIES:

1.1 Identification of Pure API:

- Identification of pure API was carried out by Melting Point (M.P.), Solubility Study, FT-IR and UV Spectroscopy method.

EXPERIMENTAL WORK:

Instrument and Apparatus:

- Melting point apparatus
- Analytical weight balance (Wensar DAB-220)
- IR spectrophotometer (Shimadzu QATR-S)
- UV-Visible spectrophotometer (Shimadzu UV-1800)
- RP-HPLC(Shimadzu LC 2010 C_{HT})
- Sonicator (Equitron)

Chemicals and Materials:

- Standard API of Spironolactone (J.K. Chemicals)
- Standard API of Dapagliflozin (Corona Remedies)
- Solvent (AR grade)

- **Identification through Melting point determination:**
  - Melting point of Spironolactone and Dapagliflozin API has been determined by open capillary method using melting point apparatus in which the Spironolactone and Dapagliflozin API were filled in Capillary tubes and kept in the melting point apparatus.

Table 1: Melting point of Spironolactone and Dapagliflozin.

Sr no.	Drug	Reported melting point	Observed melting point
1	Spironolactone	198°C-207°C	200°C-205°C
2	Dapagliflozin	62°C-68°C	64°C-66°C

- **Identification by FT-IR with QATR Spectroscopy:**
- Identification of Spironolactone by FT-IR with QATR small quantity of Spironolactone was kept in the compartment of sample of FT-IR and it was scannedin the range of 4000-400 cm^{-1}

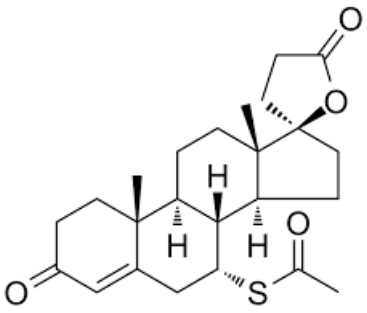


Figure 1.1 Structure of Spironolactone

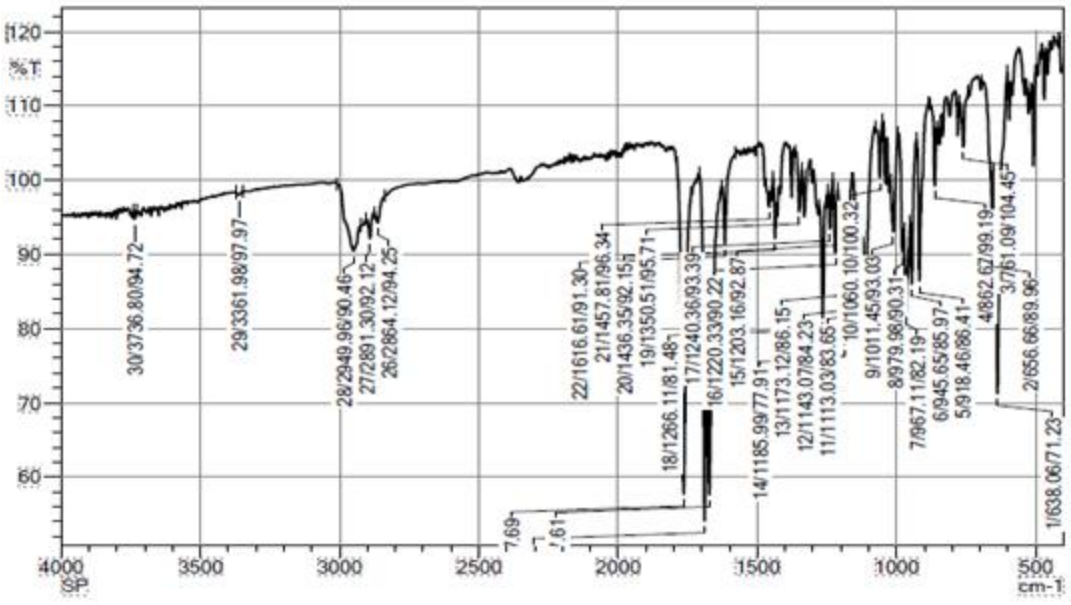


Figure 1.2 IR Spectra of Spironolactone

Table 1.2: Interpretation of FT-IR Spectra of Spironolactone.

Sr. no.	Functional Group Characteristics	Standard wave-number (cm^{-1})[57]	Observed wave-number (cm^{-1})
1.	C-O	1300-800	842.64
2.	C-C (Stretch)	750-1100	1027.19
3.	C-C (Aromatic)	1500-1600	1507.88
5.	C=C	1500-1900	1653.81
6.	C=O	1650-1780	1718.19

- Identification of Dapagliflozin by FT-IR with QATR small quantity of Dapagliflozin was kept in the sample compartment of FT-IR it was scanned in the range of 4000-400 cm-1.

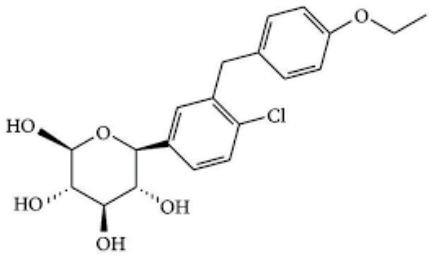


Figure 1.3 Structure of Dapagliflozin.

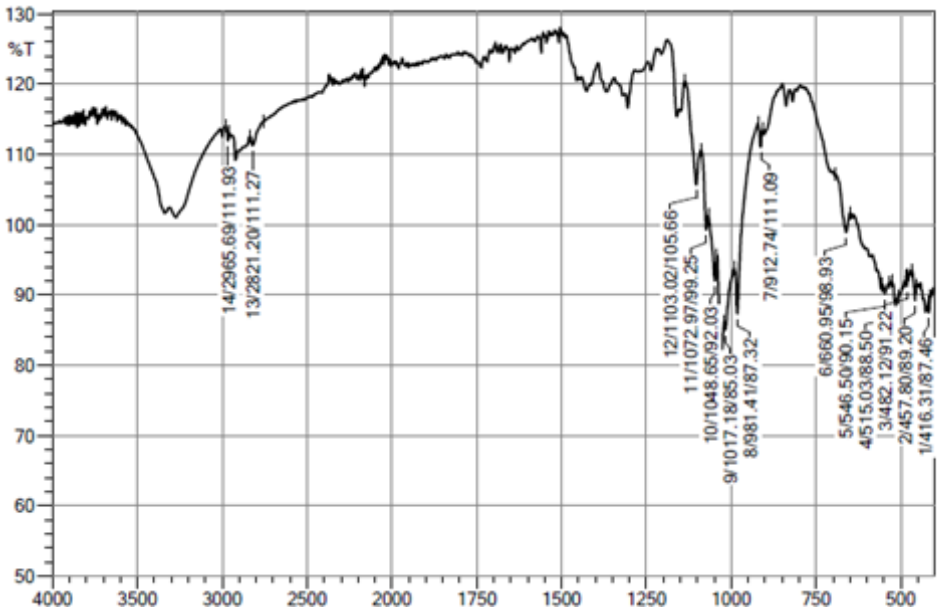


Figure 1.5 IR Spectra of Dapagliflozin

Table 1.3: Interpretation of FT-IR Spectra of Dapagliflozin.

Sr no.	Functional Group Characteristics	Standard wave-number (cm^{-1})[57]	Observed wave-number (cm^{-1})
1.	C-S	880-1030	914.17
2.	C-C (Stretch)	750-1100	1022.90
3.	C-C (Aromatic)	1500-1600	1559.39
4.	C=C	1500-1900	1772.55
5	O-H	3500-3300	3412.39
6	C-Cl	1000-700	981.28

1.1.2 Identification by Solubility Determination:

- The solubility study of Spironolactone and Dapagliflozin were practically determined taking 1 mg of drug in 10 ml volumetric flasks, adding required quantity of solvent at room temperature and shaking volumetric flask for few minutes.

Table 1.4: Solubility data of Spironolactone and Dapagliflozin

Solvent	Spironolactone	Dapagliflozin
Distilled water	Insoluble	Soluble
Methanol	Soluble	Soluble
ACN	Slightly Soluble	Soluble
0.1 N HCl	Soluble	Soluble
0.1 N NaOH	Soluble	Soluble

- Based on solubility study, in concluded that Spironolactone and Dapagliflozin were freely soluble in ACN, so ACN was selected as a solvent for further estimation of the drugs by UV spectroscopy method for identification.

1.1.3 Identification by UV-visible spectrophotometer

Preparation of Stock Solution

- Accurately weighed Spironolactone (10 mg) was transferred to a 100 ml volumetric flask, dissolved in Methanol and diluted to the mark with same solvent to obtain a

standard stock solution (100µg/ml).

- From the above 100µg/ml stock solution pipetted out 1 ml of solution and transferred to a 10 ml volumetric flask and make up volume up to 10 ml with Methanol to produce concentration10 µg/ml respectively.
- Accurately weighed Dapagliflozin (1 mg) was transferred to a 100 ml volumetric flask, dissolved in Methanol and diluted to the mark with same solvent to obtain a standard stock solution (10µg/ml).
- From the above 10µg/ml stock solution pipetted out 4 ml of solution and transferred to a 10 ml volumetric flask and make up volume up to 10 ml with Methanol to produce concentration 4 µg/ml respectively.
- The solutions were scanned in the range 200-400nm and λmax found to be 227 nm for Spironolactone and 246 nm for Dapagliflozin which match standard λmax of Spironolactone and Dapagliflozin.

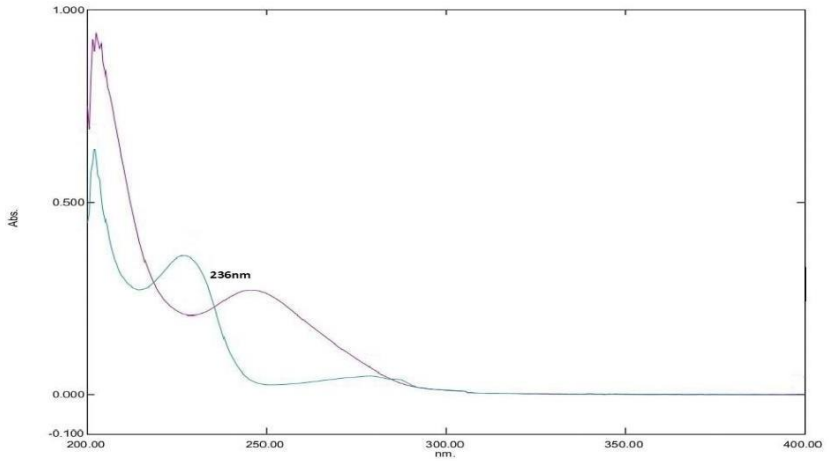


Figure 1.8 Overlay Spectra of Dapagliflozin and Spironolactone.

Table 1.5: Identification by UV Visible Spectrophotometer.

Drug	Standard λmax	Observed λmax
Spironolactone	229 nm	227 nm
Dapagliflozin	245 nm	246nm

Conclusion:

- Based on the data observed in IR, UV, Melting Point and Solubility Study it was concluded that both the API of Spironolactone and Dapagliflozin matched with the standard API data.
- Based on solubility it concluded that both the drugs are soluble in Methanol. So, Methanol was selected for the simultaneous estimation of Spironolactone and Dapagliflozin for RP-HPLC method.

STABILITY INDICATING RP-HPLC METHOD

2. STABILITY INDICATING RP-HPLC METHOD FOR SPIRONOLACTONE AND DAPAGLIFLOZIN:

EXPERIMENTAL WORK

2.1 Instrument and Apparatus:

- HPLC (Shimadzu)
  Model: HPLC_2010-C_{HT}
  Column: Shimadzu C18, (250×4.6 mm, 5 µm)
  Detector: UV-Detector
  Software: Lab Solutions
- Analytical Weighing Balance (Wensar DAB-220)
- Sonicator (Equitron)
- Digital pH Meter (Systronic)
- UV-Visible Spectrophotometer (Shimadzu UV-1800)
- High vacuum pump (Parag Engineering)
- Volumetric Flask- 10, 50, 100 ml (Borosilicate)
- Pipettes- 1, 2, 5, 10 ml (Borosilicate)

2.2 Chemicals and Materials:

- Spironolactone (J.K. Chemicals)
- Dapagliflozin (Corona Remedies)
- Methanol, Acetonitrile, Water (HPLC Analytical Grade)
- Ortho-phosphoric Acid (Analysis Purpose)

2.3 Selection of Detection Wavelength:

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. At 236nm both drugs seem to give good peak height and shape. Therefore 236nm was selected at the wavelength for detection of Spironolactone and Dapagliflozin.

2.4 Preparation of Mobile phase:

ACN, methanol and HPLC grade water was transferred in 1000 ml beaker, filtered through 0.45 µm nylon filter and sonicate for 15 minutes adjusted pH of water

through orthophosphoric acid.

- **Mobile phase:** ACN:Methanol:Buffer (35:35:30) 4.0pH adjusted with 10% v/v orthophosphoric acid.
- **Preparation of 10% ortho phosphoric acid:**

  10% ortho phosphoric acid was prepared by diluting 1 ml of concentrated ortho phosphoric acid into 10 ml HPLC grade water.

2.5 Preparation of Standard Solutions:

- **Preparation of standard solution of Spironolactone:**

  Accurately weigh 10 mg of Spironolactone API was transferred into 100 ml volumetric flask and diluted with mobile phase.

- **Preparation of standard solution of Dapagliflozin:**

  Accurately weigh 1 mg of Dapagliflozin API was transferred into 100ml volumetric flask and diluted with mobile phase.

2.6 Preparation of Calibration Curve:

- Stock solution of Spironolactone (100µg/ml) 0.25, 0.5, 0.75, 1.0, 1.25, 1.5 ml and Dapagliflozin (10µg/ml) 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 ml were pipette out in six different volumetric flasks further dilute with mobile phase to obtain the concentration of 2.5, 5, 7.5, 10, 12.5, 15 µg/ml of Spironolactone and 1, 2, 3, 4, 5, 6 µg/ml of Dapagliflozin.
- 20µl of each solution were injected into HPLC system and analyzed to obtain the respective peak area v/s concentration in µg/ml and regression equation was obtained.

2.7 Chromatographic Condition:

- **Column:** Shimadzu C18, (250 × 4.6mm, 5 µm)
- **Flow Rate:** 1 mL/min
- **Detection Wavelength:** 236 nm
- **Injection Volume:** 20 µl
- **Run Time:** 10 min
- **Retention Time:**

Spironolactone: 8.4 min

Dapagliflozin: 4.0 min

2.8 FORCE DEGRADATION STUDY:

2.8.1 Acid Degradation:

Weigh a sufficient quantity of synthetic mixture powder containing 10 mg of Spironolactone equivalent and 1 mg of Dapagliflozin equivalent, and transfer it into a 100 mL volumetric flask and made up to volume with mobile phase up to the mark. Take appropriate aliquot from the above solution of Synthetic mixture in 10 ml volumetric flask and then made up the volume with 0.1 N HCl up to the mark to obtain final concentration of spironolactone and Dapagliflozin of 10 µg/mL and 4 µg/mL respectively. Then put it into dark for reflux at 30˚C for 1.5 hrs. After reflux take 1ml from this and add 1ml of 0.1N NaOH to neutralize the solution and make up the volume with mobile phase.

2.8.2 Alkali Degradation:

Weigh a sufficient quantity of synthetic mixture powder containing 10 mg of Spironolactone equivalent and 1 mg of Dapagliflozin equivalent, and transfer it into a 100 mL volumetric flask and made up to volume with mobile phase up to the mark. Take appropriate aliquot from the above solution of Synthetic mixture in 10 ml volumetric flask and then made up the volume with 0.1 N NaOH up to the mark to obtain final concentration of spironolactone and Dapagliflozin of 10 µg/mL and 4 µg/mL respectively. Then put it into dark for reflux at 30˚C for 1 hrs. After reflux take 1ml from this and add 1ml of 0.1N HCl to neutralize the solution and make up the volume with mobile phase.

2.8.3 Oxidative Degradation:

Weigh a sufficient quantity of synthetic mixture powder containing 10 mg of Spironolactone equivalent and 1 mg of Dapagliflozin equivalent, and transfer it into a 100 mL volumetric flask and made up to volume with mobile phase up to the mark. Take 5 ml from the above solution of Synthetic mixture in 10 ml volumetric flask and then made up the volume with 3% H_2O_2 up to the mark. Then put it into dark for reflux at room temperature for 2 hrs. After reflux take 1ml and add 1ml of methanol to neutralize the solution and make up the volume with mobile phase.

2.8.4 Thermal Degradation:

For dry heat degradation the sample were placed in the oven at 40˚C for 8 hrs under the dark condition and then cooled at room temperature. Degradation sample were subjected to analysis after dilution with the mobile phase.

2.8.5 Photolytic Degradation:

For photolytic degradation the sample was put in the UV chamber with UV lamp (200-Wh/m2) at 240 nm for 1 day. Degradation sample was analyzed after its dilution with mobile phase.

2.9 RESULT AND DISCUSSIONS:

2.9.1 Selection of Elution mode

Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also better performing in terms of efficiency, stability and reproducibility. C18 column is least polar compare to C4 and C8 columns. Isocratic mode was chosen due to simplicity in application and robustness with respect to longer column stability.

2.9.2 Selection of Mobile phase

The composition and flow rate of mobile phase were changed to optimize in the separation condition using combined solution. The pKa value for Spironolactone is 17.05 and Dapagliflozin is 8.7. After number of trial experiments, it was establishing that the mobile phase ACN:Methanol:Buffer (35:35:30 %V/V) (pH-

4 adjusted with ortho phosphoric acid) shows good peak shape and resolution. Under this optimized chromatographic condition, the retention time of Spironolactone was 8.4 min and Dapagliflozin was 4.0 min. The retention time was confirmed by injecting working standard solution. Various trials for optimization of mobile phase are shown in table.

Table 2.1: Mobile phase Optimization trials of Spironolactone and Dapagliflozin

Trial	Mobile Phase	Ratio %(v/v)	Remark
1.	Methanol:Phosphate Buffer (pH 3.5)	75:25	Both the peaks got splitted.
2.	ACN:Methanol	60:40	Both the peak got splitted and merged together.
3.	ACN:Methanol:Phosphate Buffer (pH3.5)	60:20:20	One peak was obtained to be sharp but other peak got splitted.
4.	ACN:Methanol:Phosphate Buffer (pH 4)	40:40:20	Both peak were obtained to be sharp but one peak had more tailing.
5.	ACN:Methanol:Phosphte Buffer (pH 4)	35:35:30	Both peaks obtained were sharp, had acceptable tailing (<2) and retention time.

Trial 1- Methanol: Phosphate Buffer (75:25 v/v) at flow rate 1 ml/min pH 3.5

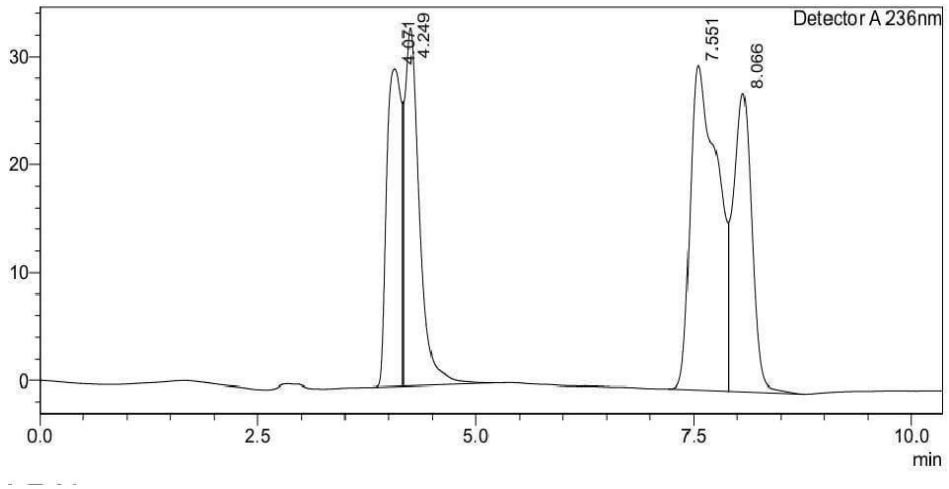


Figure 2.1

Trial 2- ACN:Methanol (60:40 v/v) at flow rate 1ml/min.

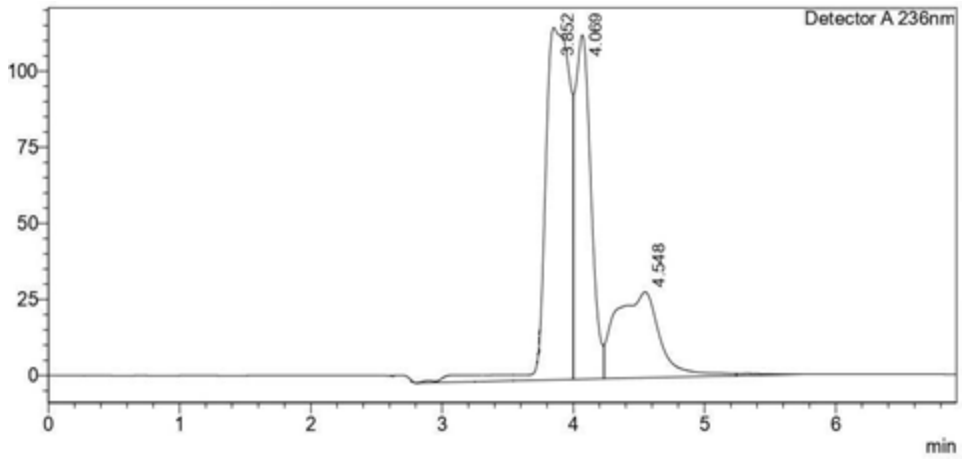


Figure 2.2

Trial 3- ACN:Methanol:Phosphate Buffer (60:20:20 v/v/v) at flow rate 1ml/min. pH 3.5

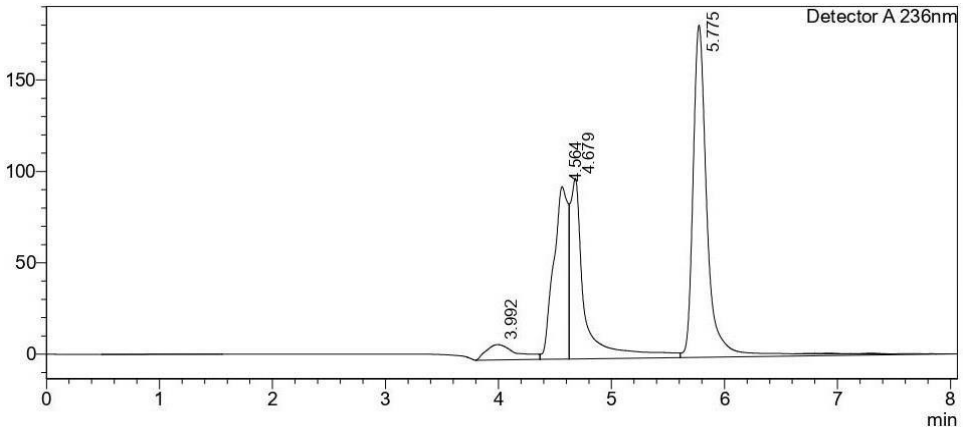


Figure 2.3

Trial 4- ACN:Methanol:Phosphate Buffer (40:20:20 v/v/v) at flow rate 1ml/min pH 4.0

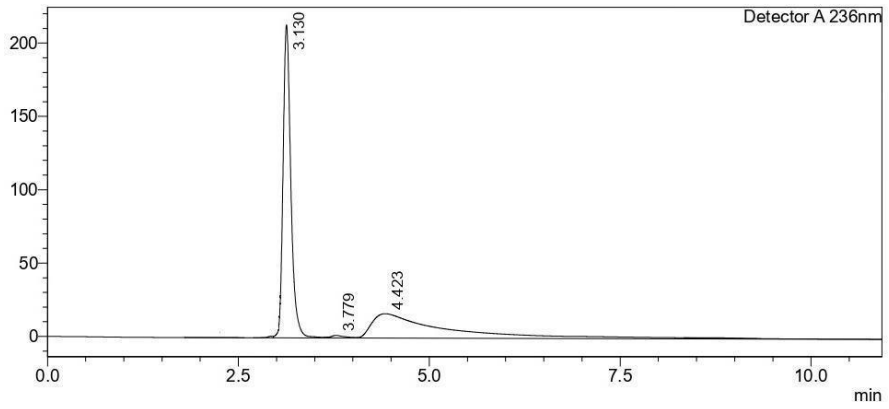


Figure 2.4

Trial 5- ACN:Methanol:Phosphate Buffer (35:35:30 v/v/v) at flow rate 1ml/min. pH 4.0

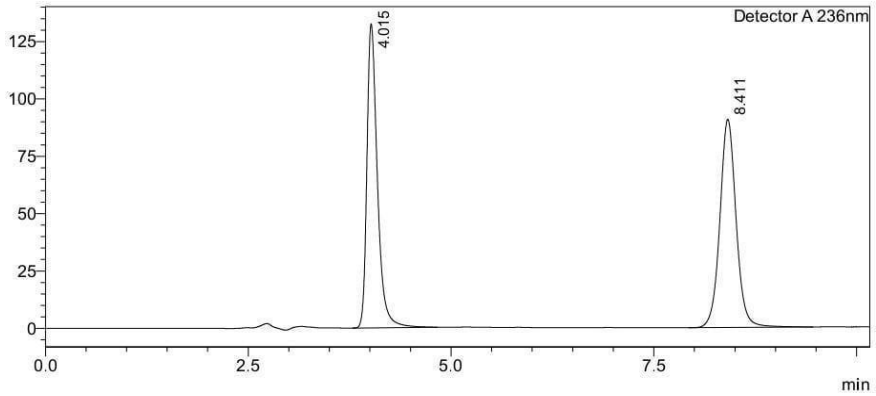


Figure 2.5

2.9.3 Chromatography of Spironolactone and Dapagliflozin.

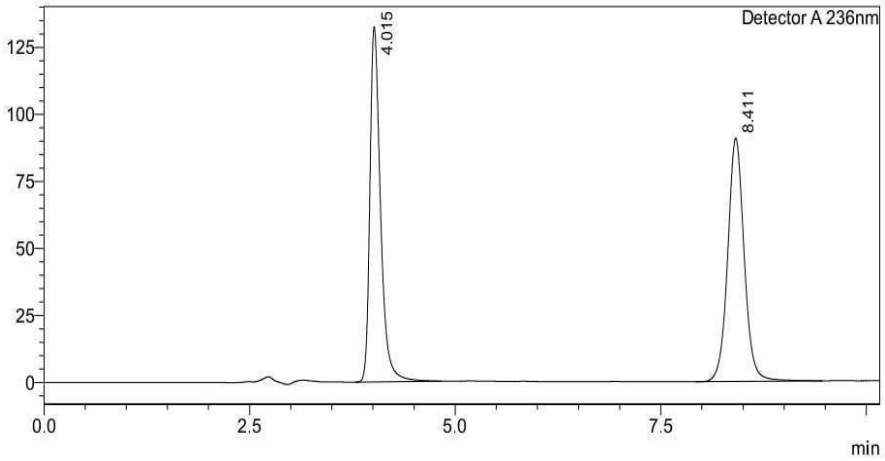


Figure 2.6 Chromatogram of Spironolactone and Dapagliflozin in ACN: Methanol: Phosphate Buffer (35:35:30) pH 4.0

Table 2.2: System Suitability Parameter

Drug	SYSTEM SUITABILITY PARAMETER				
	Peak Area	Retention Time	Theoretical Plates	Tailing Factor	Resolution
Dapagliflozin	1349810	4.015	5119	1.423	-
Spironolactone	1438190	8.411	9003	1.055	15.184

2.10 Results of Force Degradation Study

I) Acidic Condition

Acidic condition was carried out by keeping powder equivalent to 10mg of Synthetic mixture study with 0.1N HCl at 30°C for 1.5 hours.

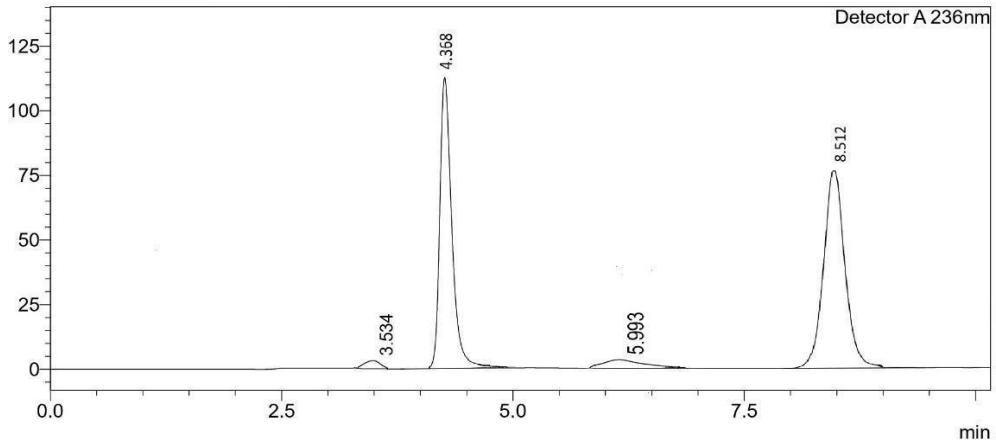


Figure 2.7 Acidic Condition

II) Alkali Condition

Alkali condition was carried out by keeping powder equivalent to 10mg of Synthetic mixture study with 0.1N HCl at 30°C for 1 hour.

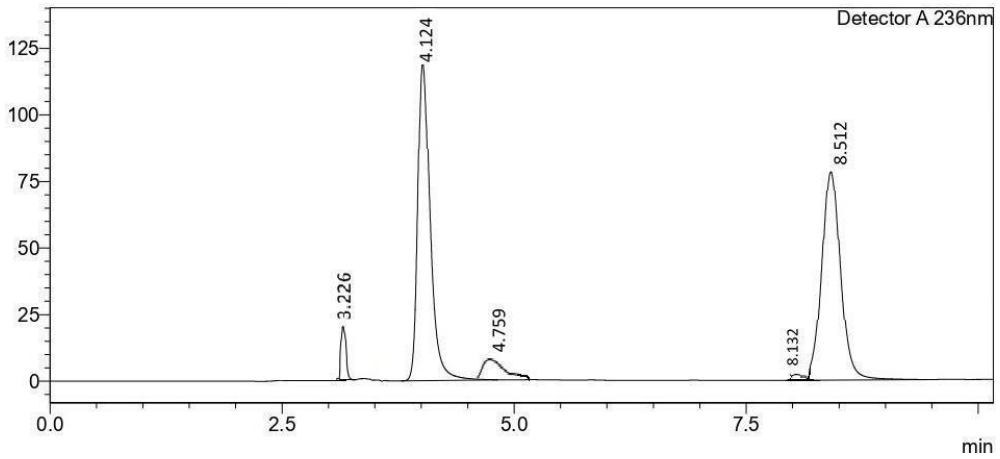


Figure 2.8 Alkali Condition

III) Oxidative Condition

Oxidative condition was carried out by keeping powder equivalent to 10mg of Synthetic mixture study with 0.1N HCl at 30°C for 2 hours..

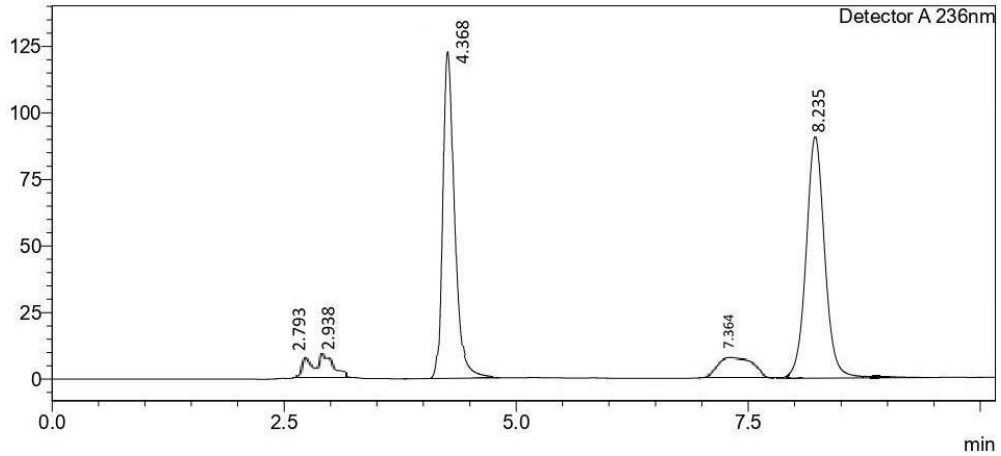


Figure 2.9 Oxidative Condition

IV) Thermal Condition

Thermal condition was carried out by keeping powder equivalent to 10mg of Synthetic mixture study with 0.1N HCl at 30°C for 30min.

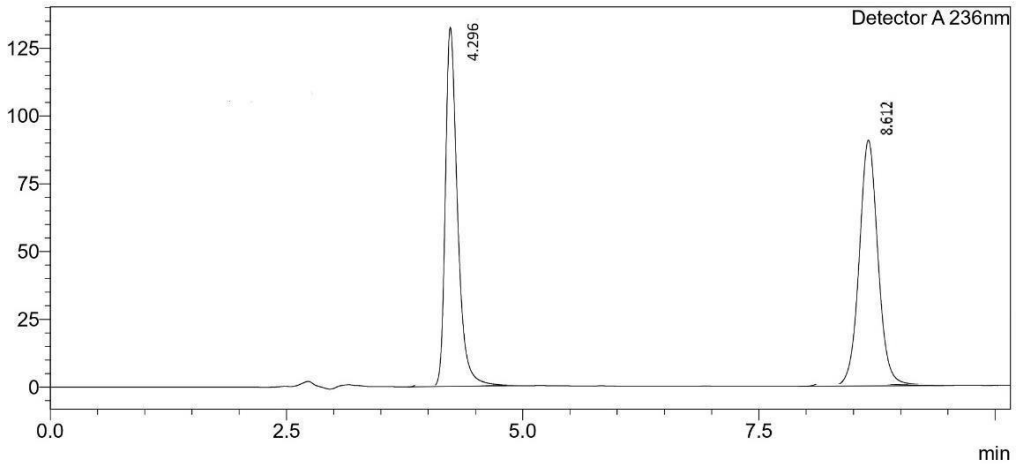


Figure 2.10 Thermal Condition

V) Photolytic Condition

Acidic condition was carried out by keeping powder equivalent to 10mg of Synthetic mixture study with 0.1N HCl at 30°C for 30min.

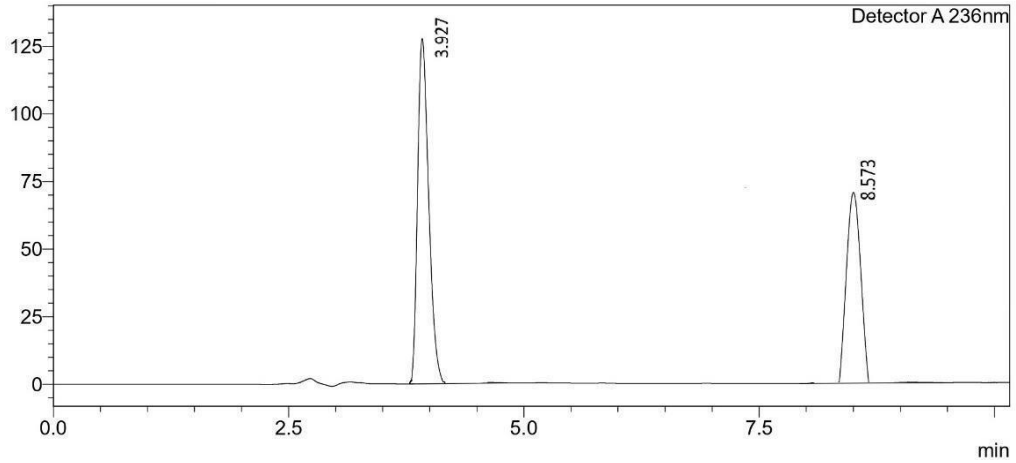


Figure 5.11 Photolytic condition

2.10.1 Stability of The Drug Under Stress Condition

Table 2.3: Summary of Stress Study of Spironolactone and Dapagliflozin by HPLC Method

Sr No	Parameter	Spironolactone		Dapagliflozin	
		Peak Area	% Drug Degradation	Peak Area	% Drug Degradation
1.	Normal	252831	-	236508	-
2.	0.1 N HCl	234223	7.36	206637	12.63
3.	0.1 N NaOH	230708	8.75	213212	9.85
4.	1% H2O2	217738	13.88	201931	14.62
5.	Thermal	249367	1.37	233954	1.08
6.	Photolytic	251693	0.45	235822	0.29

2.11 Result of Method Validation

2.11.1 Specificity

It was proven by comparing the chromatogram of mobile phase, test solution to show that there was no interference of mobile phase and excipient peaks with peak of Spironolactone and Dapagliflozin.

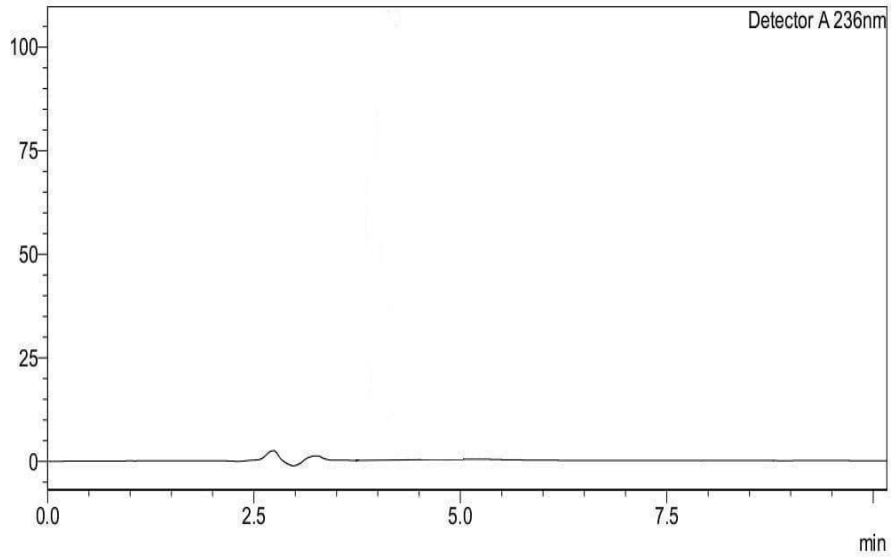


Figure 2.12 Chromatogram for Blank

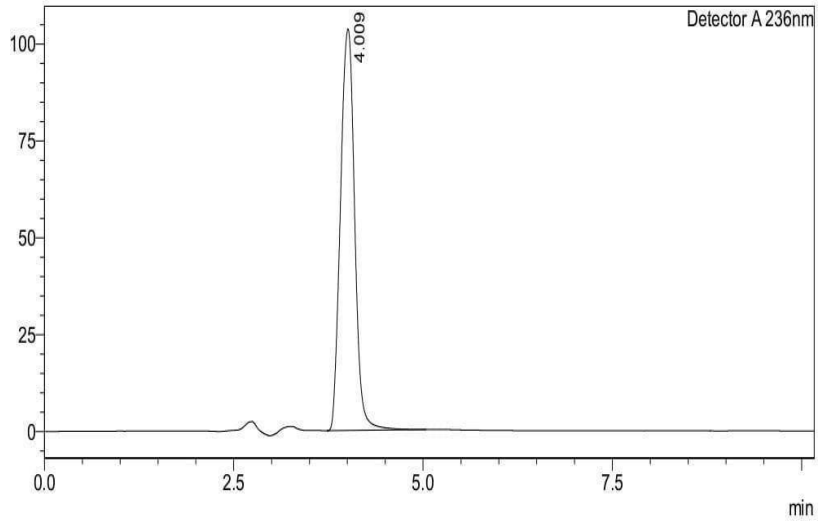


Figure 2.13 Chromatogram of Dapagliflozin

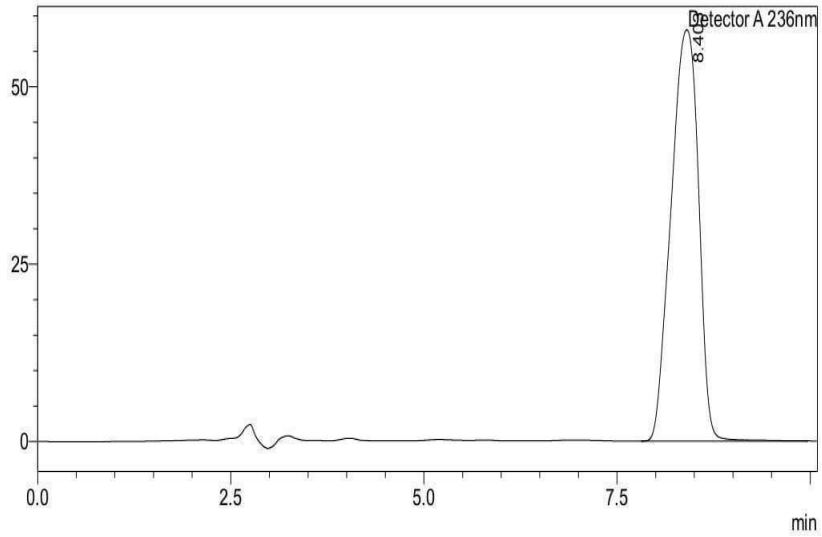


Figure 2.14 Chromatogram of Spironolactone

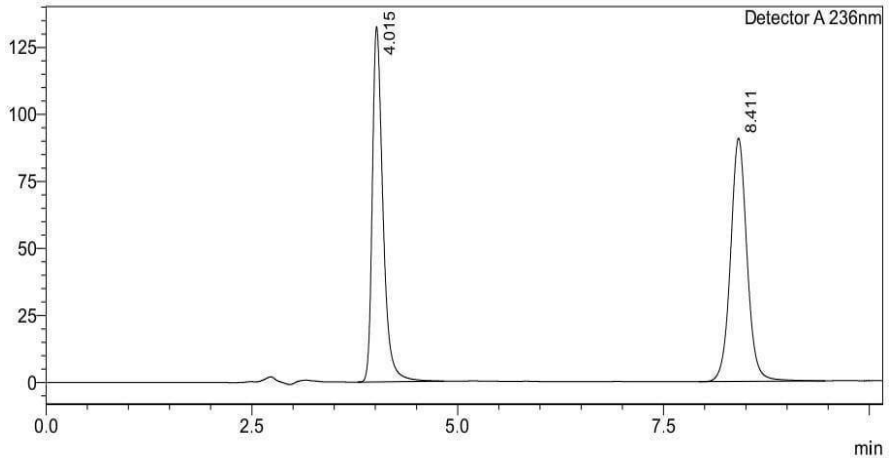


Figure 2.15 Chromatogram of Spironolactone and Dapagliflozin in ACN:Methanol:phosphate Buffer (35:35:30)

2.11.2 Linearity

The capacity of an analytical technique to produce test results that are directly equivalent to the concentration of analyte in the sample is refered to as its linearity. Linearity was studied by preparing standard solution at 6 different concentrations.

The linearity range for Dapagliflozin and Spironolactone were found to be 1-6µg/ml and 2.5-15µg/ml respectively. The calibrataion curve was plotted based on the area vs concentration (µg/ml). Linearity of both the drug was assessed with respect to slope, intercept and correlation co-efficient. The results were r^2=0.998 for Dapagliflozin and r^2=0.994 for Spironolactone.

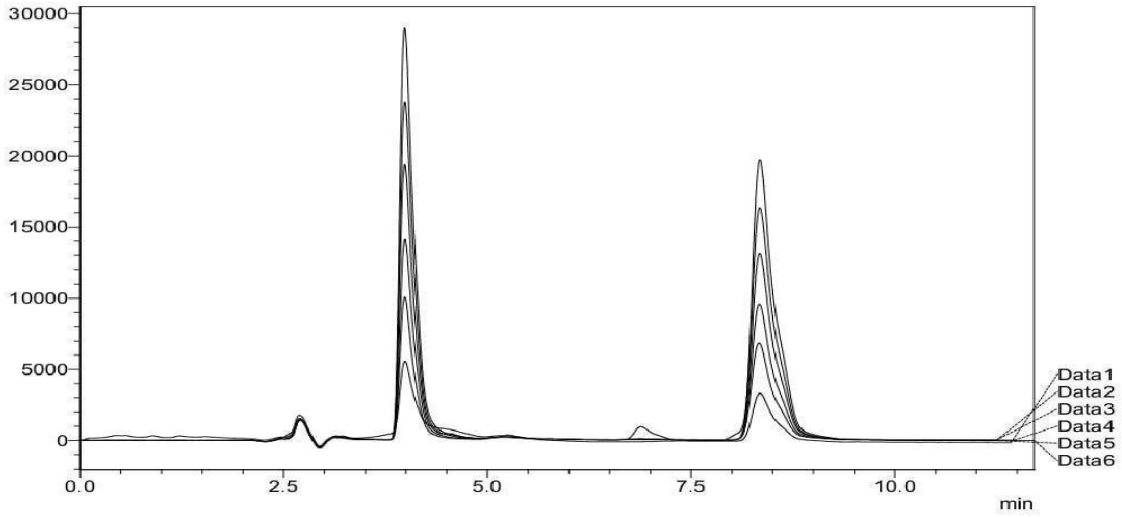


Figure 2.16 Overlay of Spironolactone (2.5-15 µg/ml) and Dapagliflozin (1-6 µg/ml)

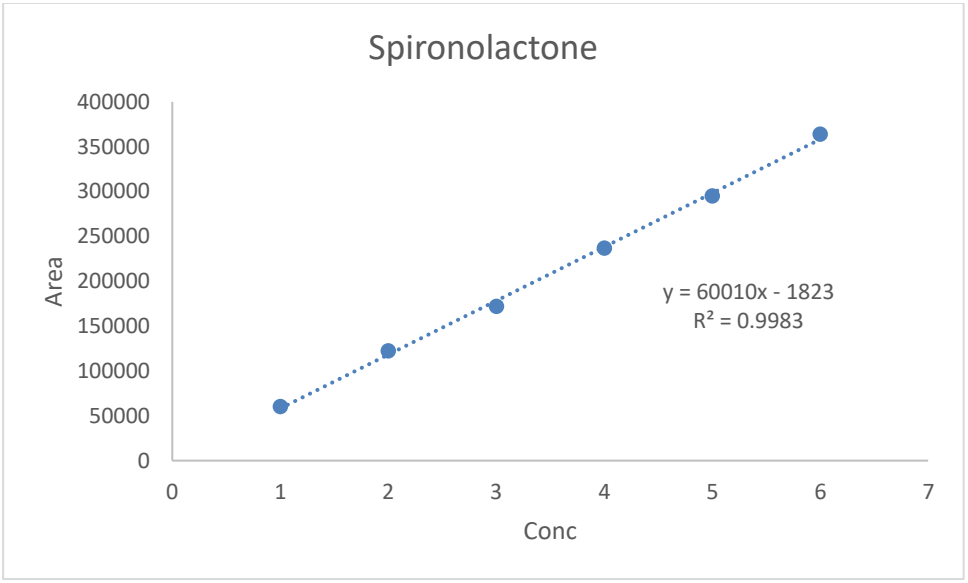


Figure 5.17 Calibration Curve of Spironolactone

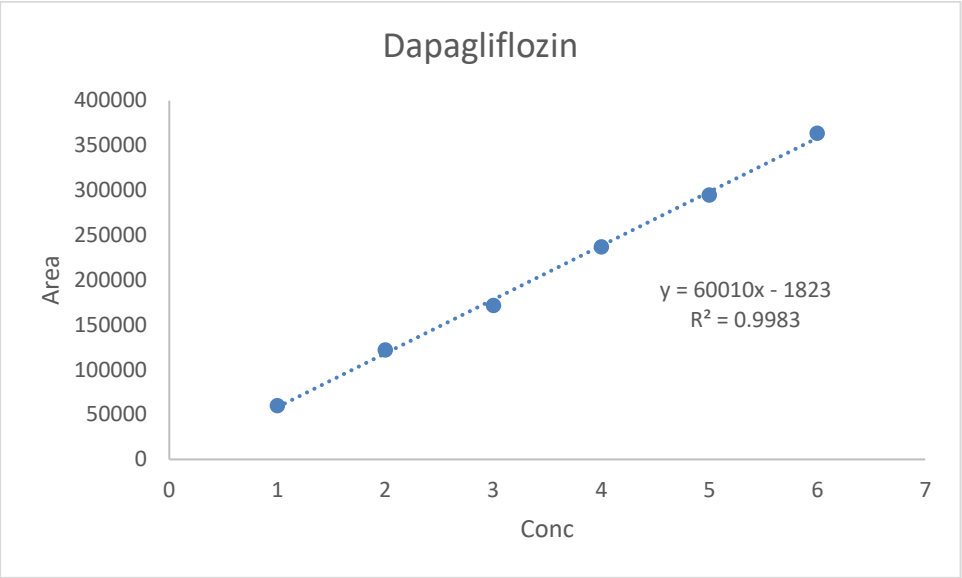


Figure 5.18 Calibration Curve of Dapagliflozin

Table 2.4: Linearity Data of Spironolactone and Dapagliflozin

Spironolactone			**Dapagliflozin**		
Concentration (µg/ml)	**Mean Area ± SD**	**%RSD**	**Concentration (µg/ml)**	**Mean Area ± SD**	**%RSD**
2.5	64490.5 ± 555.72	0.86	1	60234 ± 540.38	0.89
5	129573 ± 626.51	0.48	2	122068 ± 1031.92	0.84
7.5	172083.3 ± 1051.21	0.58	3	171656.2 ± 699.69	0.40
10	252792.3 ± 1229.00	0.48	4	236910.5 ± 1699.53	0.71
12.5	325623.2 ± 546.84	0.16	5	294758 ± 2739.49	0.92
15	385350.2 ± 1224.45	0.31	6	363636.2 ± 875.70	0.24

2.11.3. Precision

For the precision evaluation of the HPLC technique, repeatability studies, intraday and interday precision were performed. The repeatability study was executed by injecting 7.5 µg/ml Spironolactone and 43 µg/ml of Dapagliflozin (n=6). The % RSD of intraday and interday precision was calculated by injecting 2.5, 5 and 7.5 µg/ml concentration for Spironolactone and 1,2 and 3 µg/ml concentration for Dapagliflozin (n=3) for evaluation of intraday and interday precision.

Table 2.5: Repeatability Data of Spironolactone and Dapagliflozin.

Dapagliflozin		**Spironolactone**	
Concentration (3µg/ml)	Area	Concentration (7.5 µg/ml)	Area
1	171725	1	171725
2	172361	2	173811
3	170529	3	172583
4	171548	4	171562
5	172415	5	171962
6	171359	6	170857
Mean Area ± SD	171656.2 ± 699.69	Mean Area ± SD	172083.3 ± 1015.21
%RSD	0.407	%RSD	0.58

Table 2.6: Intra-day and Inter-Day Precision of Spironolactone and Dapagliflozin.

Intra-Day Precision (n=3)			
	Concentration	**Area ± SD**	**%RSD**
Spironolactone	2.5	64313.67 ± 382.50	0.59
	5	130000.7 ± 456.53	0.35
	7.5	172706 ± 1048.45	0.61
Dapagliflozin	1	60369.33 ± 87.66	0.14
	2	122610 ± 1087.84	0.88
	3	171538.3 ± 930.15	0.54
Inter-Day Precision (n=3)			
Spironolactone	2.5	64667.33 ± 729.34	1.12
	5	129702.3 ± 780.05	0.60

	7.5	172652 ± 1126.08	0.65
Dapagliflozin	1	60099.67 ± 817.19	1.35
	2	121526 ± 772.96	0.63
	3	171497.3 ± 944.02	0.55

2.11.4. Accuracy

Accuracy was calculated by performing recovery experiments at 80%, 100% and 120% levels were selected for the accuracy determination for the suggested HPLC technique. % recovery was calculated by placebo addition in the standard solution. As a result, the mean recovery ranged from 98.37%-100.9% for both drugs.

Table 5.7 Accuracy for Spironolactone and Dapagliflozin.

Drug	Level	Amount of Sample Taken (µg/ml)	Amount of Standard Spike (µg/ml)	Total Amount of Drug	Amount of Standard Recovery Mean	% Recovery ± SD (n=3)
Spironolactone	80	10	8	18	17.84	99.11 ± 0.26
	100	10	10	20	19.96	99.82 ± 0.19
	120	10	12	22	22.20	100.9 ± 0.81
Dapagliflozin	80	4	2	6	5.90	98.37 ± 0.63
	100	4	4	8	7.96	99.5 ± 0.92
	120	4	6	10	10.01	100.07 ± 1.21

5.11.5 Assay

The prepared concentration of the sample solution was injected into the HPLC. The resulting peak areas were compared with the standard peak areas and the assay was calculated for the method. % assay was found to be 99.34 for Spironolactone and 97.4 for Dapagliflozin. High % assay was found for both the drugs. Hence the method can successfully apply for the simultaneous of Spironolactone and Dapagliflozin in pharmaceutical Synthetic mixture.

Table 5.8 Assay of Dosage Form

Synthetic mixture	Dose	Amount Found (mg)	%Assay
Spironolactone + Dapagliflozin	25 mg	24.83	99.34
	10 mg	9.94	99.4

1.11.6 LOD and LOQ

Limit of Detection (LOD) and Limit of Quantification (LOQ) for Spironolactone and Dapagliflozin were determined in accordance with ICH guidelines Q2(R1). The LOD represents the smallest analyte concentration that produces a detectable response (S/N ratio of 3.3), while the LOQ is the smallest analyte concentration that can be reliably quantified (S/N ratio of 10). The LOD values for Spironolactone and Dapagliflozin were reported as 0.745 µg/mL and 0.288 µg/mL, respectively, and the LOQ values were 2.459 µg/mL and 0.951 µg/mL, respectively.

Table 2.9 LOD and LOQ data of Spironolactone and Dapagliflozin

Parameters	Spironolactone	Dapagliflozin
LOD (µg/ml)	0.745 µg/mL	0.288 µg/mL
LOQ (µg/ml)	2.459 µg/mL	0.951 µg/mL

1.11.7 Robustness

The robustness of an analytical procedure is a measure of its ability to remain unaffected minor but deliberate modification in method parameters, and its provides an indication of its dependability under normal conditions. It should demonstrate the dependability of an analysis in the face of deliberate alternative in procedure parameter. In the case of liquid chromatography, typical deviations include

- Influence of variations pH in mobile phase
- Flow rate

- Temperature
- Influence of variation in mobile phase composition
- Different columns

Table 2.10 Robustness data of Spironolactone and Dapagliflozin

Condition	Variation	%Assay ± SD (n=3)	
		Spironolactone	Dapagliflozin
Flow Rate	0.8 µg/ml	99.19 ± 0.665	9.89 ± 0.06
	1.0 µg/ml	99.10 ± 0.414	9.9 ± 0.07
Temperature	40°C	99.32 ± 0.0416	9.943 ± 0.02
	35°C	99.21 ± 0.306	9.84 ± 0.08
pH	4	99.64 ± 0.105	9.91 ± 0.04
	3.5	99.01 ± 0.272	9.913 ± 0.05

Summary of Validation Parameter

Table 5.11 Summary of Validation

Parameter	Spironolactone	Dapagliflozin
Beer's law Limit (µg/ml)	2.5-15 µg/ml	1-6µg/ml
Regression Equation (y=mx+c)	y = 60010x - 1823	y = 60010x - 1823
Correlation co-efficient (r^2)	0.9983	0.998
Accuracy (% Recovery ± RSD) n=3	99.11 ± 0.26	98.37 ± 0.63
	99.82 ± 0.19	99.5 ± 0.92
	100.9 ± 0.81	100.07 ± 1.21
Repeatability (% RSD)	0.407	0.58
Inter-Day (% RSD)	0.60-1.12	0.55-1.35

Intra-Day (% RSD)	0.35-0.61	0.14-0.88
Limit Of Detection (LOD)	0.745 µg/mL	0.288 µg/mL
Limit Of Quantification (LOQ)	2.459 µg/mL	0.951 µg/mL
%Assay	99.34%	99.4%

2.12 Conclusion

A simple, rapid, sensitive, accurate and precise stability indicating RP-HPLC method has been developed and validated for routine analysis of Spironolactone and Dapagliflozin. The stability indicating RP-HPLC method is suitable for estimation of Spironolactone and Dapagliflozin. The developed method was successfully applied in marketed dosage form. The proposed method can be utilized for the routine analysis of Spironolactone and Dapagliflozin in pharmaceutical dosage form.

SUMMARY AND CONCLUSION

3. SUMMARY AND CONCLUSION:

3.1 Summary:

Spironolactone is a potassium-sparing diuretic that functions as an aldosterone receptor antagonist. By inhibiting aldosterone, it promotes sodium and water excretion while conserving potassium, thereby reducing fluid overload and lowering blood pressure. Dapagliflozin, on the other hand, is a sodium-glucose cotransporter 2 (SGLT2) inhibitor primarily used to manage type 2 diabetes mellitus. It works by preventing glucose reabsorption in the kidneys, leading to increased glucose excretion in the urine and subsequently lowering blood sugar levels.

Recent studies suggest that the combination of these two drugs can be beneficial in treating HFpEF. For instance, a case report from rural Tanzania indicated that the combined use of spironolactone and dapagliflozin led to significant clinical improvements in a patient with HFpEF, including enhanced diastolic function and reduced hospitalization rates. The combined therapy appears to modulate myocardial fibroblast function through different signaling pathways. Research has shown that while dapagliflozin decreases fibroblast migration and reduces the secretion of pro-inflammatory cytokines, spironolactone alters the signaling pathways activated by dapagliflozin, potentially enhancing its beneficial effects on heart failure biomarkers.

Stability indicating method is capable of detecting the loss in content of the active component and subsequent increase in degradation products, and that the loss in content of the active component and subsequent increase in degradation products should be monitored by a single analytical method. Stability testing provides information about degradation mechanisms, potential degradation products, possible degradation pathways of the drug as well as interaction between the drug and excipients in the drug products. The results are applied in developing manufacturing processes and selecting proper packaging, storage conditions, product's shelf life and expiration dates.

The Identification test for Spironolactone and Dapagliflozin such as melting point, FTIR, Solubility study and UV- Spectrophotometric method were completed with their respective standards.

A simple, rapid, sensitive, accurate and precise stability indicating RP-HPLC method for analysis of Spironolactone and Dapagliflozin in pharmaceutical Synthetic mixture was developed and validated. The chromatographic condition comprised of a reverse phase: Shimadzu C18 (250 × 4.6 mm, 5µm) column, with a mobile phase composed of mixture of ACN: Methanol: Phosphate Buffer (35:35:30 %v/v/v) and pH 4.0 adjusted with orthophosphoric acid. Flow rate was adjusted to 1 ml/min. Detection was carried out at 236 nm. The retention time of Spironolactone was 8.4 ml/min and Dapagliflozin was 4.0 ml/min. The drug undergoes degradation under acidic, alkali, peroxide, thermal and photolytic condition. A peaks of degraded products were resolved from the active pharmaceutical ingredient with significantly different retention time. As the method

effectively gives the degraded peaks; it can be employed as stability indicating one. The linearity range was established for Spironolactone at 2.5-15 µg/ml and for Dapagliflozin at 1-6 µg/ml, with a correlation coefficient (R^2) of 0.9983 for Spironolactone and 0.998 for Dapagliflozin. The regression equation for both drugs was y=60010x−1823y = 60010x - 1823y=60010x−1823. Accuracy studies demonstrated recovery rates of 99.11–100.9% for Spironolactone and 98.37–100.07% for Dapagliflozin, with %RSD values below 2%, indicating high precision. The intra-day and inter-day precision studies yielded %RSD values of 0.35–0.61% and 0.60–1.12% for Spironolactone, and 0.14–0.88% and 0.55–1.35% for Dapagliflozin, respectively.

The limit of detection (LOD) and limit of quantification (LOQ) for Spironolactone were 0.745 µg/ml and 2.459 µg/ml, respectively, while for Dapagliflozin, they were 0.288 µg/ml and 0.951 µg/ml, respectively. The %assay values were 99.34% for Spironolactone and 99.4% for Dapagliflozin, indicating minimal interference from excipients.

The higher percentage of recovery study indicates that there is no interference of excipients in the presence of Synthetic mixture.

3.2 Conclusion:

A simple, rapid, sensitive, accurate and precise stability indicating RP-HPLC method has been developed and validated for routing analysis of Spironolactone and Dapagliflozin. The stability indicating RP- HPLC method is suitable for estimation of Spironolactone and Dapagliflozin. The developed method was successfully applied in marketed dosage form. The proposed method can be utilized for the routine analysis of Spironolactone and Dapagliflozin pharmaceutical dosage form.

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