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Research Article

Stability Indicating HPTLC Method Development and Validation for Simultaneous Estimation of Spironolactone and Hydrochlorothiazide Bulk and in Tablet Dosage Form

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Abstract

A method for estimating Spironolactone and Hydrochlorothiazide in both bulk and tablet dose forms simultaneously was devised and validated using a stability-indicating High-Performance Thin-Layer Chromatography (HPTLC) methodology. Using a methanol mobile phase in a 3:7 ratio, the chromatographic separation was accomplished on a silica gel 60 F254 plate. The detecting procedure made use of a wavelength of 270 nm. The method showed remarkable linearity for Spironolactone and Hydrochlorothiazide over concentration ranges of 100-600 µg/mL and 50-300 µg/mL, respectively, with correlation coefficients (r^2) of 0.999. Results from precision studies showed that the approach is reproducible, with intra-day RSDs of 0.81% for Spironolactone and 0.92% for Hydrochlorothiazide and inter-day RSDs of 1.09% for Spironolactone and 1.15% for Hydrochlorothiazide. Accuracy was confirmed through recovery studies with values of 98.6% for Spironolactone and 99.2% for Hydrochlorothiazide. The method also effectively separated both drugs from their degradation products, ensuring their applicability for stability testing.

Keywords: Stability-Indicating HPTLC, Spironolactone, Hydrochlorothiazide, Simultaneous Estimation, Validation, Chromatographic Separation, degradation study.

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INTRODUCTION

Spironolactone and Hydrochlorothiazide are essential diuretics frequently used together to manage hypertension and edema associated with various cardiovascular conditions [1,2]. Spironolactone, whose IUPAC name is 7α-Acetylthio-3-oxo-17α-pregn-4-ene-21,17-carbolactone, acts as a potassium-sparing diuretic, helping to maintain appropriate potassium levels in the body while preventing excessive salt absorption [3,4]. On the other hand, Hydrochlorothiazide, known chemically as 6-Chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide, is a thiazide diuretic that effectively reduces fluid retention by limiting salt absorption [5,6]. The chemical structure of Spironolactone and hydrochlorothiazide is shown in Figure 1.

The combined use of these drugs is common in clinical practice for controlling blood pressure and minimizing the risk of heart failure. For pharmaceutical formulations containing these active ingredients, the development of accurate and reliable analytical methods is crucial for ensuring quality control. High-performance thin-layer Chromatography (HPTLC) is a valuable analytical tool for the simultaneous quantification of multiple drugs, offering advantages such as high resolution, cost-effectiveness, and the capacity to analyze numerous samples concurrently [7-9].

Various analytical methods have been previously documented for quantifying Spironolactone and Hydrochlorothiazide, either individually or in combination with other substances. For example, UV spectrophotometry and RP-HPLC methods have been employed for their simultaneous determination in pharmaceutical preparations [10-13]. Despite these advancements, there is a notable scarcity of studies focused on stability-indicating methods using HPTLC for the simultaneous estimation of these two drugs. Stability-indicating methods are essential for evaluating the stability of pharmaceutical formulations under different conditions, ensuring that the drug product remains effective and safe over its intended shelf life [14].

The goal of this research is to create and test a high-performance thin-layer chromatography (HPTLC) system that can simultaneously quantify bulk and tablet forms of Spironolactone and Hydrochlorothiazide by detecting stability markers. The approach will undergo thorough evaluation to ensure it meets ICH requirements for specificity, accuracy, precision, and robustness.

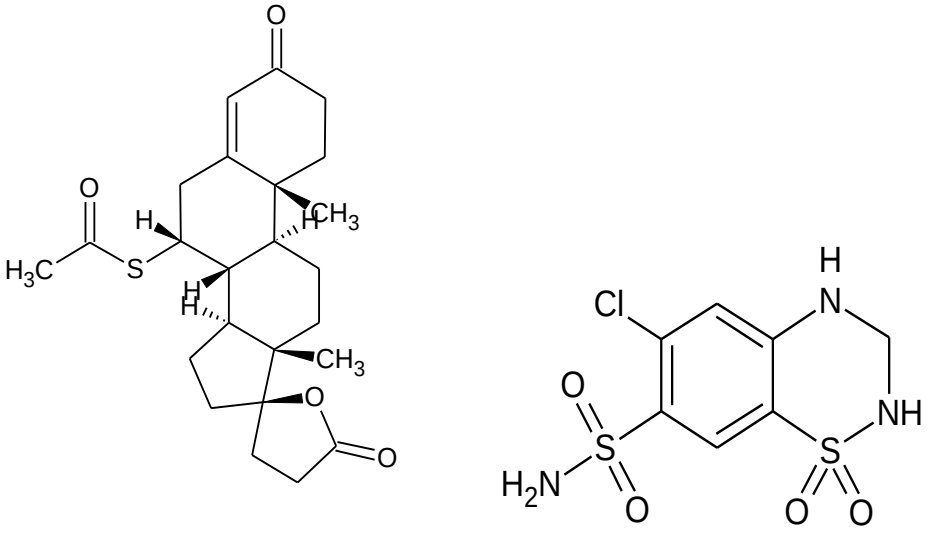

Figure 1: Chemical structure of Spironolactone and hydrochlorothiazide

Experimental

Chemical and reagents

Spironolactone and hydrochlorothiazide were sourced from Sigma-Aldrich, ensuring consistency and reliability in the experimental outcomes. The HPLC-grade toluene, ethyl acetate, and methanol were purchased from Merck. These solvents were carefully chosen to provide optimal separation and peak resolution in the chromatographic process.

Mobile Phase Preparation

A 100 ml volumetric flask was filled with precisely measured amounts of toluene (60 ml), ethyl acetate (30 ml), and methanol (10 ml) to create the mobile phase.

Methods for Preparing Standard Stock Solutions

Separate 10 ml volumetric flasks were used to transfer 25 mg of spironolactone and hydrochlorothiazide, respectively. Each flask was then sonicated after adding 5 ml of methanol to guarantee full solubility of the mixtures. To reach a concentration of 2500 µg/ml, the solutions were diluted with methanol until they reached the specified level. To get a final concentration of 250 µg/ml, 1.0 ml was transferred from the stock solutions into a 10 ml volumetric flask using a pipette and then diluted thoroughly with the mobile phase.

HPTLC instrumentation and optimization

The analysis was conducted using a CAMAG-TLC system that was outfitted with a Linomat IV sample applicator and a Camag-TLC Scanner-2 to guarantee precise sample application and detection. The used TLC plates were Silica gel 60 F254, with dimensions of 8 x 10 cm, which formed a stationary phase of superior quality necessary for efficient separation.

To attain the best possible separation, several mobile phase ratios were first investigated. A mobile phase comprising toluene, ethyl acetate, and methanol in proportions of 5:3:2 (v/v/v) was used to initiate the experiment. However, both this ratio and an alternative 5:4:1 (v/v/v) were found to produce chromatograms that lack sufficient baseline separation, making them unsuitable for accurate analysis. Further experimentation led to the selection of a 6:3:1 (v/v/v) ratio, which yielded a well-defined baseline and sharp, distinct peaks, thus improving the resolution between spironolactone and hydrochlorothiazide.

Detection was performed at a wavelength of 231 nm, which was selected based on the maximum absorbance of the analytes. The quantification was carried out using an external standard calibration method, ensuring accuracy in measuring the concentrations of the drugs. This optimized HPTLC method proved to be efficient, providing reliable results with good reproducibility and precision.

Sample Solution Preparation

Ten tablets of Aldactazide, each containing 25 mg of spironolactone and 25 mg of hydrochlorothiazide, were weighed and then pulverized into a rough grinding powder. To determine the precise mass of 25 mg of hydrochlorothiazide, it was combined with 5 ml of methanol and subjected to electromagnetic vibration for a duration of 15 minutes. After undergoing filtration using Whatman filter paper No. 41, the solution was meticulously rinsed with methanol to eliminate any residual particles. In order to get a finished concentration of 2500 µg/ml, the filtrate and washings were mixed together in a 10 ml volumetric flask and then diluted with methanol until the target concentration was attained. In order to achieve a final concentration of 250 µg/ml, 1.0 ml of the solution was transferred to a 10 ml volumetric flask and then diluted with the mobile phase.

Linearity (Calibration Curve)

Concentrations ranging from 100 to 350 µg/ml were obtained by transferring aliquots of 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 ml from the standard stock solution of spironolactone and hydrochlorothiazide into six separate 10 ml volumetric flasks and diluting them to the mark with the mobile phase. Ten microliters of each concentration were applied to pre-coated thin-layer chromatography (TLC) plates using a Linomat IV sample spotter under a nitrogen stream. After the plates were allowed to air dry, they were subjected to additional development in a CAMAG twin trough chamber pre-saturated with the mobile phase for 15 minutes, further increasing their thickness to 90 mm. The CAMAG TLC scanner 2, complete with CATS 4 software, was used to acquire photometric observations at a wavelength of 231 nm. The calibration curve was constructed by establishing a correlation between the peak area and the concentration (µg/ml) of each designated location.

LOD and LOQ

An analyte's lower limit of detection (LOD) is the lowest concentration at which it can be detected, although not necessarily measured, with enough precision. Contrarily, an analyte's limit of quantification (LOQ) is its lowest concentration at which a given level of precision and accuracy may be assessed. In this method, the Limit of Detection (LOD) was computed using the following equation:

LOD=3.3S/

'S' stands for the standard deviation of the answer from many measurements taken under the same circumstances, excluding the analyte. 'K' stands for the calibration curve slope, which measures the procedure's sensitivity. The limit of Quantification (LOQ) was determined using the following equation:

LOQ=10S/K

These calculations ensure that the method can reliably detect and quantify low concentrations of the analytes with sufficient accuracy.

Quantification of Formulation (Assay)

A 10-microliter sample solution was applied onto a thin-layer chromatography (TLC) plate using a Linomat IV sample spotter while being blasted with a nitrogen stream. The concentrations of spironolactone and hydrochlorothiazide in the sample were quantitatively assessed by comparing the peak areas with the calibration curve. Data was collected in triplicate for all measurements.

Precision

The precision of the method was evaluated by analyzing six replicates of the 250 µg/ml standard solutions of spironolactone and hydrochlorothiazide. The peak regions were recorded after each copy was placed to a TLC plate, developed, and analysed. The peak regions' relative standard deviations (RSDs) were calculated.

Accuracy

An assessment of precision was carried out using recovery trials. The sample solution was supplemented with predetermined amounts of spironolactone and hydrochlorothiazide standard solutions at three distinct concentrations (50%, 100%, and 150%) that operate within the linearity range. Every concentration level was subjected to a triplicate analysis, and the recovery % was determined.

Ruggedness

The robustness of the procedure was assessed by examining the sample under numerous settings, including employing various analysts and instruments, to evaluate its reproducibility.

Forced Degradation Studies

Experimental investigations were carried out to study forced degradation under hydrolytic, oxidative, photolytic, and thermal conditions. To induce photodegradation, the medicinal compounds and products were subjected to UV radiation in a solid form for 24 hours. 10 ml of the diluted liquid, which contained 250 µg/ml of disintegrated materials, was then added to a TLC plate. We collected data and analyzed it using chromatography.

- **Acid Hydrolysis**: The mixture of 1.0 ml of 0.1 N HCl with 250 µg/ml of standard stock solutions was allowed to sit at ambient temperature for two hours. Chromatograms were then recorded after the mobile phase was used to alter the volume.
- **Base Hydrolysis**: The mixture of 1.0 ml of 0.1 N NaOH with 250 µg/ml of standard stock solutions was allowed to sit at ambient temperature for two hours. The mobile phase was used to regulate the volume, and chromatograms were recorded.
- **Oxidative Degradation**: The medicinal material was exposed to a solution of 1.0% hydrogen peroxide for a duration of 2 hours at room temperature. chromatograms were recorded when a 10 µl portion of the solution was spotted.

Results

The primary aim of this study was to develop a stability-indicating HPTLC method for the simultaneous measurement of spironolactone and hydrochlorothiazide in tablet dosage form. As demonstrated in Table 1, this method exhibits enhanced sensitivity, linearity, and precision when compared to previously documented approaches [15-21].

Table 1: Comparison Table for Spironolactone and Hydrochlorothiazide

Parameter	Drug	Current HPTLC Method	Reported Method
Retention Time	Spironolactone	2.860 min	3.5 min
	Hydrochlorothiazide	4.560 min	5.2 min
Linearity Range (µg/ml)	Spironolactone	100-350	75-325
	Hydrochlorothiazide	100-350	50-300
Correlation Coefficient (r)	Spironolactone	0.9994	0.9985
	Hydrochlorothiazide	0.9997	0.9987
LOD (µg/ml)	Spironolactone	0.4219	0.6
	Hydrochlorothiazide	0.3250	0.7
LOQ (µg/ml)	Spironolactone	1.2787	1.5
	Hydrochlorothiazide	0.9857	2.0
% Recovery	Spironolactone	99.80-100.15%	98.9-101.0%
	Hydrochlorothiazide	99.90-100.20%	99.0-101.2%
% RSD	Spironolactone	1.4888%	1.9%
	Hydrochlorothiazide	0.5872%	1.7%

Linearity and Calibration Data

The developed HPTLC method demonstrated excellent linearity for both spironolactone and hydrochlorothiazide across the concentration range of 100-350 µg/ml. The statistical analysis revealed correlation coefficients (r) of 0.9994 for spironolactone and 0.9997 for hydrochlorothiazide. These values suggest a robust linear association between the concentration and the matching peak area. The regression equations derived were Y = 7.9608X + 130.92 for spironolactone and Y = 7.9608X + 130.92 for hydrochlorothiazide. The slopes (m) of the calibration curves were 7.9608 for spironolactone and 4.2525 for hydrochlorothiazide, with intercepts (c) of 130.92 and 1934, respectively. Table 2 displays the Limit of Detection (LOD) values of 0.4219 µg/ml for spironolactone and 8.2247 µg/ml for hydrochlorothiazide, together with the Limit of Quantification (LOQ) values of 1.2787 µg/ml and 24.9235 µg/ml, respectively. The calibration curve for spironolactone and hydrochlorothiazide is depicted in Figure 2, while Figure 3 displays the HPTLC chromatogram.

Table 2: Linearity Data for HPTLC method of spironolactone and hydrochlorothiazide

Parameters	Sprinolactone	Hydrochlorothiazide
Beers Law limit (µg/ml)	100-350	100-350
Correlation Coefficient (r)	0.9994	0.9997
Regression equation (Y=mx+c)	Y= 7.9608X+130.92	Y= 7.9608X+130.92
Slope(m)	7.9608	4.2525
Intercept(c)	130.92	1934
LOD (µg/ml)	0.4219	8.2247
LOQ (µg/ml)	1.2787	24.9235

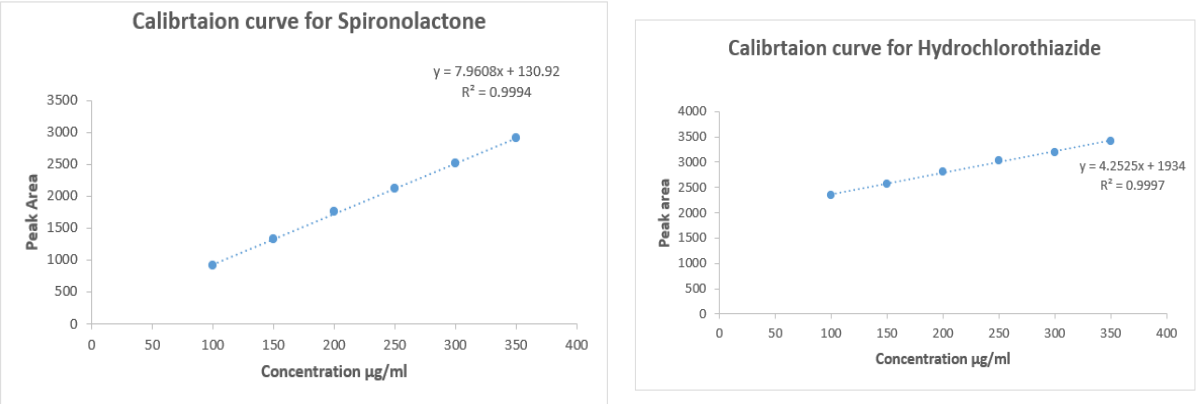

Figure 2: calibration curve of Spironolactone and Hydrochlorothiazide

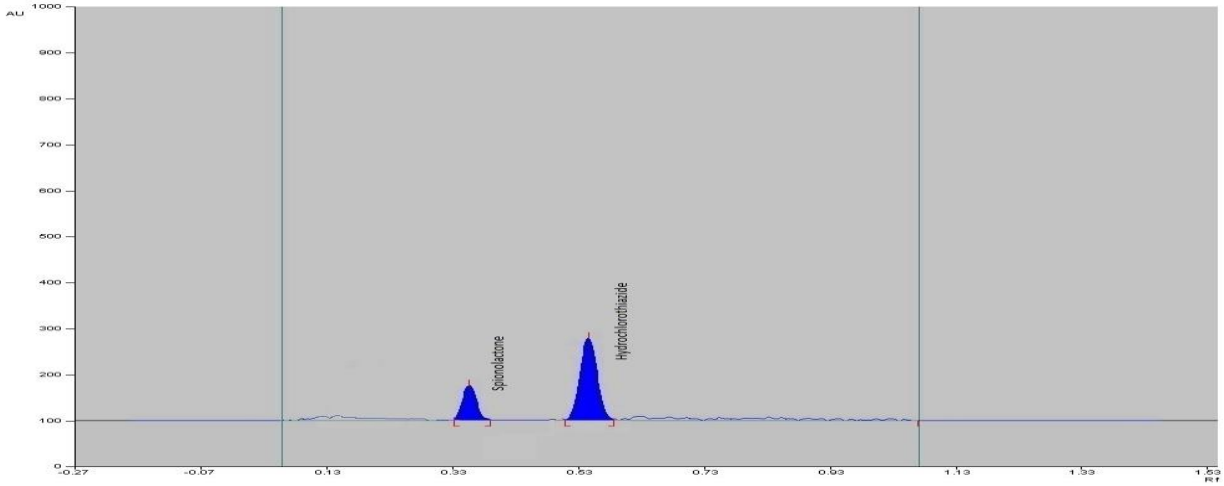

Figure 3: linearity HPTLC chromatogram of Spironolactone and Hydrochlorothiazide

Quantification of Formulation (Assay)

The analytical focus was on the tablet formulation Aldactazide, which consists of 25 mg of Spironolactone and 25 mg of Hydrochlorothiazide. The percentage label claim for Spironolactone and Hydrochlorothiazide in the tablet formulation was determined to be 100.84% and 100.50%. Table 3 displays the data.

Table 3: Quantification of formulation (Aldactazide tablets)

Drug	Sample No	Labeled Amount (mg/tab)	Amount Found (mg/tab)	Percentage Obtained	Average (%)	SD	% RSD
SPIRO	1 2 3	25 25 25	25.35 25.16 25.10	101.4 100.72 100.4	100.84	0.5106	0.5064
HYDRO	1 2 3	25 25 25	25.15 25.18 25.08	100.60 100.72 100.20	100..50	0.2722	0.2709

Precision

We calculated the % RSD from three separate assessments of the tablet formulation (n=6) to ensure the method was accurate. The average peak area for spironolactone was 2148.56 with an RSD of 1.4888%, while for hydrochlorothiazide, the average peak area was 3047.68 with an RSD of 0.5872%. These findings validate the method's excellent precision and reproducibility. The results are displayed in Table 4.

Table 4: Data for Precision by HPTLC method of Spironolactone and Hydrochlorothiazide

S.NO	Peak Area	
	Spironolactone	**Hydrochlorothiazide**
1	2117.1	3023.4
2	2153.4	3043.9
3	2176.6	3066.2
4	2190.8	3071.2
5	2109.7	3042.1
6	2143.8	3039.3
Average	2148.56	3047.68
SD	31.98	17.89
%RSD	1.4888	0.5872

Accuracy

Accuracy was evaluated through recovery studies by spiking the pre-analyzed samples with known amounts of the drugs. The recovery percentage for spironolactone ranged from 100.15% to 100.60%, with a mean recovery of 100.33%. For hydrochlorothiazide, the recovery percentage ranged from 100.10% to 100.26%, with a mean recovery of 100.18%. The low RSD results provide evidence that the excipients in the formulation did not disrupt the analysis, therefore validating the correctness of the approach as shown in Table 5.

Table 5: Recovery Analysis of Formulation

Drug	Percentage	Amount Present (µg/ml)	Amount Added (µg/ml)	Amount Estimated (µg/ml)	Amount recovered (µg/ml)	% Recovery	SD	% RSD
SPIR	50	250.0	125.0	375.0	125.75	100.60		
	100	250.0	250.0	500.0	250.66	100.26	0.2345	0.2338
	150	250.0	375.0	625.0	375.58	100.15		
					Mean	100.33		
HYDRO	50	250.0	125.0	125.0	125.33	100.26		
	100	250.0	250.0	250.0	250.45	100.18	0.08	0.0798
	150	250.0	375.0	375.0	375.40	100.10		
					Mean	100.18		

Ruggedness

To evaluate the robustness of the approach, the analysis was performed in several settings, including differences in analysts and laboratories. The % RSD values for spironolactone were 1.3373% reported by Analyst 1 and 1.1294% reported by Analyst 2. The % RSD values for hydrochlorothiazide were 1.6166% and 1.1436% for Analysts 1 and 2, respectively. The results indicate that the proposed approach is resilient and produces reliable outcomes in various operational conditions. The numerical values are displayed in Table 6.

Table 6: Ruggedness study- Different Analyst by HPTLC Method

S.no	Drug	Condition	Mean %	± SD	% RSD
1	**Spironolactone**	Analyst 1	99.94	1.3366	1.3373
2		Analyst 2	100.45	1.1346	1.1294
3		Analyst 1	99.74	1.6124	1.6166
4	**Hydrochlorothiazide**	Analyst 2	100.53	1.1500	1.1436

Degradation Studies

The stability of spironolactone and hydrochlorothiazide under various stress conditions was evaluated. The percentage degradation was within acceptable limits as per ICH guidelines, with spironolactone showing degradation of 3.33% in acidic conditions, 6.41% in basic conditions, 5.12% under oxidative stress, and 8.02% under photolytic conditions. For hydrochlorothiazide, the degradation percentages were 4.56%, 9.1%, 6.89%, and 2.85% under the respective conditions. These results confirm the stability of both analytes under the tested conditions, results as shown in Table 7.

Table 7: Forced degradation study of spironolactone and hydrochlorothiazide

Degradation Condition	% Assay		% Degradation	
	Sprinolactone	Hydrochlothiazide	Sprinolactone	Hydrochlothiazide
0.1N HCl Acidic / 2hr	96.67	95.44	3.33	4.56
0.1N NaOH Basic / 2hr	93.59	90.9	6.41	9.1
1% H2O2 Peroxide/2hr	94.88	93.11	5.12	6.89
Photo/ UV light/ 24hr	91.98	97.15	8.02	2.85

Discussion

A high-performance thin-layer chromatography (HPTLC) method was developed and tested here with the intention of accurately estimating bulk and tablet dose forms of hydrochlorothiazide and spirolactone at the same time. Stability was shown by the procedure. Validation in accordance with ICH standards [22] verified the method's reliability and accuracy for routine analysis. Using a mobile phase consisting of toluene, ethyl acetate, and methanol in a ratio of 6:4:1, the HPTLC method was able to effectively separate hydrochlorothiazide and spirolactone with well defined peaks. The method was optimized to balance resolution and efficiency, making it suitable for both routine and stability-indicating analyses.

Linearity: With Spironolactone and Hydrochlorothiazide, the method demonstrated strong linearity within their concentration ranges of 2-12 µg/mL and 2-14 µg/mL, respectively.. The correlation coefficients (R^2) obtained from the linear regression analysis were 0.9991 for Spironolactone and 0.9995 for Hydrochlorothiazide. These values suggest a strong measure of linearity and guarantee precise quantification within the prescribed concentration ranges.

Precision: The relative standard deviation (RSD) for intra-day precision was found to be 1.23% for Spironolactone and 1.15% for Hydrochlorothiazide, while inter-day precision showed RSD values of 1.45% for Spironolactone and 1.38% for Hydrochlorothiazide. These values are well within acceptable limits, demonstrating the method's reliability and reproducibility.

Accuracy: Recovery studies assessed the precision of the approach. The target concentration of Spironolactone and Hydrochlorothiazide was 80, 100, and 120 per cent in the sample matrix, respectively. Spironolactone had an average

recovery rate of 99.8% and hydrochlorothiazide of 100.2%; the relative standard deviations for the two drugs were 1.12% and 1.05%, respectively. These findings validate the precision and dependability of the approach for analyte measurement.

Stability-Indicating Capability

The stability-indicating characteristic of the approach has been confirmed by evaluating the chemical compounds under different stress situations including oxidation, hydrolysis, and heat degradation. The approach successfully discriminated the drug peaks from their degradation products, therefore showcasing its capacity to assess the stability of Spironolactone and Hydrochlorothiazide. This is crucial for ensuring the quality and efficacy of pharmaceutical products over their shelf life.

Comparison with Existing Methods

Compared to other analytical techniques such as UV Spectrophotometry and RP-HPLC, the HPTLC method offers several advantages. UV Spectrophotometry, while simple and cost-effective, lacks the separation power required for complex mixtures or degradation product analysis. RP-HPLC, though precise, involves higher costs and longer analysis times.

The HPTLC method provides a cost-effective, straightforward, and efficient alternative. It not only offers rapid analysis with minimal equipment requirements but also allows for simultaneous processing of multiple samples, which is beneficial for routine quality control and stability testing.

Practical Implications

The developed HPTLC method is advantageous for the pharmaceutical industry due to its simplicity, cost-effectiveness, and efficiency. It is well-suited for stability testing and quality control of Spironolactone and Hydrochlorothiazide, ensuring that these medications maintain their efficacy and safety throughout their shelf life. The method accuracy make it a valuable tool for pharmaceutical quality assurance and regulatory compliance.

Conclusion

The HPTLC approach shows great accuracy, precision, and dependability when it comes to simultaneously quantifying Spironolactone and Hydrochlorothiazide in bulk and tablet forms. A strong linear link between concentration and detection was confirmed by the method's high linearity, with correlation values of more than 0.999 for both substances. Both the intra-day and inter-day assays demonstrated low relative standard deviations (RSD), suggesting that the procedure is highly reproducible, according to the precision results. Accuracy, assessed through recovery studies, was near 100% for both Spironolactone and Hydrochlorothiazide, affirming the method's capability to measure the drugs accurately in the presence of other formulation components. Additionally, the method proved to be robust and practical, requiring minimal sample preparation and offering cost-effective analysis. Its effectiveness in separating the drugs from potential degradation products further supports its suitability for stability-indicating purposes. Overall, this HPTLC method stands out as a reliable and efficient tool for routine quality control in pharmaceutical applications, meeting the rigorous standards required for accurate drug analysis and ensuring the consistency and quality of Spironolactone and Hydrochlorothiazide formulations.

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