QbD Based Development and Validation of RP-HPLC method for estimation of Spironolactone: Application to Bioanalytical and Stability Study in Plasma

Jyoti Shinde[1]*, Dr. Pankaj Kumar Sharma[1], Dr. Jaya Sharma[1]

[1]Department of Pharmaceutical Sciences, Apex University, Jaipur , Rajasthan, India.

ABSTRACT

The objective of the Current research was to apply Quality by Design approach for development of more accurate, precise, specific and robust RP-HPLC method for estimation of spironolactone in its tablet dosage form and Human Plasma. The Full 3 level Factorial design was applied on mobile phase Composition & pH of buffer system as considering CQAs against dependent variables viz. retention time, peak asymmetry, theoretical plates. By using design space numerical, graphical optimization on retention time, peak asymmetry, and theoretical plates and the optimum chromatographic conditions were chosen. Optimized analytical method consisted Acetonitrile: Ammonium Formate (60:40v/v) as mobile phase, pH 3.5, flow rate 1ml/min, a wavelength 237 nm. Protein precipitation technique is used in preparing samples for bioanalysis. Spironolactone was extracted from human plasma using acetonitrile as a precipitating agent, and the supernatant was then injected. Spironolactone was eluted with retention time of 5.235 min and peak area of 920547. With a correlation coefficient of 0.999, Spironolactone had an excellent linear relationship in the range of 5-30 g/ml. RSD % for intraday, interday, and repeatability were determined to be 1.69%, 1.87%, and 0.59% respectively. The limit for detection and quantification was determined to be 0.40g/ml and 1.23g/ml. The parameters used for method validation were within the ICH-recommended range. The suggested approach is working well for the best analysis of pharmaceutical dosage forms of Spironolactone in bulk and was effectively used in a pharmacokinetic investigation.

Keywords: Spironolactone, Bioanalytical Method, RP-HPLC, 3 Level Factorial Design, QbD approach, Stability study.

Introduction

Currently, Spironolactone, a mineralocorticoid receptor antagonist, is approved to treat primary hyperaldosteronism, congestive heart failure, cirrhosis, nephrotic syndrome, essential hypertension, hypokalemia, and pregnancy-related edoema.[1] Additionally often utilized in medical gender transition is Spironolactone.[2] Spironolactone antiandrogenic action is used to

treat the conditions like hirsutism, female pattern hair loss, and adult acne vulgaris.[3] Aldactone oral tablets contain 25 mg, 50 mg, or 100 mg of the aldosterone antagonist spironolactone, 17-hydroxy-7α-mercapto-3-oxo-17α-pregn-4-ene-21-carboxylic acid γ-lactone acetate.[4]

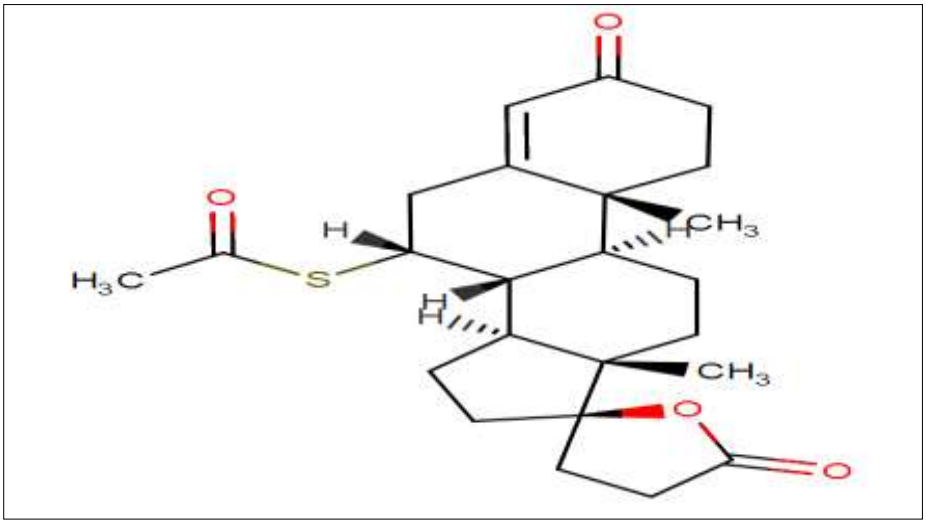

Figure 1: Structure of Spironolactone

spironolactone, a type of MRA, has been suggested to have a beneficial effect on SARS-CoV-2 outcomes through its dual action as an MRA and antiandrogen, resulting in reduced transmembrane protease receptor serine type 2 (TMPRSS2)-related viral entry to host cells. In this study, we sought to investigate the association between MRA antagonist therapy and mortality in SARS-CoV-2 patients via systematic review and meta-analysis.

Spironolactone prior study was done and the literature details are Ram *et al.* developed and validated stability-indicating HPLC assay method for simultaneous determination of Spironolactone and Furosemide in tablet formulation. Pallavi *et al.* developed analytical method and validated for simultaneous estimation of Metolazone and Spironolactone in bulk and pharmaceutical dosage form by RP-HPLC. Chaure *et al.* developed RP- HPLC method for the simultaneous estimation of Losartan and Spironolactone in tablet dosage form. El-Shahawi *et al.* given analytical method of spironolactone residues in industrial waste water and in drug formulations by cathodic stripping voltammetry. We investigated the quality by design approach to quantification of Spironolactone in bulk, pharmaceutical formulation, and plasma because, in accordance with previous studies, literature, and the stated hypothesis, there isn't a database of reverse phase-high performance liquid chromatography on C18 Column at different mobile phase with different pH. The suggested method was validated in accordance with ICH guidelines and was found to be precise, accurate, and reproducible. This study was designed to demonstrate the application of quality by design in the development of bioanalytical methods and its validation to method accuracy, profits, yield, and stability, reduce process variability, and reduce

process design and developing time and cost. It also stated the drug compatibility or estimation in plasma with/without its constituents.

METHODS

Preliminary analysis of drug

Melting Point was determined to be 244°C. Spironolactone color was matched to the reported characteristics listed in the drug bank. Spironolactone is practically insoluble in water, soluble in alcohol, and freely soluble in benzene and in chloroform. Maximum absorbance (max) in methanol was observed at 237 nm.[9]

Instrumentation and Software

The high-pressure liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) was used for estimation. The instrument equipped with a UV detector (SPD 20 AV) and analysis was at 237 nm. The chromatographic separation of Spironolactone was performed by using the C18 column (average particle size 5 µm, 250 x 4.6 mm I.D) with a flow rate of 1 ml/min. The study was performed at a column temperature of 25°C and injection volume (20 µL) was injected.
Jasco UV Spectroscopy (Model Jasco.V 630) with 1cm quartz cell and connected to computer with UV Win5 software v6 was used. Leela Sonic Sonicator Ultrasonic Bath was used.

Preparation of Mobile Phase

60 ml of Acetonitrile and 40 ml of ammonium formate were combined in the ratio of 60:40 v/v. As a peak modifier, one drop of triethylamine (0.01 ml/ml) was added, and the pH was subsequently adjusted to 3.5 using orthophosphoric acid. The mixture was sonicated for 10 minutes after being filtered via a 0.45 µ membrane filter.[10]

Preparation of stock solutions of Spironolactone

10 mg of Spironolactone is solubilized in 10ml Methanol in a volumetric flask to get the concentration of 1000 µg/ml. From the resulting solution, 0.1 ml was diluted to 10 ml with Methanol to obtain a concentration of 10 µg/ml of Spironolactone.

Design of experiment

Three level Factorial designs by Design Expert version 8 software

Using Design Expert 8 Software, an experiment 3-level factorial design was created. Simply said, any numerical element has three levels of variation. Miscellaneous factorial design is a synonym for the design. It works with up to four variables. The duplicates of the centre points are also shown in the 3^k plus runs of the tests. The quadratic model is the correct one. This design is open to the addition of up to 10 category factors. The number of categorical factor levels must be multiplied by the number of runs produced.[11-12]

Mobile phase composition and buffer system pH were chosen as dependent variables. Due to the fact that these designs make it easy to add an organic or aqueous phase, we chose an organic phase range of 60% to 70% by volume. The pH range for the buffer system was also

chosen to be between 3.5 and 5.5 mmol/l. Independent factors were chosen retention time, theoretical plates, and peak asymmetry. The software gave 9 runs of an experiment.

After the completion of the above trials, we turned to optimization, which is based on desirability. It is an objective function with a zero limit and a maximum value of one. The numerical optimization locates a position where the desirability function is maximized. As close the lower and higher limits are established in relation to the actual optimum determines the value in its entirety. Simply using mathematics, the best solution was found.[13-15]

Analytical Method Validation

Method validation was done after the RP-HPLC method development by QbD approach to ensure that the results were very accurate. We demonstrated that no other element, including formulation additives, the atmosphere, equipment, glassware, people, or maybe slight modifications to our established method, altered the results in any way. We investigated robustness, specificity, system suitability, precision, recovery, the limit of detection, the limit of quantitation, and linearity. [16-17]

Bioanalytical method Validation

In the analysis and interpretation of bioavailability, bioequivalence, and the investigation of toxicity, quantization of the drug molecule in biological fluid is crucial. We can utilize human plasma, serum, urine, etc. for the relevant study. However, we choose to use human plasma in order to create a user-friendly bioanalysis approach. To assess the interactions and reactivity of a drug or its metabolite with blood elements, an estimation of Spironolactone in human plasma is crucial.[18,19,22,23]

Preparation of spiked plasma samples

Spironolactone 10µl was added to each Eppendrof tube. Then, added 100 µl of plasma, 100 µl of Acetonitrile for mixing, and 0.9 ml of Acetonitrile as a precipitating agent for the drug extraction. Allow the mixture to vortex for 5 minutes. The aforementioned solution centrifuged for 15 minutes at 2000 rpm to ensure good mixing then placed nitrogen gas to it to concentrate it, then separated the supernatant into Eppendorf tubes. The final sample was 20 µl of the supernatant.[18,20]

Stability of Spironolactone in human plasma

The purpose of this study was to determine spironolactone stability in human plasma. By analyzing freeze and thaw, short-term, long-term, and stock solution stability, we were able to evaluate the storage conditions.[21]

Result and discussion

Optimization result

Screening design for sound chromatographic condition

The best peak parameter plays a significant role to determine a suitable solvent system and pH of buffer. These mobile phases were screened by varying the organic phase composition from 60 to 80v/v and pH of buffer from 3.5 to 4.5 mmol/l. The flow rate was selected 1 ml/min. After performing the trials, we optimized chromatographic conditions according to the desirability value.

Desirability value

The initial basis of this system is the desirability value assigned to each response. The scale of desirability function runs from zero to one; a desirability value close to one is considered as desired response, while zero is thought to represent the entirely undesired response. The trials were chosen based on their highest desire value. In order to optimize the approach, a first experiment with desirability one (i=0.946) was chosen. The table 1 shows the outcome as follows. .

Table 1 Optimized trials suggested by software based on desirability value

Sr. no	Amount of Acetonitrile	pH of Buffer	Retention Time	Tailing Factor	Theoretical Plates	Desirability
1	60	3.5	5.23	1.16	10489.8	0.946
2	60	3.61	5.24	1.19	10145.8	0.904

Optimized chromatographic conditions

Mobile phase: Acetonitrile : Ammonium formate buffer (60:40%v/v), pH of buffer: 3.5, Analytical column: C_{18} (4.6× 250mm id. particle size 5µm), UV detection: 237nm, Injection volume: 20 µl, Flow rate: 1.00 ml min $^{-1}$, Temperature: Ambient, Run time: 10 min

Effect of independent variables on retention time (X):

After applying experimental design, the suggested Response Surface Quadratic Model was found to be significant with a model F value was 921.34, a p-value less than 0.005, and an R^2 value was 0.9993. The % C.V. and adjusted R^2 were 0.72 and 0.9983 respectively.

The quadratic model shows the equation of retention time is as follows.

Retention Time (X) = 61.052 - 1.614A + 0.464B – 00.0140000AB + 0.011 A^2 -0.0411 B^2 ……(1)

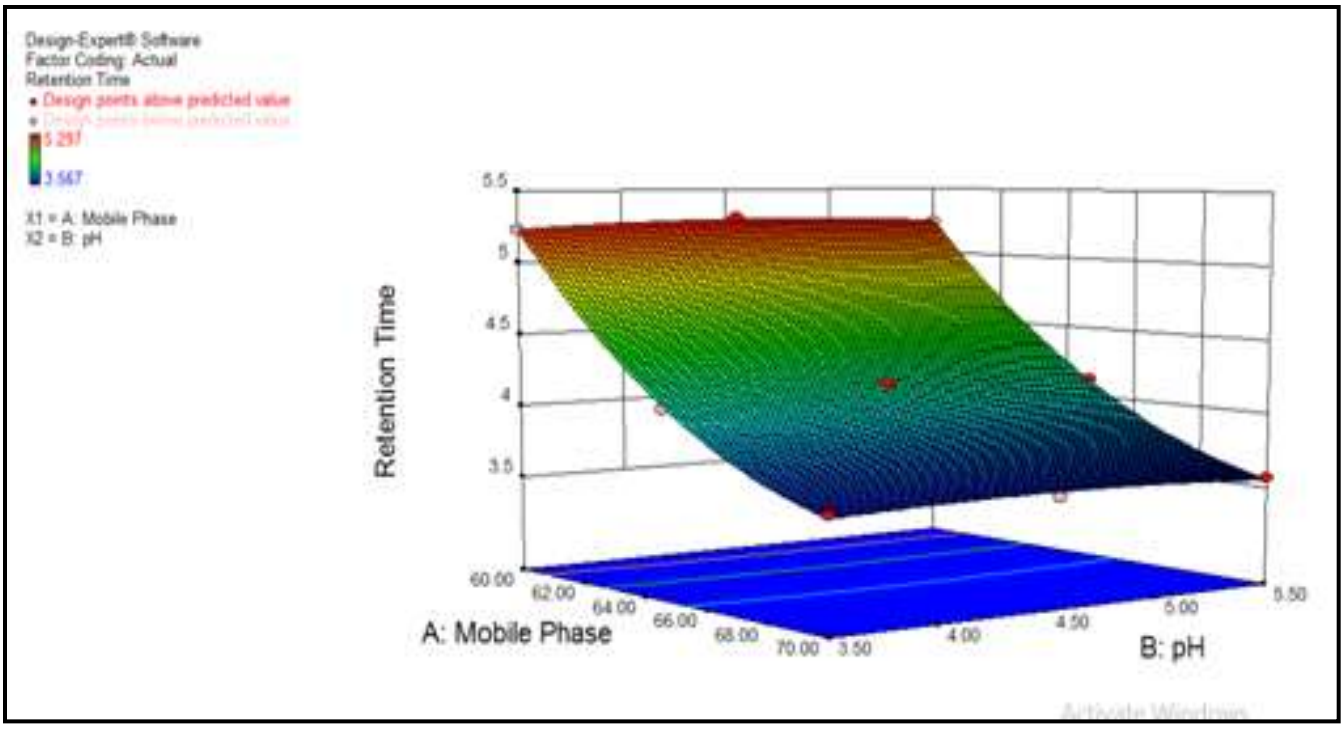

Figure 2: 3D surface plot for effect of combination of factors on retention time of Spironolactone by using three level factorial design.

From Figure 2 and equation 1, it indicated that as β_1 negative coefficient (-1.614) suggests that as the amount of Acetonitrile in mobile phase (A) decrease and β_2 positive coefficient (0.464) suggests that as pH of buffer (B) increase, the value of retention time will increase.

Effect of independent variables on Theoretical Plates (Y):

The presented Response Surface Quadratic Model was found to be significant after using experimental design with a model F value was 671.66, a p-value less than 0.005, and an R^2 value was 0.9991. The % C.V. and adjusted R^2 were 0.98 and 0.9976 respectively.

The quadratic model shows the equation of theoretical plates is as follows.

Theoretical Plates (Y) = 90206.56 - 1723.76A - 9624.0B + 24.20AB + 12.173 A^2 + 692.83 B^2 ……(2)

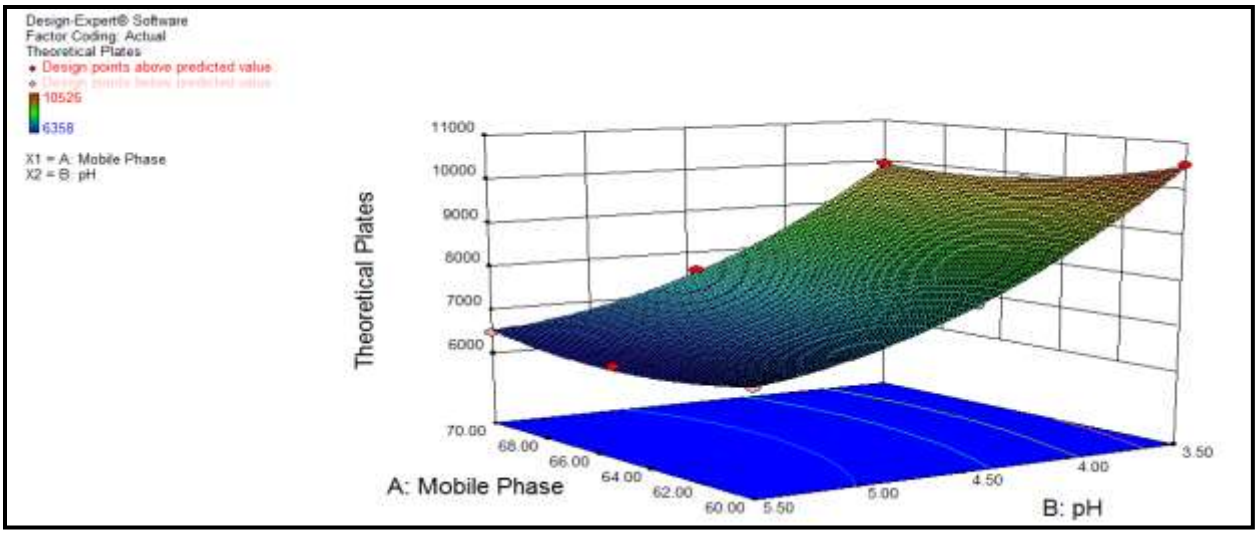

Figure 3: 3D surface plot for effect of combination of factors on theoretical Plates of Spironolactone by using three level factorial design.

From Figure 3 and equation 2, it indicated that as β_1 negative coefficient (-1723.76) suggests that as the amount of Acetonitrile in mobile phase (A) decrease and β_2 negative coefficient (-9624.0) suggests that as pH of buffer (B) decrease, the value of theoretical plates will increase.

Effect of independent variables on Asymmetric factor (z):

After applying experimental design, the suggested Response Surface Quadratic Model was found to be significant with a model F value was 66.62, a p-value less than 0.005, and an R^2 value was 0.9569. The % C.V. and adjusted R^2 were 4.14 and 0.9425 respectively. The amount of Acetonitrile in mobile phase (A) decrease and pH of buffer (B) decrease, the value of asymmetric factor will decreased.

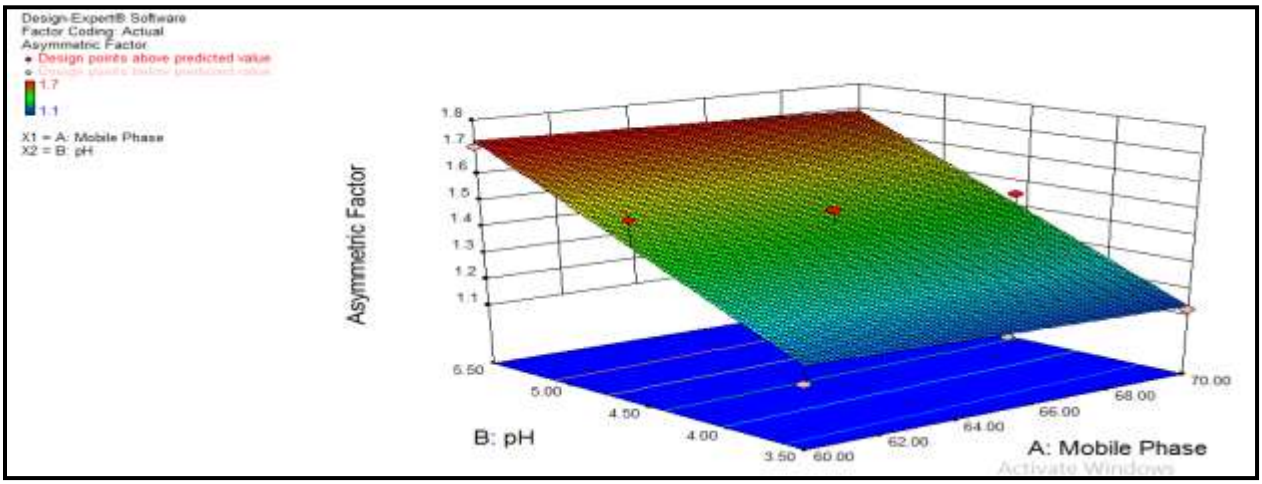

Figure 4: 3D surface plot for effect of combination of factors on Asymmetric factor of Spironolactone by using three level factorial design.

Analytical Method Validation

The RP-HPLC technique for Spironolactone estimation in bulk and formulation was developed using a 3 Level Factorial Design approach. By using the design expert 8 software, we were able to optimize the chromatographic conditions, which were as follows:

Mobile phase: Acetonitrile : Ammonium formate buffer (60:40v/v), pH of buffer: 3.5, Analytical column: C_{18} (4.6× 250mm id. particle size 5µm), UV detection: 237nm, Injection volume: 20 µl, Flow rate: 1.00 ml min^{-1}, Temperature: Ambient, Run time: 10 min

Chromatogram of Spironolactone

Spironolactone was successfully separated under the given chromatographic conditions in a retention period of 5.235 min. (Fig. 5); no drug degradation was seen during the study. The parameters listed below were used to validate the LC technique.

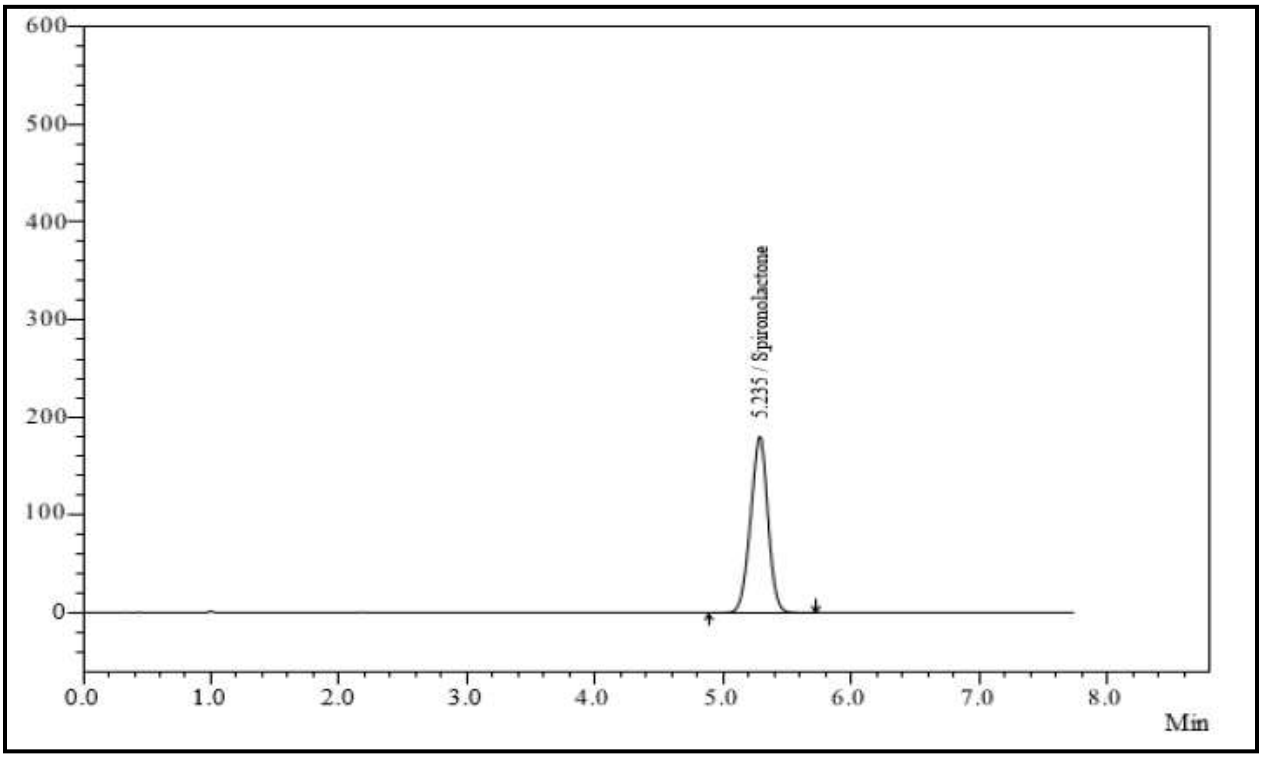

Figure 5: A typical chromatogram of Spironolactone

System suitability study

System suitability factors are essential component of analytical techniques, according to USP. As a result, the theoretical plates, peak area, and retention time for standard solutions were specified. The results obtained after running a sample of 5 µg/ml at 237 nm was compared to the prescribed limit. Table 2 showed the results.

Table 2: System suitability parameters

Sr. No.	Parameters	Observation
1	Retention time	5.233min
2	Peak area	965281
3	Theoretical plates	10526
4	Asymmetric Factor	1.12

Calibration Curve Preparation

Accurately 10mg of Spironolactone were dissolved in 10ml of methanol to produce the stock solution, and subsequent solutions were diluted with the mobile phase. The Final solutions were also sonicated for approximately 5 minutes. The calibration curve was plotted by taking concentration vs. peak area. The curve was found to be linear with R^2 of 0.999 across the linearity range of 5 to 25 µg/ml. Figure 6 shows a calibration curve, while table 3 has data.

Table 3: Details of Linearity

Sr. No	Concentration (µg/ml)	Peak Area
1	5	965148
2	10	1930296
3	15	2845444
4	20	3860592
5	25	4825740
6	30	5790888

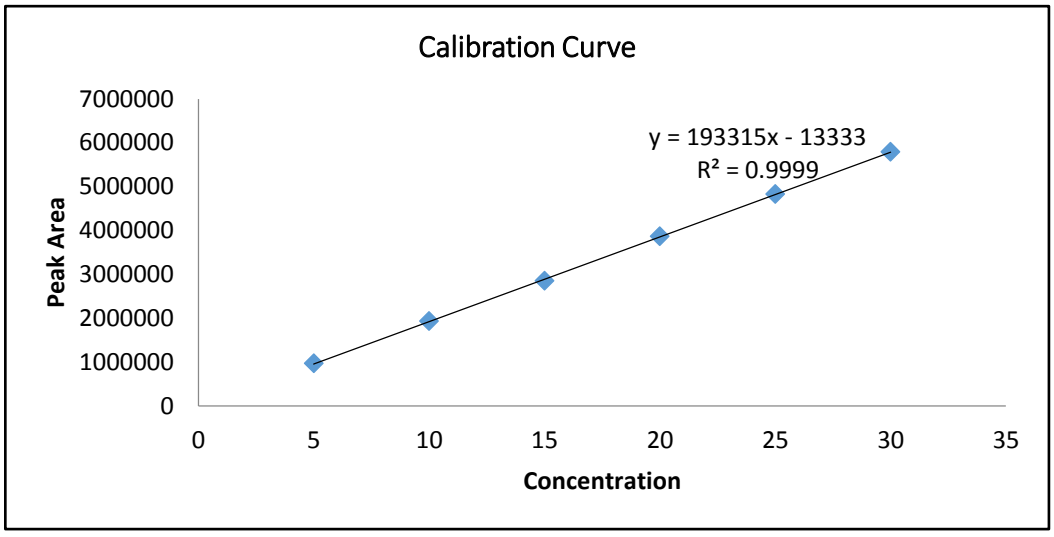

Figure 6: Linearity graph for Spironolactone

Selectivity

Aldactone 50 is the brand name of the Spironolactone formulation. 10 Spironolactone tablets were weighted & crushed in a mortar and pestle. Powder equivalent to 10 mg was transferred to a 10 ml volumetric flask and made up the volume with methanol. Further diluted to get 10 µg/ml solution. Retention time and another peak parameter of formulation were compared to the drug's API. In figure 7 a chromatogram is shown. The retention time, Theoretical plates, asymmetric factor and peak area were discovered to be 5.237 min, 6789422, 1.11, and 1918189, respectively. According to these results, the tablet excipients have no effect on drug separation in the chosen mobile phase.

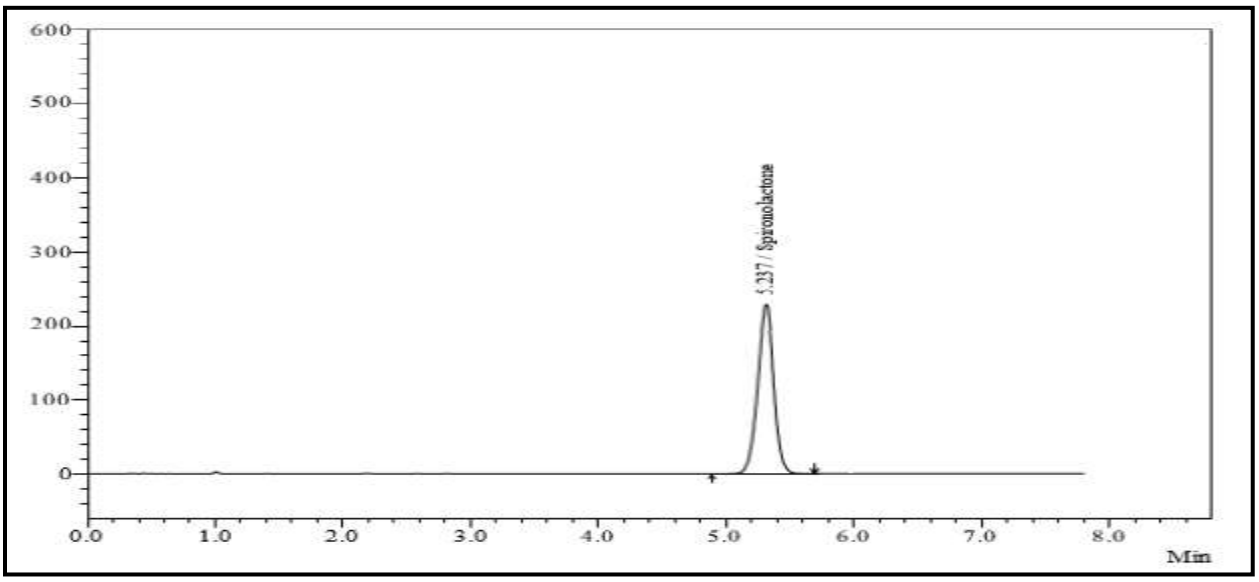

Figure 7: A typical chromatogram of Spironolactone formulation

Sensitivity

The limit of quantitation & limit of detection can be used to estimate the method's sensitivity. In the calculation, the signal to noise ratio is of utmost importance. The noise level for the blank sample was calculated using six repetitions and is three times the LOD and ten times the LOQ, respectively. Limits of Quantitation and Limit of Detection were discovered to be 0.3862µg/ ml and 1.1704µg/ml respectively.

Accuracy

Accuracy has importance validation parameter while developing a routine analytical method. It is simply a comparison of the reference value and the obtained value. Accuracy was found between the acceptable ranges of 99.92 and 100.36%. Five replicates of each concentration were prepared, and the estimated concentration and recovery percentage were calculated. The results obtained are shown in table 4.

Table 4: Accuracy results of Spironolactone by RP-HPLC

Sr. No.	Concentration µg/ml	Peak area	Found Concentration	% Recovery
1	16	3114077.6	15.99	99.92
2	20	3892597	19.99	99.95
3	24	4671116.4	24.09	100.36

Precision

Precision is the result of several measurements taken every day in various settings. The repeatability, intraday and interday precision tests were conducted. The 20 µg/ml solution was made in six replicates, and six readings were taken throughout the day from the morning to the evening. The method's intraday accuracy demonstrated its stability under various climatic circumstances throughout the day. Additionally, 0.604% relative standard deviation % was detected. The sample analysis for three days indicated a relative standard deviation percent of 1.6197% for interday precision. Analytical accuracy produces reliable outcomes and meets with ICH guidelines. Six replicates were prepared for repeatability testing, and the RSD% was 1.790%. The result was shown in table 5.

Table 5 System Precision results for Spironolactone by RP-HPLC.

Sr. No	Concentration µg/ml	Intraday Precision Peak Area	Interday Precision Peak Area	Repeatability
1	20	3885068	3873431	3865810
2	20	3910093	3914341	3845810
3	20	3826706	3899754	3865823
4	20	3892073	3799384	3858936
5	20	3813828	3860294	3915810
6	20	3867279.25	3826015	3863598
Average		3865841.21	3862203	3869298
Standard Deviation		38092.0039	43621.08	23996.48
RSD%		0.98	1.13	0.62

Specificity

A peak was observed on the chromatogram of Spironolactone in tablet form at a retention period of 5.237 minutes. The drug was extremely well resolved by the mobile phase used for the method, and the retention time of Spironolactone was 5.237 minutes. The wavelength that was detected was 237 nm. When a formulation containing the drug had its peak qualities compared to the standard, it was found that the peak was already satisfactorily resolved without the excipients interference. At 99.28%, recovery was achieved. The results obtained are shown in table 6.

Table 6: specificity results of Spironolactone by RP-HPLC

Sample	Label Claim (mg)	Amount Found (mg)	Recovery %	Retention Time
Tablet	50	49.91	99.82	5.233

Robustness

For the method conditions to be reliable, robustness is important. Simply said, it means that small adjustments or variations in the method, experimental, operating, or laboratory settings have no impact on the method. We ran a sample at a concentration of 20µg/ml, and showed that small modifications to the flow rate, detecting wavelength, mobile phase concentration and pH of buffer all produced the anticipated results. The results obtained are shown in table 7.

Table 7: Results of Robustness

Sr. No	Parameter		Response	Parameter	Response
Acetonitrile: Ammonium Format Buffer			**Retention Time (min)**	**Detection Wavelength**	**Peak Area**
(%V/V)				(nm)	
1	59	41	5.136	235	3802465
2	60	40	5.233	237	3860780
3	61	39	5.232	239	3894044
Average			5.234	Average	5.234
Standard Deviation			0.05571654	Standard Deviation	46357.0314
RSD%			1.06	**RSD%**	1.20
Flow Rate			**Retention Time (min)**	**pH of Buffer**	**Peak Area**
(ml/min)				(mmol/l)	
1	0.9		5.264	3.3	3888852

2	1	5.233	3.5	3860953
3	1.1	5.119	3.7	3785332
Average		5.20533333	5.20533333	3845045.67
Standard Deviation		0.07635662	0.07635662	53561.9272
RSD%		1.47	1.47	1.40

Recovery

The addition of standards to the mixture at three different levels like 80, 100, and 120% was used to evaluate the method's recovery. The mean quantity of drug recovered and the percentage of recovery were determined. It found that the results were seen within the acceptable range. The result was shown in table 5.

Table 8: Recovery results of Spironolactone by RP-HPLC

Sr. No	**Amount of Sample** (µg/ml)	**Amount of Drug Added** (µg/ml)	**Amount of Drug Recovered** (µg/ml)	**Recovery %**
1	20	10	9.989	99.89
2	20	20	19.99	99.96
3	20	30	30.01	100.03

BIOANALYTICAL METHOD DEVELOPMENT

The RP-HPLC method was designed and validated using the QbD approach in accordance with the above given. This study gives development and validation of bioanalytical methods by applying Quality by Design approach. To estimate the amount of drug in human plasma, optimized mobile phase by design expert software was used. Details are as follows.[22,23]

Chromatographic Conditions Optimized

Mobile phase: Acetonitrile: Ammonium formate buffer (60:40%v/v), pH of buffer: 3.5, Analytical column: C_{18} (4.6× 250mm id. particle size 5µm), UV detection: 237nm, Injection volume: 20 µl, Flow rate: 1.00 ml min^{-1}, Temperature: Ambient, Run time: 10 min

System Suitability Parameter

At 237 nm, five samples containing 5 g/ml were assessed, and the obtained data was compared with an optimized method. In spiked plasma, it was found that the peak's retention time was slightly changed from 5.233 min to 5.235 min.

Table 9: Details of System Suitability Parameter in Human Plasma

Sr. No.	Parameters	Observation
1	Retention time	5.235 min
2	Peak area	920547
3	Theoretical plates	8926
4	Asymmetric Factor	1.15

Selectivity

The blank plasma samples were taken from six different people to ensure selectivity. Each blank sample was examined for Spironolactone peak interference. The Spironolactone and plasma peaks were well defined. Spironolactone peak was found to be unaffected by the peak from blank plasma. As a result, the new approach is selective, and Spironolactone alone is responsible for the peak at 5.2 minutes. Figures 8 and 9 shown typical chromatograms of human plasma that haven't been spiked or adjusted.

Blank (Spiked Plasma)

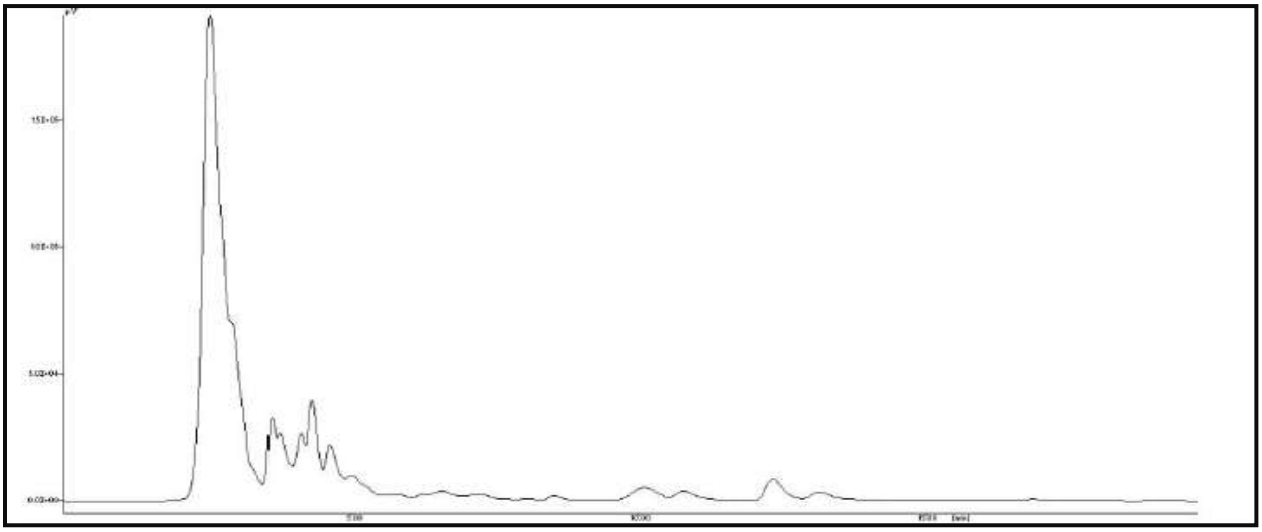

Figure 8: A typical chromatogram of blank human plasma

Sensitivity

When looking for drugs in human plasma, bioanalysis sensitivity is important. It offers vital information on how to quantify the smallest concentration. It was found that the limits of detection and quantitation were 0.4049µg/ ml and 1.2270µg / ml, respectively.

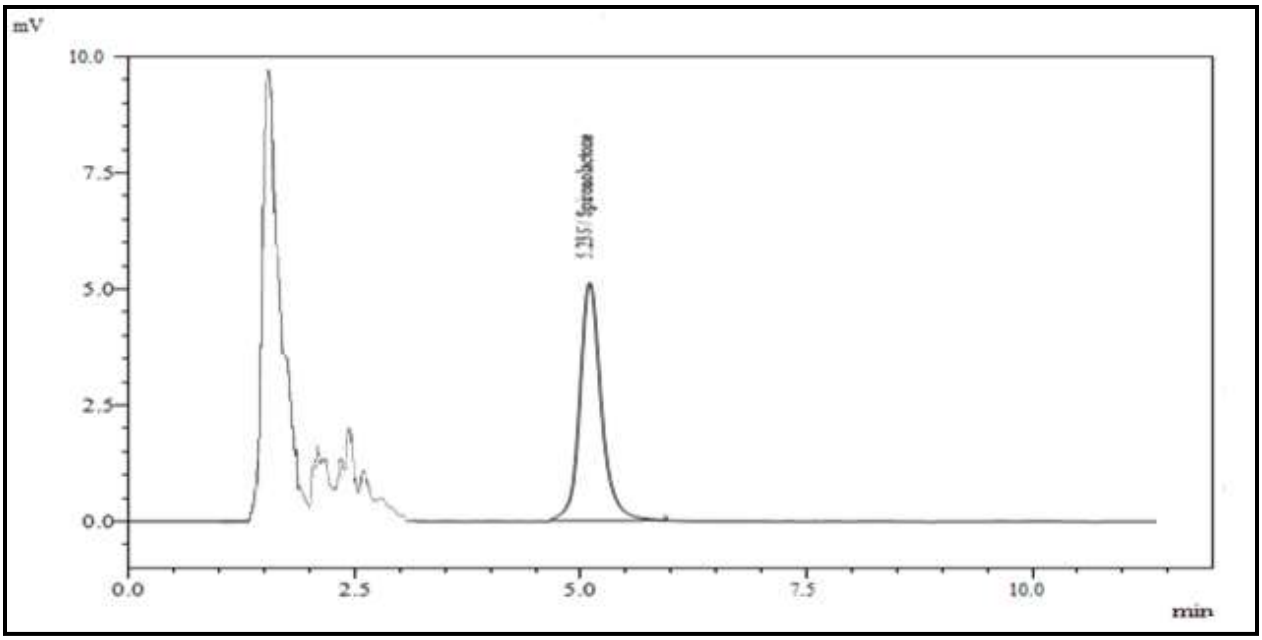

Figure 9: Chromatogram of Spironolactone spiked in human plasma

Linearity

The capacity to get responses that are directly proportionate to the concentration of drug is known as the linearity of the bioanalytical approach. Spironolactone was showed linearity from 5µg/ml to 30µg/ml. The results of the slope and correlation coefficient were determined to be satisfactory, and the concentration range was found to be linear with peak area.

Table 9: Details of Linearity

Sr. No	Concentration (µg/ml)	Peak Area
1	5	920547
2	10	1841094
3	15	2711641
4	20	3682188
5	25	4602735
6	30	5523282

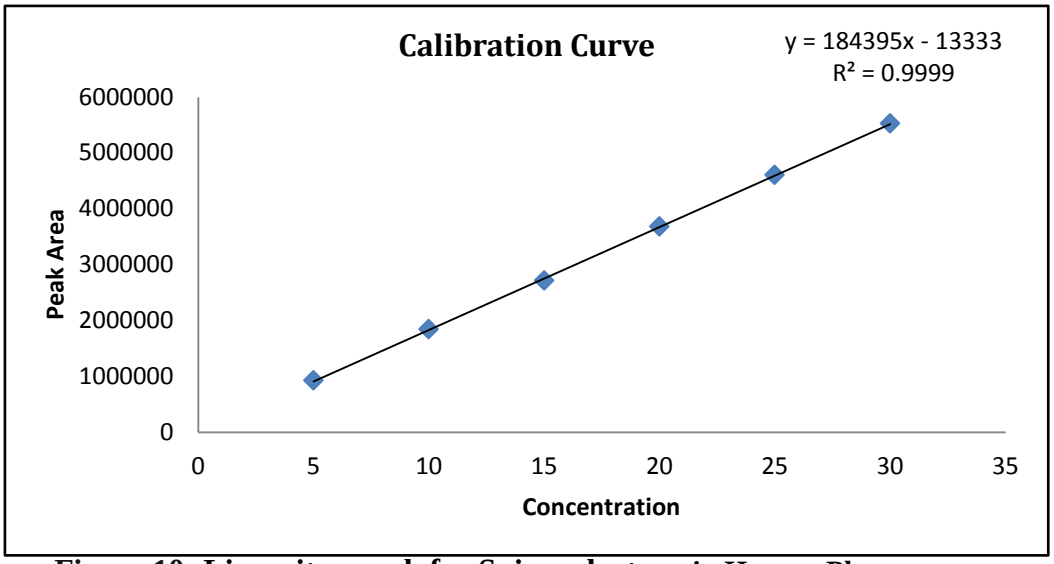

Figure 10: Linearity graph for Spironolactone in Human Plasma

Precision

System precision and method precision are the two components of analytical precision. Repetition only defines system precision. There are two types of method precision: intraday precision and interday precision. The average value and individual value were not significantly different according to relative standard deviations. It proved the method's reproducible and accuracy. The results obtained are shown in table 10.

Table 10: System & Method Precision results in Spiked Plasma

Sr. No	Concentration µg/ml	Intraday Precision Peak Area	Interday Precision Peak Area	Repeatability
1	20	3706665	3695027	3687406
2	20	3731689	3735937	3667406
3	20	3658302	3721350	3687419
4	20	3713669	3600980	3680532
5	20	3745424	3681890	3737406
6	20	3718875	3647611	3685194
Average		3712437	3680466	3690894
Standard Deviation		29885.35	49719.4	23996.48
RSD%		0.80	1.35	0.65

Specificity

We have shown through a selectivity research that the peak of Spironolactone is effectively resolved in human plasma. The separation of Spironolactone is unaffected by plasma peaks. A formulation was used to create six duplicates (Aldactone 50 Tablet). The results of the specificity test indicated that the tablet's excipients had no impact on the ability to detect Spironolactone. The tablet's chromatogram for the drug Spironolactone was discovered at 5.235 minutes. 99.82% of the drug was discovered to have been recovered. The result was shown in table 11.

Table 11: Recovery results of Spironolactone in Human Plasma

Sample	**Label Claim** (mg)	**Amount of Drug Found** (mg)	**Recovery (%)**	**Retention Time (min)**
Tablet	50	49.91	99.82	5.235

Robustness

To evaluate the robustness of the bioanalytical method, the effect of mobile phase composition, detection wavelength, and pH of the buffer was employed to assess the ability of the method to remain unaffected by small changes in chromatographic conditions. Serial dilutions of 20µg/ml solution were prepared and generated the relative standard deviation. The results were shown in table 12.

Table 13: Results of Robustness in Spiked Plasma

Sr. No	**Parameter**		**Response**	**Parameter**	**Response**
Acetonitrile: Ammonium Format Buffer			**Retention Time (min)**	**Detection Wavelength**	**Peak Area**
(%V/V)				(nm)	
1	59	41	5.434	235	3624061
2	60	40	5.531	237	3682376
3	61	39	5.61	239	3715640
Average			5.525	Average	3674026
Standard Deviation			0.08815	Standard Deviation	46357
RSD%			1.59	**RSD%**	1.26
Flow Rate			**Retention Time (min)**	**pH of Buffer**	**Peak Area**
(ml/min)				(mmol/l)	
1	0.9		5.562	3.3	3710448

2	1	5.531	3.5	3682549
3	1.1	5.417	3.7	3606928
Average		5.50333	5.50333	3666642
Standard Deviation		0.07636	0.07636	53561.9
RSD%		1.39	1.39	1.46

Recovery

Recovery of the developed bioanalytical method was determined by adding standard with 80, 100, and 120% to the spiked plasma. The percentage of recovery was found to be 99.87 to 100.08%. The result is shown below in table 13.

Table 13 Recovery results of Spironolactone by RP-HPLC

Sr. No	Amount of Sample (µg/ml)	Amount of Drug Added (µg/ml)	Amount of Drug Recovered (µg/ml)	Recovery %
1	10	8	7.99	99.87
2	10	10	9.98	99.80
3	10	12	12.01	100.08

Stability of Analyte in the Human Plasma

Freeze and Thaw Stability:

Three freeze-thaw cycles at a temperature of -20°C±1°C were used to evaluate the stability of Spironolactone in spiking plasma. The drug is still stable during freeze/thaw cycles, as shown by comparing stability samples to freshly prepared samples. The result is shown below in table 14.

Table 14: Results of Freeze and Thaw Stability

Replicate No.	Actual Concentration			
	LQC (8 µg/ml)		HQC (24 µg/ml)	
	Comparison Sample	Stability Sample	Comparison Sample	Stability Sample
1	1492132	1389624	4478026	4256941
2	1492521	1387241	4528136	4259924
3	1482418	1352133	4476998	4149987

Mean	1489024	1376333	4494387	4222284
SD	5723.981	20991.37	29232.3	62628.801
% RSD	0.38	1.52	0.65	1.48

Short Term Stability

The storage time of the spiked plasma samples at room temperature was calculated to be 8 hours. Stability was evaluated by comparing stability samples with freshly prepared quality control samples that were spiked. The result is shown below in table 15.

Table 16 Results of Short Term Stability

Replicate No.	**Actual Concentration**			
	LQC (2 µg/ml)		**HQC (60 µg/ml)**	
	Comparison Sample	**Stability Sample**	**Comparison Sample**	**Stability Sample**
1	393168	359312	12395213	11795067
2	393641	353274	12388297	11997852
3	388897	351101	12554187	12099321
Mean	391902	354562.3	12445899	11964080
SD	2613.13	4254.407	93843.89	154912.99
% RSD	0.67	1.20	0.75	1.29

Long Term Stability

A sample of spiked plasma was evaluated for stability over a period of seven days at room temperature by comparing it to a freshly weighed stock solution. The result is shown below in table 16.

Table 16: Results of Long Term Stability

Replicate No.	**Actual Concentration**			
	LQC (8 µg/ml)		**HQC (24 µg/ml)**	
	Comparison Sample	**Stability Sample**	**Comparison Sample**	**Stability Sample**
1	1492064	1389993	4479314	4256900

2	1489354	1387923	4470297	4259199
3	1479714	1355297	4477632	4149039
Mean	1487044	1377737.67	4475747.67	4221712.67
SD	6490.971	19461.728	4794.748	62947.738
% RSD	0.44	1.41	0.11	1.49

Conclusion

Our recent experiment demonstrated the development and validation of a bioanalytical and RP-HPLC method for the accurate measurement of Spironolactone in its pure form, dosage forms, and spiked human plasma. The drawbacks of the previous published approach, such as the need for the isocratic process, longer retention times, and complex extraction for this simple method, are all eliminated by the developed experiment. Additionally, this procedure reduces costs because it uses inexpensive instruments, solvents, and reagents. This simple approach may be used for pharmacokinetic analysis of Spironolactone as well as quality control and quality assurance because to its excellent accuracy, precision, and sensitivity.

Future prospects

For the estimation of Spironolactone in human plasma, HPTLC technique development and validation using central composite design or any statistical methodology would be challenging research study.

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