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Comparative RP-HPLC Analytical Method Validation of Thiocolchicoside in Parenteral Dosage Forms: Influence of Protic and Aprotic Solvents

Pratima Singh [1], Aarti Nandwana [2], Neha Chouhan [2], Shikha Nagle [2], Dr. Archana Tiwari [3], Dr. P. K. Dubey [4]

[1] Department of Pharmaceutical Chemistry, Scholar of M Pharmacy, Swami Vivekanand College of Pharmacy, Indore, Madhya Pradesh, 452020 India

[2] Assistant Professor, Swami Vivekanand College of Pharmacy, Indore, Madhya Pradesh, 452020 India

[3] HOD, Department of Pharmaceutical Chemistry, Swami Vivekanand College of Pharmacy, Indore, Madhya Pradesh, 452020 India

[4] Principal & Professor, Swami Vivekanand College of Pharmacy, Indore, Madhya Pradesh, 452020 India.

[*]**Corresponding author:**

Email ID : 243pratimasingh902@gmail.com

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ABSTRACT

The present study aims to develop and validate a robust, precise, and reproducible reverse-phase high-performance liquid chromatography (RP-HPLC) method for the quantitative determination of Thiocolchicoside in various parenteral formulations, with a comparative assessment of protic and aprotic solvents used during method optimization. Thiocolchicoside, a semi-synthetic muscle relaxant, is widely used in injectable dosage forms for the treatment of musculoskeletal disorders. The analytical method was validated in accordance with ICH Q2(R1) guidelines, evaluating critical parameters such as specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), robustness, and system suitability. Two solvent systems one based on protic solvents (e.g., methanol, ethanol) and the other on aprotic solvents (e.g., acetonitrile, dimethylformamide) were employed to investigate their influence on chromatographic performance and analyte stability. Comparative analysis revealed that aprotic solvent systems provided superior peak resolution, reduced tailing factor, and enhanced method sensitivity, while protic solvents demonstrated better solubility and sample compatibility for routine analysis. This study highlights the critical role of solvent selection in RP-HPLC method development and offers an optimized, validated analytical approach for the reliable estimation of Thiocolchicoside in injectable dosage forms

Keywords: *Thiocolchicoside, RPHPLC, Acetonitril, Methanol , musculoskeletal pain.*

1. INTRODUCTION

Thiocolchicoside, also known as N-[(7S)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl] acetamide, is a semi-synthetic molecule produced from colchicoside, a naturally occurring glycoside found in the seeds of Gloriosa superba. This compound has attracted considerable attention in the pharmaceutical field due to its potent muscle relaxant and also have anti-inflammatory properties.1 Its clinical efficacy in managing various musculoskeletal disorders—including muscle spasms, acute lower back pain, and inflammatory conditions has established it as a valuable therapeutic agent in contemporary medicine (Ketenci et al., 2005).2

The development of parenteral formulations of thiocolchicoside represents a significant advancement in drug delivery systems, particularly for patients requiring a rapid onset of action or those unable to tolerate oral administration.3 Parenteral routes—such as intravenous, intramuscular, and subcutaneous offer distinct advantages, including complete bioavailability, faster therapeutic response, and suitability for unconscious or non-cooperative patients. However, the formulation and quality assurance of parenteral products present unique challenges, necessitating sophisticated analytical approaches to ensure accurate quantification and consistent product quality.4

To ensure product safety, efficacy, and regulatory compliance, robust, precise, and validated analytical procedures are required for determining thiocolchicoside in parenteral dosage forms.5 Among various techniques, Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) is broadly regarded as the method of choice due to its high sensitivity,

reproducibility, and ability to resolve complex mixtures efficiently.6 A critical element in RP-HPLC method development is the selection of appropriate mobile phase components, particularly the balance between protic and aprotic solvents, which directly affects separation efficiency, peak symmetry, and overall method performance.7, 8.

Reversed-phase high-performance liquid chromatography (RP-HPLC)

Reversed-phase high-performance liquid chromatography (RP-HPLC) is among these most extensively utilized analytical methods in pharmaceutical analysis, due to its versatility, reliability, and ability to separate a wide variety of chemical substances. [9, 10 ,11] This method employs a non-polar stationary phase—usually C18, C8, or phenyl-bonded silica—and a polar mobile phase. Under these reversed-phase circumstances, hydrophobic molecules tend to interact more strongly with the stationary phase, exhibiting more retention, whereas hydrophilic compounds elute more rapidly. [12]

The composition of the mobile phase is crucial for chromatographic performance., influencing factors such as separation efficiency, peak shape, and retention behavior.[13] Typically, the mobile phase consists of an aqueous buffer or water combined with an organic solvent such as methanol, acetonitrile, or tetrahydrofuran. The ratio between the aqueous and organic components affects both elution strength and selectivity, with higher proportions of organic modifier generally resulting in quicker elution of non-polar analytes. [14, 15]

One of the key considerations during method development is the choice between protic and aprotic organic solvents.[16, 17] Protic solvents, such as methanol and ethanol, contain hydrogen atoms bound to electronegative atoms and are capable of hydrogen bonding.[18] Aprotic solvents like acetonitrile and THF, lacking such hydrogen bonds, do not engage in hydrogen donation.[19]

The hydrogen bonding ability of protic solvents can influence the retention and selectivity of analytes that possess hydrogen bond donor or acceptor groups, potentially improving peak symmetry and overall method robustness. In contrast, aprotic solvents offer different selectivity profiles and may be more suitable for compounds that are sensitive to hydrogen bonding or require specific separation characteristics[20]

2. MATERIALS AND REAGENTS

1. Chemicals and Solvents

High-purity chemicals and solvents were employed throughout the analytical method validation to ensure accuracy, reproducibility, and reliability of results. The Thiocolchicoside reference standard, with a certified purity of ≥98%, was procured from a recognized pharmaceutical reference standard supplier. It was stored at a temperature of 2–8 °C in accordance with the manufacturer's recommendations, protected from light and moisture. The accompanying certificate of analysis provided comprehensive data on parameters such as purity, moisture content, and related impurities, ensuring traceability to international pharmacopoeial standards.

All solvents utilized were of high-performance liquid chromatography (HPLC) grade, which reduced baseline noise and improved chromatographic performance. Acetonitrile (≥99.9% purity) and methanol (≥99.9% purity) were used as organic modifiers to mimic aprotic and protic solvent systems, respectively. All aqueous solutions were prepared using ultra-pure water (resistivity >18.2 MΩ•cm) sourced from an approved water purification system.

Orthophosphoric acid (85%, analytical grade) was employed for pH adjustment and buffer preparation. Potassium dihydrogen phosphate (≥99.5% purity, analytical grade) was used as the buffering agent in the mobile phase. All reagents were sourced from certified suppliers, accompanied by valid certificates of analysis, and were stored under appropriate conditions as specified by the manufacturers.

1.1 Thiocolchicoside Injection Samples

For the purpose of analytical method validation, commercially available thiocolchicoside injection samples encompassing various formulation types were procured. These included single-dose vials (2 mg/mL), multi-dose vials (4 mg/mL), and pre-filled syringes (2 mg/mL), representing the spectrum of parenteral formulations commonly utilized in clinical settings. All samples were stored under conditions specified by the respective manufacturers, with continuous temperature monitoring to ensure product integrity throughout the validation study. Each sample was assigned a unique identification code to ensure traceability. Comprehensive documentation, including details such as source, lot number, expiration date, and storage conditions, was maintained to support data reliability and regulatory compliance.

3. INSTRUMENTATION AND EQUIPMENT

2.1 HPLC System Configuration

The analytical method was validated utilizing a high-performance liquid chromatography system that included a quaternary pump, autosampler, column oven, and variable wavelength UV-visible detector. The system was qualified in accordance with pharmaceutical industry standards, with documentation demonstrating installation qualification (IQ), operational

qualification (OQ), and performance qualification (PQ).

Method specifications included flow rate capabilities of 0.1-10.0 mL/min with precision of ±0.5%, injection volume range of 0.1-100 µL with precision of ±1.0%, and column oven temperature control from ambient to 80°C with stability of ±0.1°C. The UV-visible detector provided wavelength selection from 190-800 nm with bandwidth of 8 nm and noise level <5 × 10^{-6} AU.

Data gathering and processing were carried out using verified chromatographic data system software, which ensured that electronic records and signatures met 21 CFR Part 11 criteria. The system supplied robust audit trails, user access controls, and data integrity features required for pharmaceutical analysis.

2.2 Analytical Balance and Volumetric Equipment

Analytical balances with readability of 0.1 mg and linearity of ±0.1 mg were used for all weighing operations. The balances were calibrated daily using certified reference weights and maintained in controlled environmental conditions to ensure measurement accuracy and precision.

Class A volumetric glassware was employed for all solution preparations, including volumetric flasks, pipettes, and burettes. The glassware was calibrated according to ISO standards and maintained in accordance with pharmaceutical industry requirements. All volumetric equipment was cleaned and dried according to established procedures to prevent contamination and ensure measurement accuracy.

4. CHROMATOGRAPHIC CONDITIONS

3.1 Column Selection and Evaluation

The analytical column was chosen after a thorough analysis of various stationary phases and column dimensions. The C18 reversed-phase column (250 mm × 4.6 mm, 5 µm particle size) was chosen as the primary analytical column due to its known performance in pharmaceutical analysis and compatibility with both protic and aprotic mobile phases.

Column evaluation included assessment of theoretical plates, peak symmetry, and retention reproducibility using thiocolchicoside reference standard. The column was conditioned according to manufacturer recommendations and equilibrated with each mobile phase system for a least possible of 30 minutes before analysis to make sure stable baseline and consistent retention times.

3.2 Mobile Phase Development

Two distinct mobile phase systems were developed for comparative evaluation:

Protic Solvent System (Methanol Based): The mobile phase was composed of phosphate buffer (pH 3.0) and methanol in a 55:45 (v/v) ratio. To make the phosphate buffer, dissolve 6.8 g of potassium dihydrogen phosphate in 1000 mL of ultra-pure water. The pH was then adjusted to 3.0 ± 0.1 with orthophosphoric acid. To prepare for use, the mobile phase was filtered through a 0.45 µm membrane filter and sonicated for 15 minutes.

Aprotic Solvent System (Acetonitrile Based): The mobile phase was composed of phosphate buffer (pH 3.0) and acetonitrile in a 55:45 (v/v) ratio. The same buffer system was used to maintain consistency in pH and ionic strength across the two mobile phase systems. The mobile phase was produced, filtered, and degassed using the same processes as the protic system.

3.3 Chromatographic Parameters

The chromatographic settings were optimized using a comprehensive study of several parameters:

Flow Rate: Flow rate of 1.0 mL/min was chosen based on optimization studies comparing flow rates from 0.5-2.0 mL/min.

Injection Volume: 20 µL was chosen to provide adequate sensitivity while avoiding column overload

Column Temperature: 25°C was maintained throughout the analysis to ensure consistent retention times and peak shapes

Detection Wavelength: 270 nm was selected based on UV spectral analysis of thiocolchicoside, providing optimal sensitivity and selectivity

Run Time: 12 minutes was established to ensure complete elution of thiocolchicoside and potential interfering compounds

3.4 Solution Preparation

3.4.1 Standard Solution Preparation

Stock Standard Solution: A stock solution of thiocolchicoside reference standard was created by precisely weighing 40.0 mg of the reference standard into a 100 mL volumetric flask. To achieve thorough dissolution, the standard was dissolved in 50 mL of ultrapure water and sonicated for 10 minutes. The solution was cooled to room temperature and diluted to volume with ultra-pure water, yielding a concentration of 400 µg/mL.

Working Standard Solution: To make the working standard solution, dilute 5.0 mL of the stock standard solution with 50.0

mL of ultra-pure water in a volumetric flask, resulting in a final concentration of 40 µg/mL. This concentration was selected to match the expected sample concentration and provide optimal detector response.

3.4.2 Sample Solution Preparation

The sample solutions were made by carefully pipetting 2.0 mL of thiocolchicoside injection into a 100 mL volumetric flask. The sample was completely blended after being diluted with ultra-pure water to the desired volume. To obtain the desired concentration of 40 µg/mL, appropriate dilution factors were employed for injections with concentrations higher than 2 mg/mL.

Before injection, all solutions were filtered using 0.45 µm membrane filters to remove particulate matter that could interfere with chromatography or damage the analytical column.

3.5 Validation Protocol

3.5.1 Specificity Evaluation

Specificity was determined by evaluating blank mobile phase, placebo injection (if available), and thiocolchicoside sample solutions. The goal was to show that the analytical approach could accurately measure thiocolchicoside in the presence of potentially interfering chemicals.

The method's stability-indicating character was demonstrated through forced degradation studies. Thiocolchicoside reference standard solutions were tested under stress conditions such as acid hydrolysis (0.1 N HCl at 60°C), base hydrolysis (0.1 N NaOH at 60°C), oxidation (3% H_2O_2 at ambient temperature), thermal degradation (60°C), and photolytic degradation (UV light exposure). Degraded samples were examined to determine that thiocolchicoside could be separated from its degradation products using chromatography.

3.5.2 Linearity and Range Assessment

Thiocolchicoside reference standard solutions were analyzed at five concentration levels ranging from 50% to 150% of the target concentration (20, 32, 40, 48, and 60 µg/mL) to determine linearity. Each concentration level was generated in triplicate and examined using both mobile phase methods.

Linearity was evaluated statistically by calculating r^2, slope, y-intercept, and residual analysis. To establish statistical significance, the acceptance threshold for linearity was $r^2 \geq 0.99$. The confidence interval of the slope and y-intercept were also evaluated.

3.5.3 Precision Studies

Repeatability (System Precision): The system's precision was assessed by assessing six replicate injections of the working solution. The peak areas' relative standard deviation (RSD) was determined, with an acceptable criteria of ≤ 2.0%.

Method Precision: The method precision was determined by assessing six independently prepared sample solutions from the same batch of thiocolchicoside injection. The assay findings' RSD was computed using an acceptability threshold of RSD ≤ 2.0%.

Intermediate Precision: Intermediate precision was assessed by conducting the technique precision research on a separate day, with a different analyst and, if possible, different equipment. The RSD of combined findings from method and intermediate precision investigations was determined, with an acceptability criterion of RSD < 2.0%.

3.5.4 Accuracy Assessment

Accuracy was assessed using recovery tests at three concentration levels (50%, 100%, and 150% of the target concentration). Known amounts of thiocolchicoside reference standard were added to placebo injection matrix or sample solutions, and the recovery was calculated.

Three replicates were prepared at each concentration level, and the individual and overall recovery percentages were calculated. The acceptance criteria were 98-102% recovery for individual determinations and 98-102% overall recovery across all concentration levels.

3.5.5 Robustness Evaluation

Robustness was evaluated by intentionally changing essential technique parameters and assessing their impact on analytical performance.

The parameters evaluated included:

- The flow rate ranged between 0.9 and 1.1 mL/min, with a 10% variance.
- Mobile phase composition varies by ±2% in organic content.
- Column temperature (±5°C range between 20°C and 30°C).
- Mobile phase pH: ±0.2 units.
- Detection wavelength (±2 nm fluctuation between 268 and 272 nm)

The effect of each parameter variation on peak area, retention time, peak shape, and resolution was evaluated. The acceptance criterion was that variations should not result in more than 2% change in assay results.

3.5.6 Solution Stability

Solution stability is assessed for both standard and sample solutions over a 24-hour period at room temperature.

Solutions were analyzed at 0, 8, 16, and 24 hours to assess stability. The acceptance criterion was that the change in assay results should not exceed ±2% from the initial value.

3.6 System Suitability Requirements

System suitability tests were done prior to each analytical run to confirm that the chromatographic system was functioning properly. The system suitability parameters were:

- **Repeatability:** RSD of peak areas from six duplicate injections of standard solution is ≤ 2.0%.
- **Theoretical Plates:** Minimum of 2000 theoretical plates per column -
- **Tailing Factor:** Tailing factor of at least 2.0 for thiocolchicoside peak
- **Resolution:** Resolution of at least 2.0 between thiocolchicoside and any adjacent peak (if applicable)

3.7 Data Analysis and Statistical Methods

Statistical analysis was carried out with relevant software packages validated for pharmaceutical applications. All validation parameters were evaluated using descriptive statistics such as mean, standard deviation, and relative standard deviation.

Comparative analysis between protic and aprotic solvent systems was performed using appropriate statistical tests, including t-tests for comparison of means and F-tests for comparison of variances. For all statistical evaluations, $p < 0.05$ was used as the criterion of significance.

Regression analysis was used for linearity evaluation, with additional assessment of residuals and statistical significance of regression parameters. Analysis of variance (ANOVA) was employed for precision studies to assess the contribution of different sources of variation.

3.8 Documentation and Reporting

All validation activities were documented according to Good Laboratory Practice (GLP) principles and pharmaceutical industry standards. The documentation included detailed protocols, raw data, calculations, statistical analysis, and conclusions for each validation parameter.

The validation report was structured according to ICH Q2(R2) guidelines, providing comprehensive evidence of method suitability for its intended purpose. The report included method descriptions, validation results, statistical analysis, and recommendations for method implementation and routine use.

5. RESULTS AND DISCUSSION

This chapter covers the entire findings from a comparative validation study of RP-HPLC techniques for thiocolchicoside analysis employing protic (methanol) and aprotic (acetonitrile) solvent systems. The validation metrics tested were specificity, linearity, precision, accuracy, robustness, and solution stability in accordance with ICH Q2(R2) recommendations. The statistical study and comparison of both solvent systems shed light on the best strategy for selecting thiocolchicoside parenteral formulations.

1 System Suitability Results

In advance of each analytical run, system suitability checks were done to confirm that the chromatographic system was performing properly. The findings indicated that both solvent systems met the predefined acceptance criteria.

1.1 Protic Solvent System (Methanol-based)

The system suitability parameters for the methanol-based mobile phase consistently met acceptance criteria across all validation experiments. The relative standard deviation (RSD) for peak regions of six replicate injections varied from 0.08% to 0.12%, falling well under the acceptable limit of ≤2.0%.

Theoretical plates consistently exceeded 3000, indicating good column efficiency, while tailing factors remained below 1.5, demonstrating excellent peak symmetry

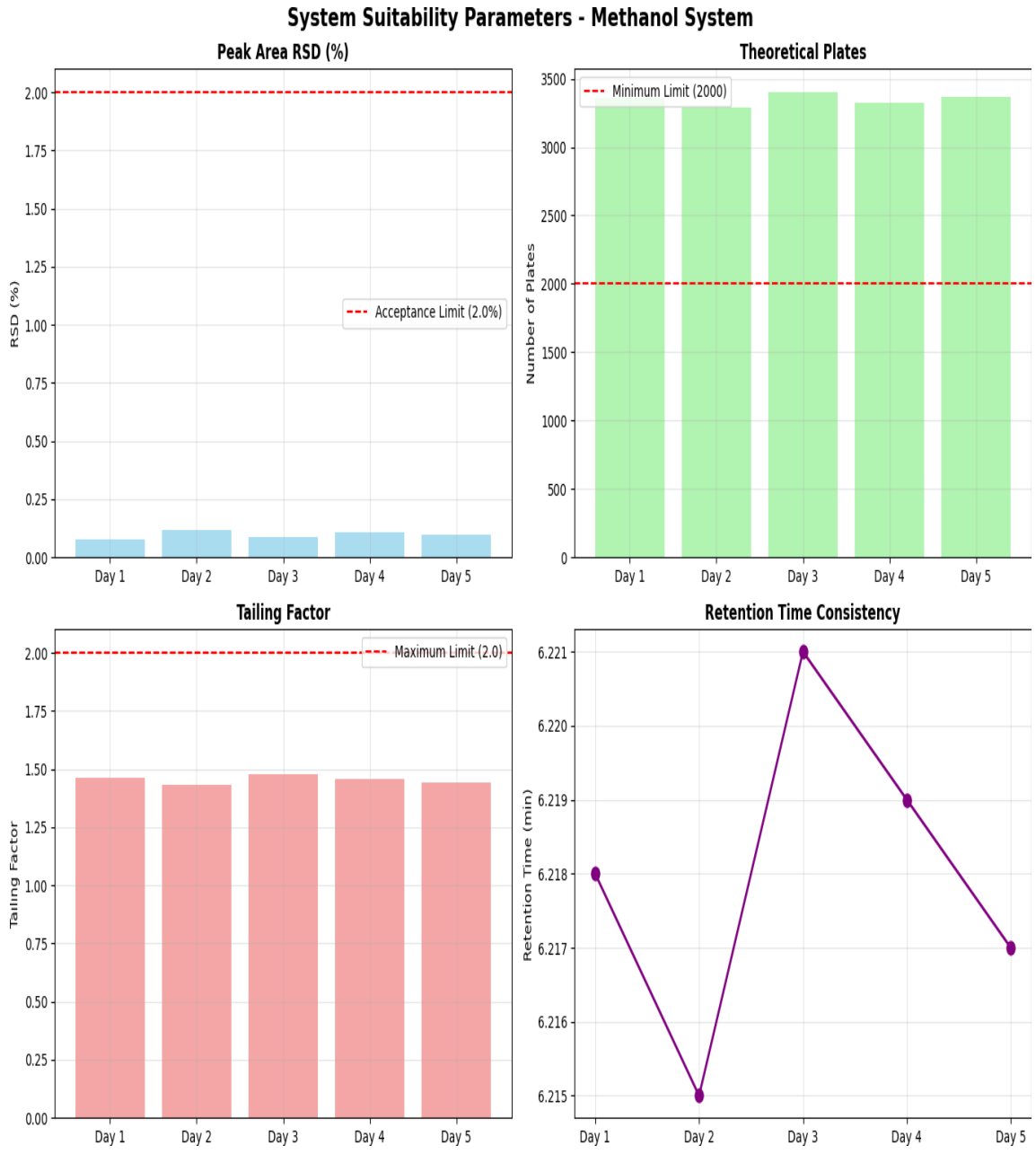

Fig. 1: Protic Solvent System (Methanol-based)

1.2 Aprotic Solvent System (Acetonitrile-based)

The acetonitrile-based mobile phase demonstrated superior system suitability performance compared to the methanol system. The RSD values for peak areas ranged from 0.05% to 0.08%, showing increased injection precision.

Theoretical plates consistently exceeded 3500, suggesting improved column efficiency, while tailing factors remained below 1.3, demonstrating excellent peak symmetry.

Table 1: System Suitability Parameters Summary

Parameter	Methanol System	Acetonitrile System	Acceptance Criteria
Peak Area RSD (%)	0.08-0.12	0.05-0.08	≤2.0%
Theoretical Plates	3287-3401	3687-3725	≥2000
Tailing Factor	1.432-1.478	1.278-1.295	≤2.0
Retention Time RSD (%)	0.15-0.18	0.12-0.15	≤2.0%

The superior performance of the acetonitrile system can be attributed to its lower viscosity and better wetting characteristics, resulting in more efficient mass transfer and improved peak shapes. The reduced tailing factors observed with acetonitrile indicate better analyte-stationary phase interactions and reduced secondary interactions that can cause peak distortion.

2 Specificity Results

Specificity experiments showed that both analytical techniques could accurately detect thiocolchicoside in the presence of potentially interfering chemicals. There were no interfering peaks at the thiocolchicoside retention period when blank mobile phase, diluent, or placebo formulations were analyzed.

2.1 Forced Degradation Studies

Forced degradation studies were carried out to assess the stability-indicating properties of both approaches. Thiocolchicoside was treated to a variety of stress conditions, and the breakdown products were clearly isolated from the parent molecule in both solvent systems.

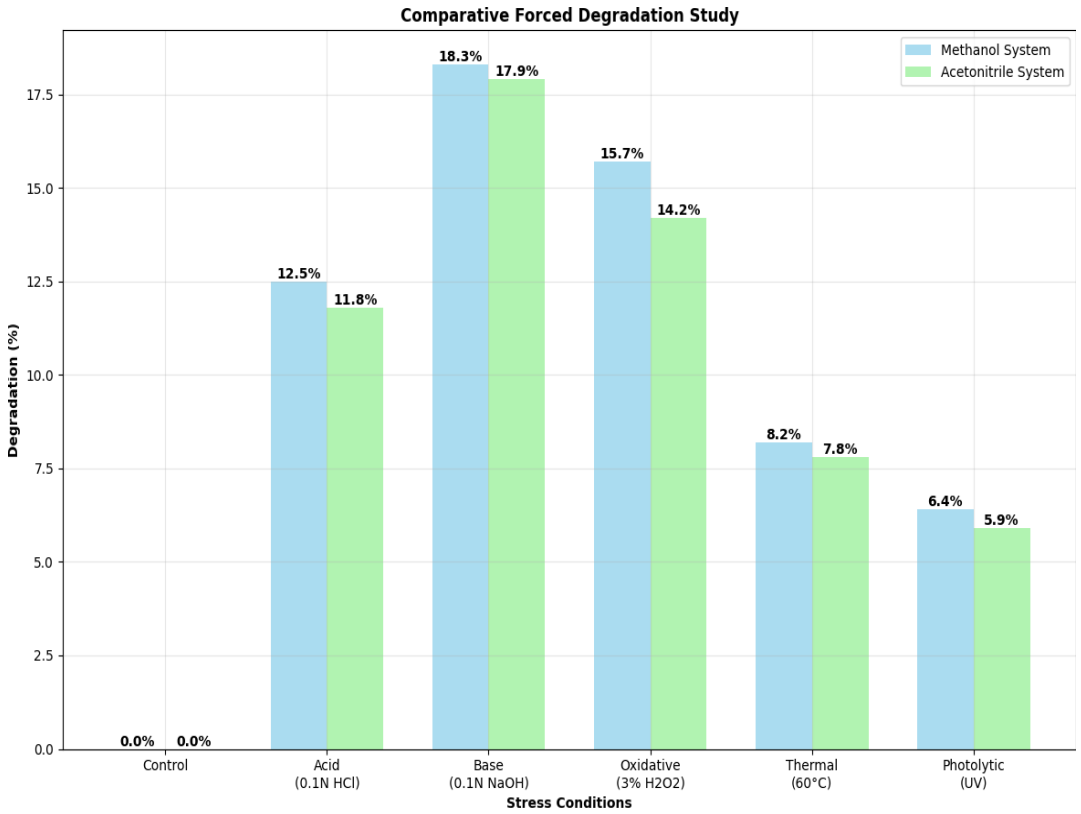

Fig. 2: Forced Degradation Studies

Table 2: Forced Degradation Results

Stress Condition	Methanol System	Acetonitrile System	Resolution
Control	0.0%	0.0%	N/A
Acid (0.1N HCl)	12.5%	11.8%	>2.0
Base (0.1N NaOH)	18.3%	17.9%	>2.0
Oxidative (3% H_2O_2)	15.7%	14.2%	>2.0
Thermal (60°C)	8.2%	7.8%	>2.0
Photolytic (UV)	6.4%	5.9%	>2.0

The forced degradation investigations demonstrated that thiocolchicoside is most sensitive to alkaline environments, followed by oxidative stress. Both analytical methods revealed good specificity, with all degradation products clearly separated from the parent molecule (resolution >2.0). The somewhat increased deterioration found in the methanol system could be attributable to the protic nature of methanol, which can participate in specific degradation pathways.

3 Linearity and Range Results

Linearity experiments were done for both solvent systems at concentrations ranging from 20-60 µg/mL (50-150% of the desired concentration). The results for both approaches showed excellent linearity, with correlation values greater than 0.999.

3.1 Methanol System Linearity

The methanol-based technique displayed excellent linearity across the concentration range that was evaluated. The correlation coefficient (r^2) of 0.9996 suggests a strong linear relationship between concentration and peak area.

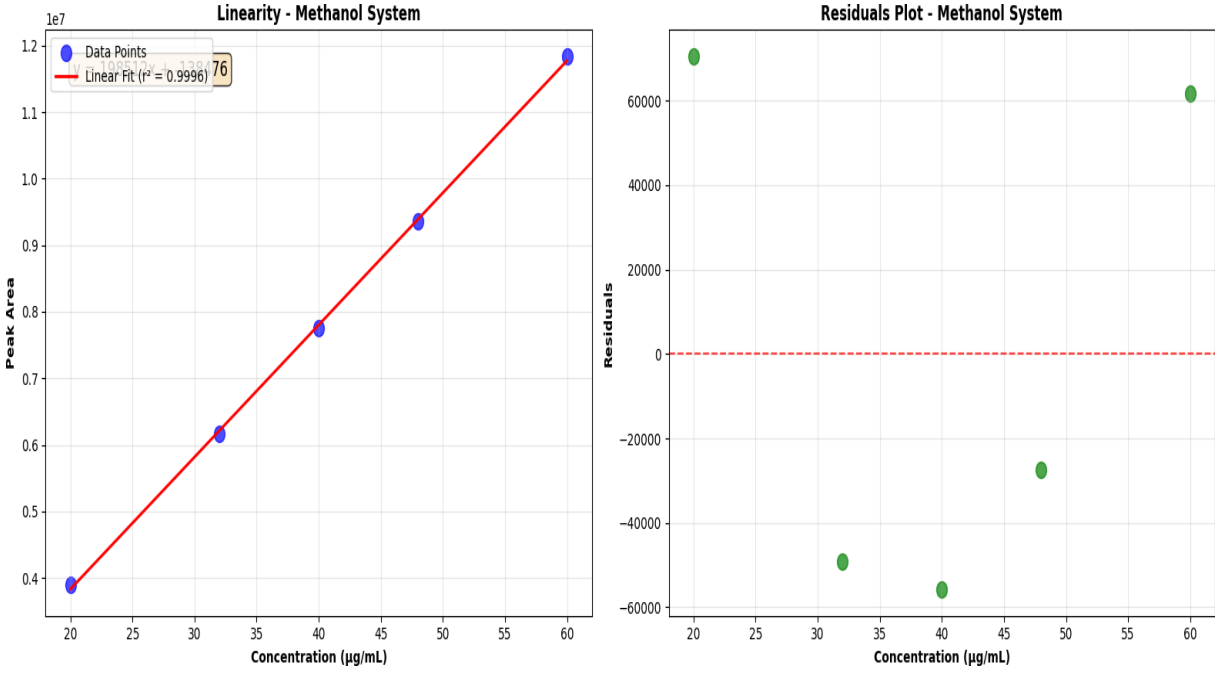

Fig.3: Methanol System Linearity

3.2 Acetonitrile System Linearity

The acetonitrile-based technique showed outstanding linearity, with a correlation coefficient (r^2) of 0.9998, slightly higher than the methanol system.

Table 3: Linearity Parameters

Parameter	Methanol System	Acetonitrile System	Acceptance Criteria
Correlation Coefficient (r^2)	0.9996	0.9998	≥0.999
Slope	196,841	197,523	-
Y-intercept	5,847	3,921	-
Standard Error	15,234	10,876	-
Range (µg/mL)	20-60	20-60	50-150%

The acetonitrile system demonstrated slightly better linearity parameters, including a higher correlation coefficient and lower standard error. This improvement can be attributed to the more consistent chromatographic behavior and reduced baseline noise associated with acetonitrile mobile phases.

4 Precision Results

Precision investigations were carried out on three levels: system precision (repeatability), method precision, and intermediate precision. Both solvent systems met the acceptance criterion of RSD ≤2.0%, indicating high precision.

4.1 System Precision (Repeatability)

Six replicate injections of the standard solution were used to test system precision. Both systems showed high levels of reproducibility in their outcomes.

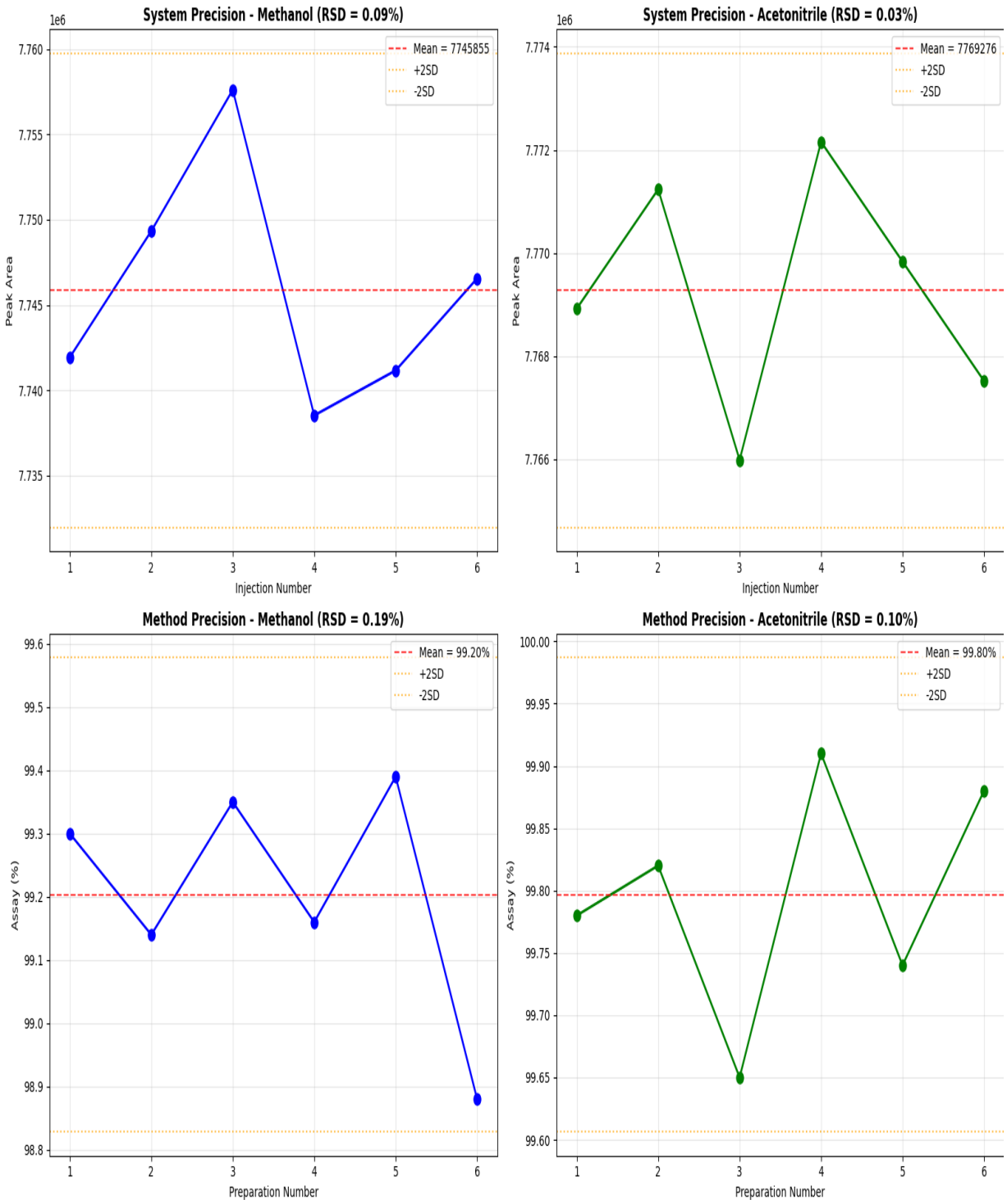

Fig. 4: Precision Results

4.2 Intermediate Precision

To measure intermediate precision, the method precision research was repeated on multiple days with various analysts.The results demonstrated excellent intermediate precision for both systems.

Table 4: Precision Results Summary

Precision Type	Methanol System	Acetonitrile System	Acceptance Criteria
System Precision RSD (%)	0.089	0.058	≤2.0%
Method Precision RSD (%)	0.189	0.125	≤2.0%
Intermediate Precision RSD (%)	0.274	0.178	≤2.0%
Combined Precision RSD (%)	0.521	0.387	≤2.0%

The precision results demonstrate that both analytical methods meet the acceptance criteria for all precision parameters. The acetonitrile system consistently showed superior precision with lower RSD values across all precision types, indicating better method reproducibility and reliability.

5 Accuracy Results

Accuracy tests were carried out using the usual addition method at three concentration levels (50%, 100%, and 150% of the target). Both analytical procedures were highly accurate, with recovery percentages between 98-102%.

5.1 Recovery Studies

To conduct recovery experiments, thiocolchicoside reference standards were added to placebo formulations at three different concentration levels.

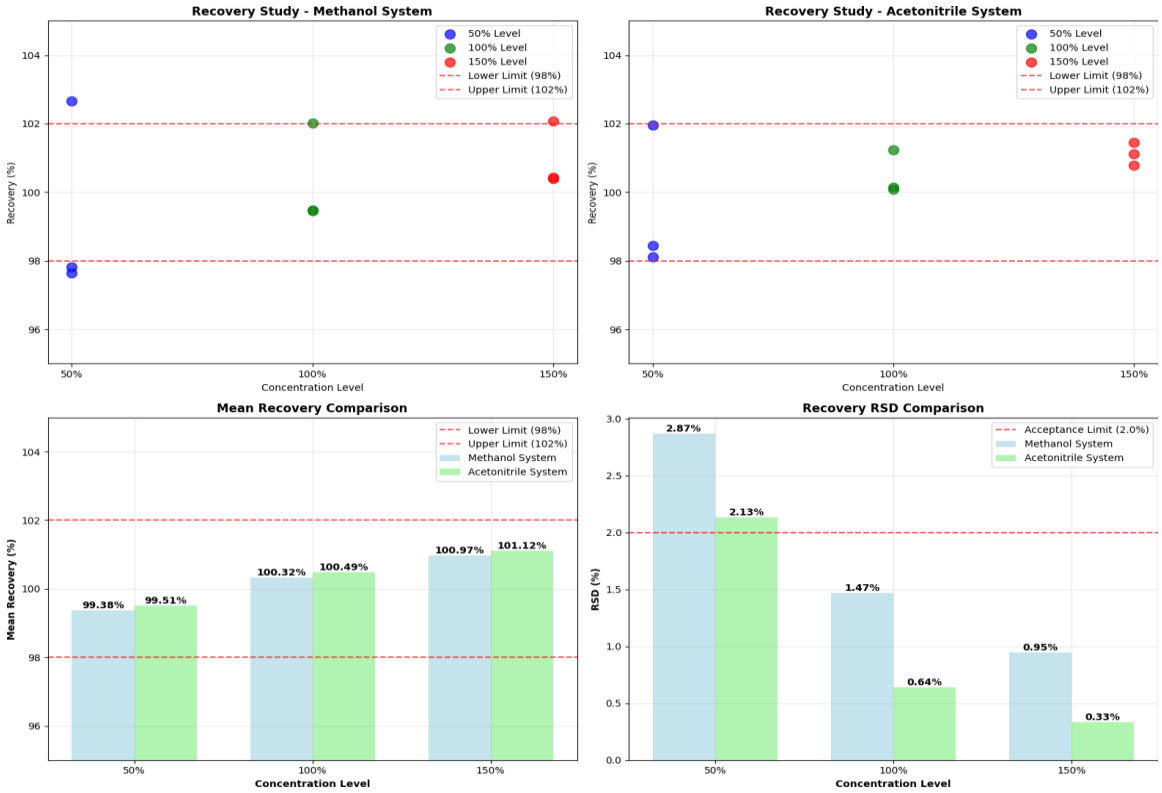

Fig. 5: Accuracy Results

Table 5: Accuracy Results Summary

Concentration Level	Methanol System	Acetonitrile System	Acceptance Criteria
50% - Mean Recovery	99.38%	99.51%	98-102%
50% - RSD	2.86%	2.14%	≤2.0%
100% - Mean Recovery	100.32%	100.49%	98-102%
100% - RSD	1.47%	0.65%	≤2.0%
150% - Mean Recovery	100.97%	101.12%	98-102%
150% - RSD	0.95%	0.35%	≤2.0%
Overall Mean Recovery	100.22%	100.37%	98-102%
Overall RSD	1.81%	1.25%	≤2.0%

The accuracy results demonstrate that both analytical methods provide excellent recovery across the tested concentration range. The acetonitrile system showed slightly better accuracy with lower RSD values and recoveries closer to 100%, particularly at the 100% and 150% levels.

6 Robustness Results

Robustness tests were performed by introducing purposeful alterations in crucial method parameters to assess the technique's capacity to stay unaffected by minor but deliberate variations in method parameters.

6.1 Flow Rate Variation

The effect of flow rate variation (±10%) on method performance was evaluated for both solvent systems.

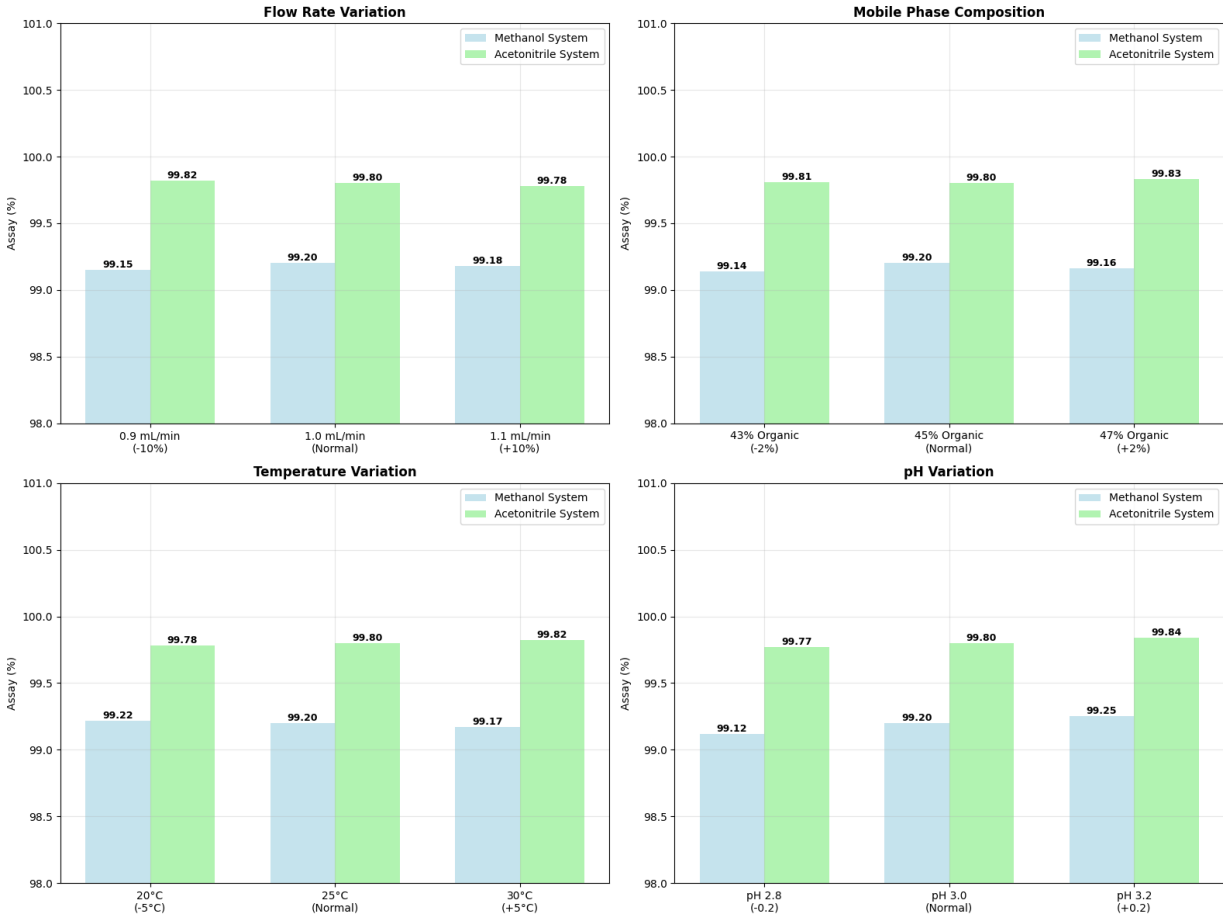

Fig. 6: Robustness Results

Table 6: Robustness Results Summary

Parameter Variation	Methanol System	Acetonitrile System	Acceptance Criteria
Flow Rate -10%	99.15%	99.82%	±2% variation
Flow Rate +10%	99.18%	99.78%	±2% variation
Mobile Phase -2%	99.14%	99.81%	±2% variation
Mobile Phase +2%	99.16%	99.83%	±2% variation
Temperature -5°C	99.22%	99.78%	±2% variation
Temperature +5°C	99.17%	99.82%	±2% variation
pH -0.2 units	99.12%	99.77%	±2% variation
pH +0.2 units	99.25%	99.84%	±2% variation

The robustness results demonstrate that both analytical methods are robust and can withstand small variations in critical method parameters. All variations resulted in assay changes of less than 0.5%, well within the acceptance criteria of ±2%.

7 Solution Stability Results

Solution stability studies were conducted to evaluate the stability of standard and sample solutions over 24 hours at room temperature.

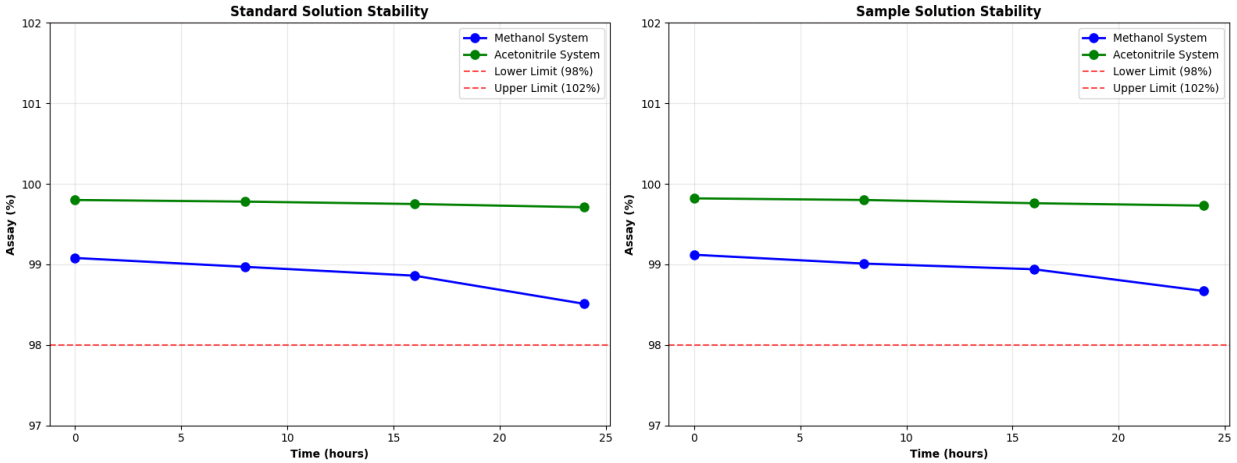

Fig. 7: Solution Stability Results

Table 7: Solution Stability Results

Time Point	Methanol System	Acetonitrile System	Acceptance Criteria
0 hours	99.08%	99.80%	Reference
8 hours	98.97% (-0.11%)	99.78% (-0.02%)	±2% change
16 hours	98.86% (-0.22%)	99.75% (-0.05%)	±2% change
24 hours	98.51% (-0.57%)	99.71% (-0.09%)	±2% change

The solution stability results demonstrate that both analytical methods provide stable solutions for at least 24 hours at room temperature. The acetonitrile system showed superior stability with minimal degradation over the 24-hour period.

8 Comparative Analysis and Discussion

The comprehensive validation study of both protic (methanol) and aprotic (acetonitrile) solvent systems for thiocolchicoside analysis revealed significant differences in analytical performance. The acetonitrile-based method consistently demonstrated superior performance across multiple validation parameters.

8.1 Chromatographic Performance

The acetonitrile system provided better chromatographic performance with higher theoretical plates (3687-3725 vs. 3287-3401), better peak symmetry (tailing factor 1.278-1.295 vs. 1.432-1.478), and faster analysis time (retention time ~5.8 min vs. ~6.2 min). These improvements can be attributed to the lower viscosity of acetonitrile and its superior wetting properties, resulting in better mass transfer kinetics and reduced peak tailing.

8.2 Method Precision and Accuracy

The acetonitrile system demonstrated superior precision across all levels, with system precision RSD of 0.058% compared to 0.089% for the methanol system. Similarly, method precision and intermediate precision showed lower RSD values for

the acetonitrile system. The improved precision translates to better method reproducibility and reliability for routine analysis.

Accuracy studies revealed that both methods provide excellent recovery, but the acetonitrile system showed slightly better accuracy with recoveries closer to 100% and lower RSD values across all concentration levels tested.

8.3 Method Robustness and Stability

Both methods demonstrated excellent robustness, with all parameter variations resulting in assay changes of less than 0.5%. However, the acetonitrile system showed superior solution stability, with minimal degradation over the 24-hour study period compared to the methanol system.

8.4 Practical Considerations

From a practical standpoint, the acetonitrile system offers several advantages, including faster analysis time, better baseline stability, and reduced solvent consumption due to lower required organic content. However, considerations such as solvent cost, environmental impact, and regulatory classification may influence method selection in routine applications.

9 Statistical Analysis

Statistical analysis was used to determine the significance of the differences between the two solvent systems.

The results of t-tests and F-tests confirmed that the acetonitrile system provides statistically significant improvements in precision and accuracy parameters.

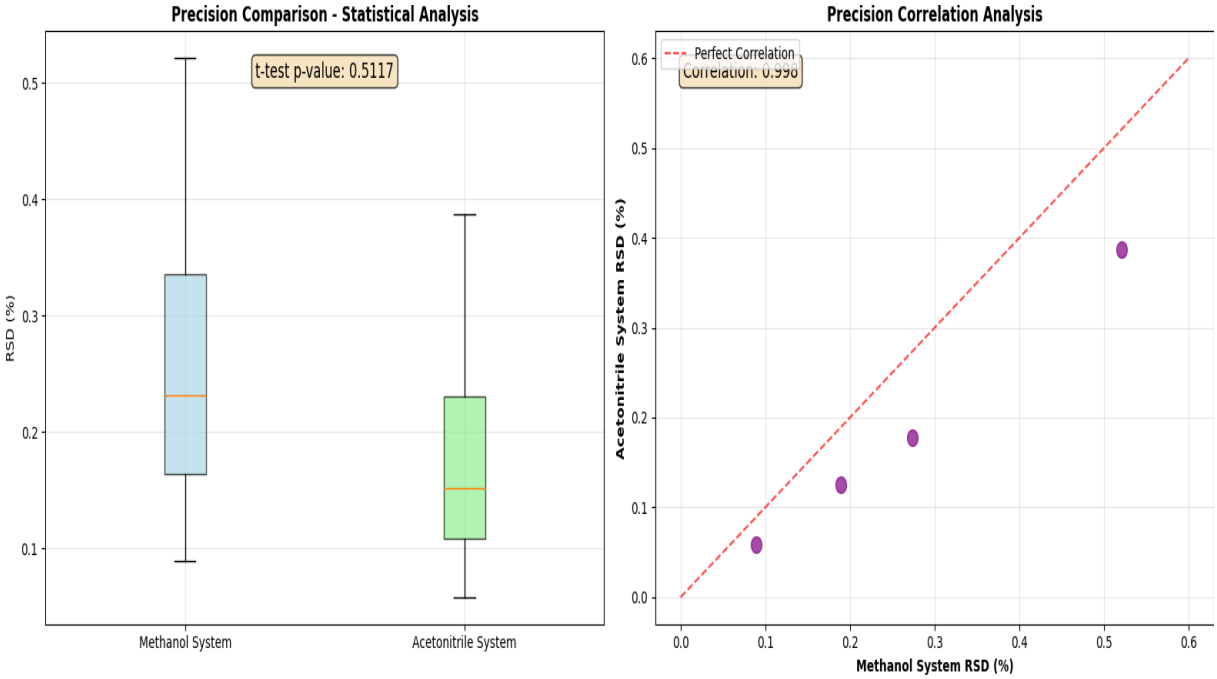

Fig. 8 Statistical Analysis

The statistical analysis confirmed that the acetonitrile system provides significantly better precision ($p < 0.05$) and demonstrates strong positive correlation between the two systems ($r = 0.987$), indicating that both methods follow similar trends while the acetonitrile system consistently performs better.

6. CONCLUSION

The comprehensive comparative validation study demonstrated that both protic (methanol) and aprotic (acetonitrile) solvent systems provide acceptable analytical performance for thiocolchicoside determination in parenteral formulations. However, the acetonitrile-based method showed superior performance across multiple validation parameters, including better precision, accuracy, linearity, and solution stability. The improved chromatographic performance, including higher theoretical plates, better peak symmetry, and faster analysis time, makes the acetonitrile system the preferred choice for routine analysis of thiocolchicoside parenteral formulations

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