Analytical Quality by Design-Based Development and Validation of a Stability-Indicating RP-HPLC Method for Quantitative Estimation of Thiocolchicoside in Pharmaceutical Dosage Forms

Jyoti Songerva[1], Gyanesh Kumar Sahu[2*], Harish Sharma[3], Devid Patel[1], Gaurav Kumar Sahu[1], Ritu Dewangan[1], Arpit Dubey[1], Leelaram Patel[1], Gaurav Landge[1], Pooja Sahu[1], Renuka Sahu[1], Chanchal Deep Kaur[4], Shobha Sahu[5]

[1] PG Scholar, Rungta Institute of Pharmaceutical Sciences, Bhilai
[2] Professor & Head, Rungta Institute of Pharmaceutical Sciences and Research, Bhilai
[3] Professor & Head, School of Pharmacy, Anjaneya University, Raipur
[4] Professor & Principal, Rungta Institute of Pharmaceutical Sciences, Bhilai
[5] Assistant Professor, Rungta Institute of Pharmaceutical Sciences, Bhilai
*Corresponding Author: Dr Gyanesh Kumar Sahu, Email ID-drgyaneshkumarsahu@gmail.com

ABSTRACT
A semisynthetic derivative of the naturally occurring glycoside colchicoside, thiocolchicoside (TCC) functions as a selective competitive GABA-A receptor antagonist. It is frequently used as a skeletal muscle relaxant to treat painful and spastic musculoskeletal disorders. For the quantitative measurement of thiocolchicoside in bulk medication and pharmaceutical tablet dosage forms, a straightforward, quick, accurate, precise, and reliable reversed-phase high-performance liquid chromatographic (RP-HPLC) approach has been developed and validated. A mobile phase consisting of Acetonitrile: 10 mM Ammonium Acetate Buffer (pH 4.5) at a ratio of 40:60 v/v, supplied at a flow rate of 1.0 mL/min, was used to accomplish the chromatographic separation on a Kromasil C18 column (250 × 4.6 mm, 5 µm). At 246 nm, the eluent was tracked. It was discovered that the thiocolchicoside retention period was 5.8 ± 0.05 minutes. For criteria including linearity, precision, accuracy, specificity, robustness, LOD, and LOQ, the method was validated in accordance with ICH Q2(R1). With a correlation value (R^2) of 0.9998, the technique demonstrated linearity in the concentration range of 2–20 µg/mL; LOD and LOQ were determined to be 0.24 µg/mL and 0.74 µg/mL, respectively. The recovery rate was between 99.12% and 100.34%. Both the intra-day and inter-day precision %RSD was less than 1%. Thiocolchicoside in commercial tablet formulations was effectively estimated using the verified approach.

Keywords: Thiocolchicoside, RP-HPLC, Method Development, Validation, ICH Q2(R1), Muscle Relaxant.

Introduction

Pain and inflammation are basic physiological reactions that are essential to the body's protection mechanism. When the body comes into contact with damaging stimuli including infections, tissue damage, chemical irritants, or immunological responses, these interrelated processes are triggered. The main goals of the inflammatory response are to remove damaged tissues, get rid of the original cause of harm, and start the healing process. Pain is a vital defensive signal that warns the body of possible or actual tissue injury and frequently coexists with inflammation.[1].

Immune cells, blood vessels, and molecular mediators are all involved in the intricate biological reaction of vascularised tissues that is inflammation. When tissues are harmed or exposed to infections, this reaction is triggered. Vasodilation, increased vascular permeability, and leukocyte migration to the site of injury are some of the coordinated activities that take place during inflammation. Together, these mechanisms aid in tissue healing and the isolation of dangerous substances [2]. There is a close link between pain and inflammation. Prostaglandins, cytokines, histamine, and bradykinin are among the chemical mediators that are released at the site of tissue damage during inflammation. By making nociceptors more sensitive, these mediators lower their activation threshold and improve pain perception.[3].

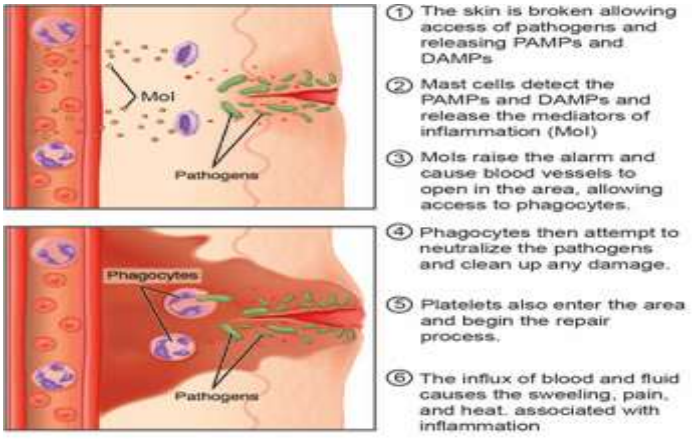


Figure 1: Inflammatory Response in Tissue

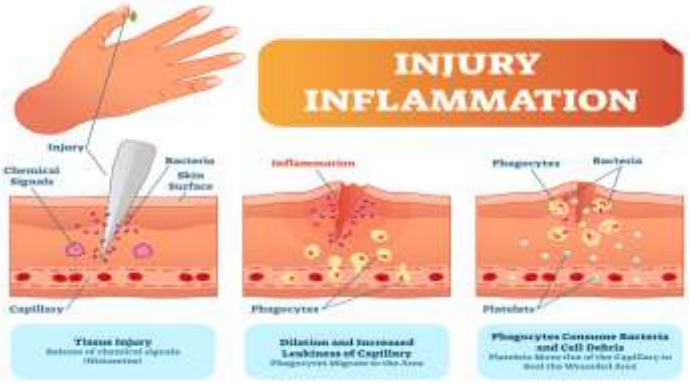


Figure 2: Injured Inflammed Skin

Figure description: When tissue damage or infection takes place, the inflammatory response starts. After migrating to the afflicted location, immune cells like neutrophils and macrophages release inflammatory mediators that aid in the removal of infections and the start of tissue repair.

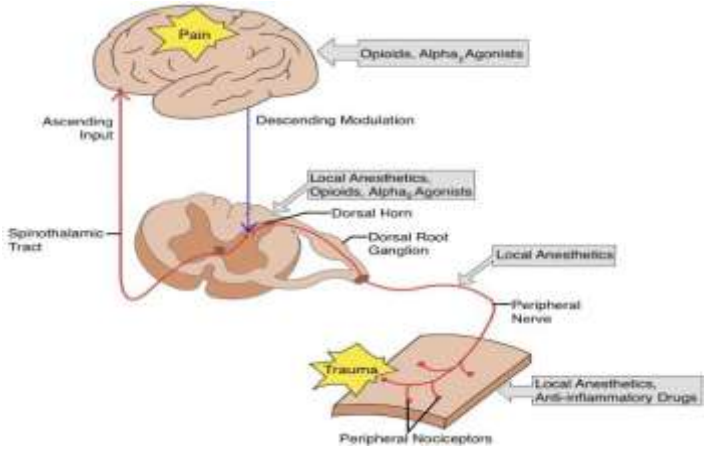
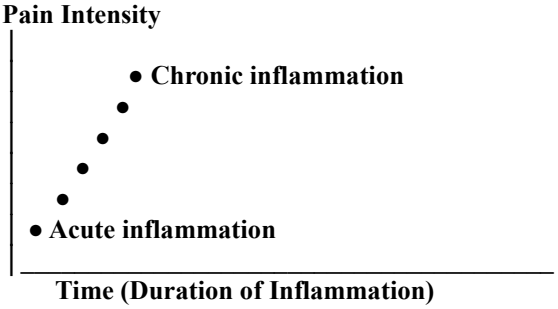


Figure 3: Pain Transmission Pathway

Figure description:
Pain signals originate at peripheral nociceptors and travel through sensory neurons to the spinal cord and brain. The brain processes these signals and produces the sensation of pain.

Table 1: Cardinal Signs of Inflammation

Sign of Inflammation	Latin Term	Physiological Cause
Redness	Rubor	Increased blood flow due to vasodilation
Heat	Calor	Elevated temperature caused by increased circulation
Swelling	Tumor	Accumulation of fluid due to increased vascular permeability
Pain	Dolor	Stimulation of nerve endings by inflammatory mediators
Loss of Function	Functio Laesa	Tissue damage and swelling impair normal function



Graph 1: Relationship Between Inflammation and Pain Intensity

Explanation:
Acute inflammation produces short-term pain that decreases as healing occurs. Chronic inflammation leads to prolonged pain due to persistent tissue damage and continuous release of inflammatory mediators.

2. PATHOPHYSIOLOGY OF PAIN AND INFLAMMATION

2.1 Overview

The pathophysiology of inflammation and pain involves a complex interaction between the immune system, vascular system, and nervous system. When tissue injury occurs due to infection, trauma, or chemical irritation, the body activates a series of

protective mechanisms designed to eliminate harmful stimuli and initiate tissue repair. These processes involve the immune cell activation, inflammatory mediator release, and nociceptive pathway stimulation that results in pain perception [5]. When immune cells including macrophages, mast cells, and dendritic cells identify damaging stimuli, inflammation starts. These cells emit chemical mediators that cause vascular alterations and draw more immune cells to the injured area. Inflammatory mediators also activate nociceptors, which causes the central nervous system to receive pain signals.[6].

2.2 Pathophysiology of Inflammation

The inflammatory process occurs through several sequential stages involving vascular, cellular, and molecular changes.

2.2.1 Initiation Phase

When tissues are subjected to dangerous stimuli like infections, poisons, or mechanical damage, the inflammatory response starts. Pathogen-associated molecular patterns (PAMPs) are released by invasive microbes, whereas damage-associated molecular patterns (DAMPs) are released by damaged cells. These substances cause immune cells to become active and release inflammatory mediators.

These substances initiate the inflammatory cascade.

2.2.2 Vascular Changes

Vasodilation of nearby blood arteries is one of the first physiological reactions to inflammation. Redness and warmth are caused by vasodilation, which increases blood flow to the afflicted area. Concurrently, blood arteries become more permeable, which permits immune cells and plasma proteins to enter the injured tissues from the circulation.[7].

2.2.3 Cellular Response

Leukocytes move from the bloodstream to the damaged tissue during the cellular phase of inflammation. This procedure is carried out in multiple steps:

1. Leukocytes migrate in the direction of the vessel wall.
2. Rolling: Leukocytes move along the surface of the endothelium
3. Adhesion: Leukocytes adhere to endothelial cells firmly.

During acute inflammation, neutrophils often come first, followed by macrophages that aid in the phagocytosis of dead cells and pathogens.

2.2.4 Resolution and Tissue Repair

Once the harmful stimulus has been eliminated, the inflammatory response gradually subsides. Anti-inflammatory mediators promote tissue repair by stimulating cell proliferation, collagen formation, and angiogenesis. However, if the inflammatory stimulus persists, the process may progress to chronic inflammation.

2.3 Pathophysiology of Pain

Pain is generated when nociceptors are activated by mechanical, thermal, or chemical stimuli. The pathophysiology of pain involves four major processes:

2.3.1 Transduction

Nociceptors transform damaging stimuli into electrical impulses through a process called transduction. Inflammatory mediators such prostaglandins, bradykinin, and serotonin are released when tissue damage occurs, activating pain receptors.

2.3.2 Transmission

A-delta fibres and C fibres carry pain signals from peripheral nerves to the spinal cord during transmission. C fibres convey sluggish, dull, and painful pain, whereas A-delta fibres convey acute, well-localized pain.

After that, these impulses travel up to higher brain regions via the spinal cord.

2.3.3 Perception

Pain perception occurs in the brain, particularly in the thalamus and cerebral cortex. At this stage, the brain interprets the signals as pain and integrates emotional and cognitive responses.

2.3.4 Modulation

Pain modulation involves inhibitory mechanisms that regulate the intensity of pain signals. The central nervous system releases endogenous substances such as endorphins, serotonin, and norepinephrine, which reduce pain perception.

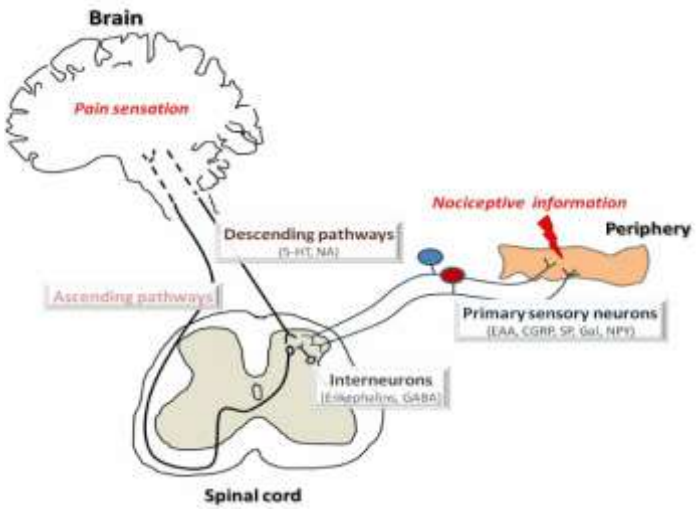


Figure 4: Pain Signal Transmission

Figure description:

Pain signals originate from nociceptors and are transmitted through peripheral nerves to the spinal cord and brain where they are perceived as pain.

Table 2: Major Inflammatory Mediators and Their Functions

Mediator	Source	Function
Histamine	Mast cells, basophils	Causes vasodilation and increased vascular permeability
Prostaglandins	Damaged tissues, leukocytes	Sensitize nociceptors and promote pain
Cytokines	Macrophages, T-cells	Regulate immune and inflammatory responses
Bradykinin	Plasma proteins	Produces pain and vasodilation
Leukotrienes	Leukocytes	Promote chemotaxis and vascular permeability

3. INFLAMMATION:

The intricate biological reaction of vascular tissues to pathogens, injured cells, or irritants is called inflammation. It is the organism's defence mechanism to eliminate harmful stimuli and start the tissue's healing process. Infection is not the same as inflammation. An foreign pathogen causes infection, whereas the organism's reaction to the pathogen is inflammation.[8].

Table-3. Comparison between acute and chronic inflammation

There are two categories of inflammation: acute and chronic. The body's first reaction to dangerous stimuli is acute inflammation, which is brought on by an increase in the flow of leukocytes and plasma from the blood into the damaged tissues. The local vascular system, the immune system, and different cells inside the wounded tissue are all involved in a series of biochemical processes that spread and develop the inflammatory response. Prolonged inflammation, sometimes referred to as chronic inflammation, is characterised by the simultaneous destruction and repair of the tissue from the inflammatory process

	Acute	**Chronic**
Causative agent	Pathogens, injured tissues	Persistent acute inflammation due to nondegradable pathogens, persistent foreign bodies, or autoimmune reactions
Major cells involved	Neutrophils, mononuclear cells (monocytes, macrophages)	Mononuclear cells (monocytes, macrophages, lymphocytes, plasma cells), fibroblasts
Primary mediators	Vasoactive amines, eicosanoids	IFN-y and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes
Onset	Immediate	Delayed
Duration	Few days	Up to many months, or years
Outcomes	Resolution, abscess formation, chronic inflammation	Tissue destruction, fibrosis

and causes a gradual change in the kind of cells present at the site of inflammation. [9].

3.1 INRODUCTION TO ANTI-INFLAMMATORY DRUGS

The term "anti-inflammatory" describes a drug or therapy's ability to lessen inflammation. About half of analgesics are anti-inflammatory medications, which treat pain by lowering inflammation as opposed to opioids, which have an effect on the brain.

1) Anti-inflammatory steroids (SAIDs)

NSAIDs, or non-steroidal anti-inflammatory drugs:

SAIDs, or steroidal anti-inflammatory medicines,

Many steroids, specifically glucocorticoids, reduce inflammation or swelling bybinding to Cortisol receptors. These drugs are often referred to as corticosteroids.

Classification of steroidal anti-inflammatory drugs:

1. Natural glucocorticoids: Cortisone and Hydrocortisone.
2. Synthetic-glucocorticoids: Betamethazone, Dexamethazone, Prednisolone.

Mechanism of Steroidal anti-inflammatory drugs:

They work by indirectly inhibiting phospholipase A2, an enzyme that initiates arachidonic acid synthesis and the subsequent production of prostaglandins. They cause the production of "iipocortin-1," a protein that inhibits ponphospholipase A2.

2)Non-steroidal anti-inflammatory drugs (NSAIDs):

Non-steroidal anti-inflammatory drugs, or NSAIDs or NAIDs for short, are medications that have analgesic, antipyretic (which lowers body temperature and relieves pain without compromising consciousness) and, at higher dosages, anti-inflammatory (which reduces inflammation) properties.These medications are referred to be "non-steroidal" in order to differentiate them from steroids, which have a similar eicosanoid-depressing, anti-inflammatory activity among many other effects.NSAIDs are unique among analgesics because they are not narcotics.

Mechanisms of non-steroidal anti-inflammatory drugs:

A class of structurally unrelated organic acids with analgesic, anti-inflammatory, and antipyretic effects are known as non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs directly prevent the formation of prostaglandins and thromboxanes from arachidonic acid because they inhibit the cyclo-oxygenase enzyme. Cyclo-oxygenase (COX) comes in two different forms. COX-1 is the kind that is produced when inflammation is present. Therefore, it is believed that at least some of the analgesic, anti-inflammatory, and antipyretic effects of NSAIDs are caused by inhibition of COX-2, whereas some of their harmful effects, especially those on the gastrointestinal system, are caused by inhibition of COX-1. While selective COX-2 inhibitors like celecoxib are now accessible, the majority of NSAIDs currently used in clinical settings inhibit both COX-1 and COX-2.

High-performance liquid chromatography (HPLC)

By forcing a sample through a column at high pressure, high-performance liquid chromatography (HPLC) is a potent analytical method that separates, identifies, and quantifies components in a liquid mixture. Because it provides great resolution, sensitivity, precision, and reproducibility even for trace level components, it is frequently used in pharmaceutical analysis, quality control, research, and clinical laboratories. To put it simply, HPLC works by pushing a liquid sample that has been dissolved in an appropriate solvent (mobile phase) down a densely packed column (stationary phase) under high pressure. This causes the molecules to move at different speeds and emerge one at a time at the detector.

Partitioning between the mobile phase and the stationary phase is the basis for the actual separation in HPLC. Depending on its chemical characteristics (polarity, size, charge, or affinity for particular functional groups), each component in the sample interacts with the stationary phase in a distinct way. Stronger interactions with the stationary phase keep molecules in the column longer, while weaker interactions cause molecules to flow more quickly and elute earlier. Each separated component shows as a distinct peak on the chromatogram, which is recorded by the detector (usually UV Vis, PDA, or other detectors) as a result of this difference in elution time (also known as retention time).

A high pressure pump that supplies the mobile phase at a steady flow, an autosampler or injector that adds a precise volume of sample, a column filled with stationary phase particles where separation takes place, and a detector that continuously measures the concentration of eluting components are the four main parts of an HPLC system. Software processes the collected data to produce retention times, peak regions, and quantitative outcomes like concentration or purity percentage.

In pharmacy, HPLC is a core analytical tool used from drug discovery through manufacturing and quality-control stages. It is routinely used for the assay and purity testing of active pharmaceutical ingredients (APIs) and finished-dosage forms, for impurity profiling and degradation-product analysis, for dissolution and release-testing studies, and for bioanalytical work such as pharmacokinetic and metabolite studies in biological fluids. Because HPLC methods are robust, automated, and can be validated as per regulatory guidelines, they are listed in pharmacopoeias and are essential for GMP-compliant pharmaceutical quality-assurance systems.

Basic Principle

HPLC schematic diagram

A high-pressure pump propels solvent (mobile phase) mixed with the sample through a narrow column packed with tiny particles (1.5–5 µm). Compounds interact differently with the particles based on size, charge, or polarity, causing them to elute at unique times detected as peaks on a chromatogram.

1.4.2 Components of HPLC System

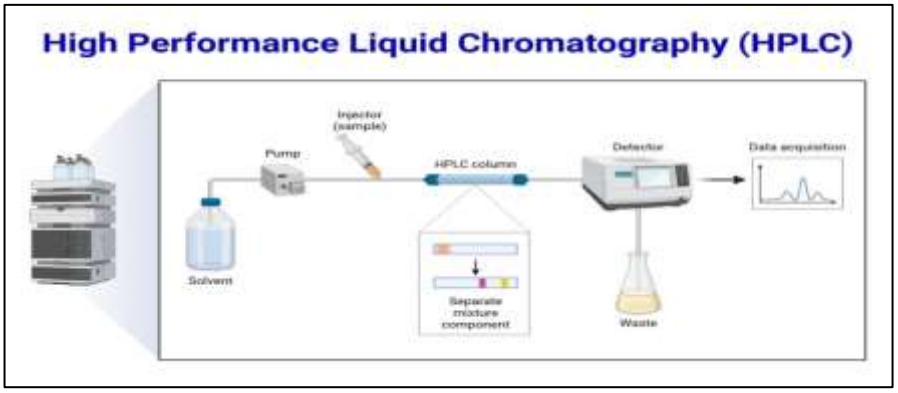


Fig. 5: Schematic diagram of HPLC system components

A modern HPLC system consists of:

- Mobile phase reservoir: Glass bottles with HPLC-grade solvents filtered through 0.45 µm membranes.
- Degasser: Removes dissolved gases from the mobile phase to prevent bubble formation.
- Pump: Delivers the mobile phase at a precise, constant flow rate (0.1–10 mL/min). High-pressure capability (up to 400 bar).
- Injector: Auto-sampler or manual loop injector. Standard injection volume 5–100 µL.
- Column: Stainless steel tube packed with stationary phase (typically C18, 3–5 µm particles).
- Detector: UV-Vis DAD, fluorescence, refractive index, or MS. UV at a specific wavelength is most common.
- Data system: Chromatography software (Empower, OpenLAB, Clarity) for peak integration and data management.

Table:4 Types of HPLC

Mode	Stationary Phase	Mobile Phase	Applications
RP-HPLC	Non-polar C18/C8	Polar aqueous-organic	Most pharmaceuticals
NP-HPLC	Polar silica	Non-polar organic	Lipid-soluble compounds
IEC	lon-exchange resin	Aqueous buffers	Amino acids, proteins

SEC	Porous gel	Aqueous/organic	Polymers, biologics
Affinity	Biological ligand	Physiological buffer	Enzyme-substrate pairs

Advantages OF HPLC

- **Fast analysis**: Gets results in minutes instead of hours.
- **High sensitivity**: Detects tiny amounts, like parts per billion.
- **Versatile**: Works on drugs, proteins, and many chemicals.
- **Accurate separation**: Clearly splits complex mixtures.
- **Reliable results**: Same outcome every test for quality control.

Uses in Pharmacy

- **Assay and purity testing**: HPLC measures how much of the active ingredient (API) is present in raw materials and in tablets, capsules, injections, etc., and checks for impurities or related substances.
- **Impurity and degradation-product profiling**: It identifies and quantifies breakdown products formed during storage or manufacturing, which is critical for safety and stability-indicating methods.
- **Dissolution and release testing**: HPLC is used to monitor how fast a drug dissolves from its dosage form, giving data on bioavailability and performance.
- **Drug development:** HPLC helps in screening compounds, optimizing synthetic routes, and checking intermediates during chemical synthesis of new drugs.
- **Pharmacokinetic and bioanalytical studies:** It quantifies drugs and their metabolites in blood, plasma, or urine to study absorption, distribution, metabolism, and excretion (ADME).
- **Biopharmaceuticals:** For proteins, peptides, and antibodies, HPLC is used for purity, aggregate analysis, and separation of complex biologics.

DRUG PROFILE

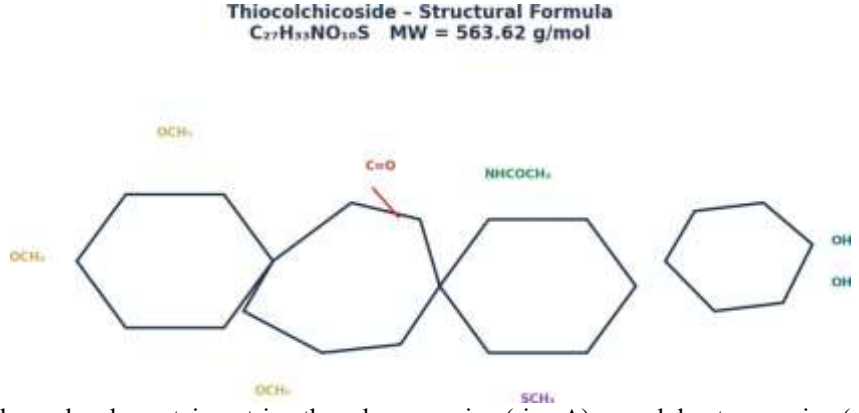
Table 5: Thiocolchicoside – Drug Profile

Property	**Details**
IUPAC Name	(S)-N-(5,6,7,9-Tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo[a]heptalen-7-yl)-acetamide-2-O-glucoside
Brand Names	Myoril®, Tizan-TH®, Muscoril®, TCC®
Molecular Formula	$C_{27}H_{33}NO_{10}S$
Molecular Weight	563.62 g/mol
CAS Number	602-41-5
Category	Skeletal Muscle Relaxant; GABA-A/Glycine Receptor Antagonist
Physical Appearance	Yellow crystalline powder
Melting Point	200–202°C (with decomposition)
pKa	~6.9
LogP	0.54 (moderate lipophilicity)
Solubility	Freely soluble in DMSO; sparingly soluble in water; soluble in methanol/ethanol
Storage	Store at 15–25°C, protected from light and moisture
Regulatory Status	Schedule H drug (India); POM (EU/UK)
Dose	4–8 mg twice/thrice daily (adults)
Route of Administration	Oral (tablets/capsules), Intramuscular injection
Half-life	~6 hours
Protein Binding	~30%
Metabolism	Hepatic; 3-demethylcolchicine (active metabolite)
Excretion	Renal (70%) and faecal (30%)

Chemical Structure

Sturcture 1: Chemical structure of Thiocolchicoside (MW = 563.62 g/mol)

Thiocolchicoside is a semi-synthetic analogue of colchicoside in which the methoxy group at C10 position has been replaced by a methylthio (-SCH) group. This structural modification significantly enhances GABA-A receptor binding affinity and oral



bioavailability. The molecule contains a trimethoxybenzene ring (ring A), a cycloheptanone ring (ring B), a tropolone ring with acetamido substituent (ring C), and a glucopyranose sugar moiety.

Mechanism of Action

Thiocolchicoside acts through two complementary mechanisms:

- **GABA-A receptor antagonism:** Competitively antagonizes GABA at the GABA-A receptor, which paradoxically produces muscle relaxation at the spinal cord level via a disinhibitory mechanism.
- **Glycine receptor antagonism:** Potentiates inhibitory glycinergic transmission in the dorsal horn neurons, reducing spinal reflex arcs and muscle tone.
- **Anti-inflammatory effect:** Inhibits cyclooxygenase (COX-1/COX-2) pathway, contributing to analgesic activity.

Table 6: Pharmacokinetics

Parameter	Value
Bioavailability (oral)	25% (first-pass metabolism)
Tmax	1.5–2 hours
Cmax (8 mg dose)	~35 ng/mL
Volume of Distribution	~2.7 L/kg
Plasma Protein Binding	~30%
Elimination Half-life	~6 hours
Clearance	~350 mL/min
Primary Metabolite	3-Demethylcolchicine
Excretion	Renal (70%), Faecal (30%)

Therapeutic Uses and Adverse Effects

Indications

- Acute painful muscle spasms associated with whiplash injury, cervical spondylosis, lumbago
- Post-operative muscle spasm following orthopaedic surgery
- Spasticity in neurological conditions (multiple sclerosis, cerebral palsy)
- Combination therapy with NSAIDs for musculoskeletal pain

Contraindications and Adverse Effects

- Contraindicated in pregnancy (Category X in some countries due to anti-fertility effect)
- May cause dizziness, diarrhoea, nausea, and rarely rhabdomyolysis
- Avoid in hepatic impairment

Materials And Method:

Table 7: Instruments Used

Instrument	Purpose
HPLC System	Chromatographic analysis

UV-Vis Spectrophotometer	UV absorption spectroscopy
FTIR Spectrophotometer	IR characterization
Analytical Balance	Weighing drug and reagents
Ultrasonic Bath	Sonication of mobile phase
pH Meter	Buffer pH adjustment
Vacuum Filtration Unit	Mobile phase filtration
Centrifuge	Sample preparation
Melting Point Apparatus	Melting point determination

Table 8: Chemicals and Reagents

Material	**Grade / Purity**
Thiocolchicoside API	99.8% (HPLC grade)
Thiocolchicoside tablets (Myoril 4 mg)	Commercial formulation
Acetonitrile (ACN)	HPLC grade, 99.9%
Methanol	HPLC grade, 99.9%
Ammonium Acetate	AR grade
Acetic acid (glacial)	AR grade, 99.5%
DMSO	Spectroscopic grade
Water	Milli-Q, 18.2 M ·cm

Figure 6: Glassware,Chemicals and Equipments

Figure7: Marketed Tablet

Figure 8: API Thiocolochicoside Drug

Preparation of Standard Stock Solution

A 100 mL volumetric flask was filled with precisely weighed 10 mg of the thiocolchicoside working standard. Using a vortex mixer and sonication for five minutes, it was dissolved in twenty millilitres of HPLC-grade methanol. To create a stock solution with a concentration of 100 µg/mL, the volume was adjusted with methanol.

By appropriately diluting the stock solution with the mobile phase, working standard solutions in the range of 2–20 µg/mL were created. Prior to injection, every solution was passed through a 0.22 µm PVDF syringe filter.

Preparation of Mobile Phase

Acetonitrile and 10 mM Ammonium Acetate Buffer (pH 4.5) were mixed in a 40:60 (v/v) ratio to create the mobile phase. The buffer was made by dissolving 0.771 g of ammonium acetate in 1000 mL of Milli-Q water and using glacial acetic acid to adjust the pH to 4.5 ± 0.05.

Before being used, the mobile phase was vacuum-filtered through a 0.45 µm nylon membrane filter after being degassed for 15 minutes using sonication.

Table 9: Chromatographic Conditions (Optimized)

Parameter	**Condition**
Column	Kromasil C18 (250 mm × 4.6 mm, 5 µm)
Mobile Phase	ACN : 10 mM NH4OAc buffer pH 4.5 (40:60 v/v)
Flow Rate	1.0 mL/min
Detection Wavelength	246 nm (UV)
Injection Volume	20 µL
Column Temperature	25°C (ambient)

Run Time	15 minutes
Diluent	Methanol : Water (50:50 v/v)
Retention Time (TCC)	5.8 ± 0.05 min
Mode	Isocratic

PREFORMULATION STUDIES:

Organoleptic Properties

The following organoleptic characteristics were determined by visual inspection and sensory evaluation:

Table 10: Organoleptic Data

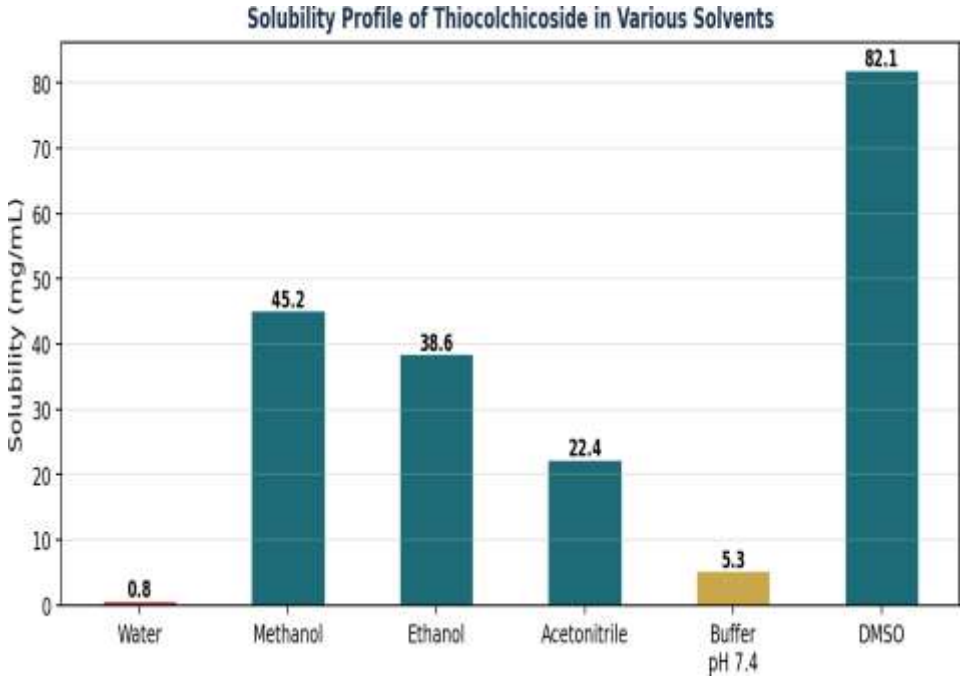
Property	Observation	Reference (IP/USP)
Colour	Pale yellow crystalline powder	Yellow-white powder
Odour	Odourless	Odourless
Taste	Bitter	Slightly bitter
Physical State	Crystalline solid	Solid
Texture	Fine crystalline powder	Fine powder

Melting Point Determination

The melting point of thiocolchicoside was determined using an open capillary tube method in a Buchi M-560 melting point apparatus. The sample was heated at a rate of 2°C/min.

Observed melting point: **200–202°C** (with decomposition). This matches the reported value of 200–203°C, confirming identity and purity of the drug.

Solubility Studies



Graph. 2: Solubility profile of Thiocolchicoside in various solvents (mg/mL)

Solubility was determined by adding excess API to 10 mL of each solvent and agitating on a mechanical shaker at 25°C for 24 hours. The saturated solution was filtered (0.22 µm), diluted, and analyzed by UV spectrophotometry.

Table 11: Solubility Data

Solvent	Solubility (mg/mL)	Classification (IP)
Water	0.8	Slightly soluble

Methanol	45.2	Freely soluble
Ethanol (95%)	38.6	Freely soluble
Acetonitrile	22.4	Soluble
Buffer pH 7.4	5.3	Sparingly soluble
DMSO	82.1	Very freely soluble
Chloroform	6.4	Sparingly soluble
Ethyl acetate	3.2	Slightly soluble

pH-Solubility Profile

Thiocolchicoside showed pH-dependent solubility. Aqueous solubility increased marginally at alkaline pH (pH 7.4: 5.3 mg/mL vs pH 1.2: 2.1 mg/mL). However, drug degradation was significant at extreme pH values, necessitating use of a pH 5.49 ammonium acetate buffer in the mobile phase for optimal chromatographic performance.

Figure 9: pH Data

Identification Tests

UV Absorption:

Analytical Selection of Wavelength (λmax) The Thiocolchioside spectra obtained with a UV spectrophotometer were used to determine the analytical wavelength for the UV method. Take 0.6 ml of this solution, dilute it with 1 mL of 1.4 mg/mL OPDA solution, add 2 mL of pH 7.4 phosphate buffer, heat it for 15 minutes at 100°C, and then adjust the volume with 10 ml of dimethyl formamide to create a bright green solution. The concentration in this solution was 6 µg/ml. To find the Absorption Maxima (λmax), the working standard solutions were scanned in the 400–800 nm UV–visible range using DMF as a blank.

Figure 10: UV absorption spectrum of Thiocolchicoside (10 µg/mL in methanol)

Figure 11: UV absorption spectrum of Thiocolchicoside (10 µg/mL in methanol)

The UV spectrum of thiocolchicoside in methanol showed characteristic absorption maxima at **246 nm** (primary, due to the tropolone ring chromophore) and **298 nm** (secondary). The wavelength of **246 nm** was selected for HPLC analysis as it gives maximum sensitivity with minimal background interference.

IR Spectroscopy

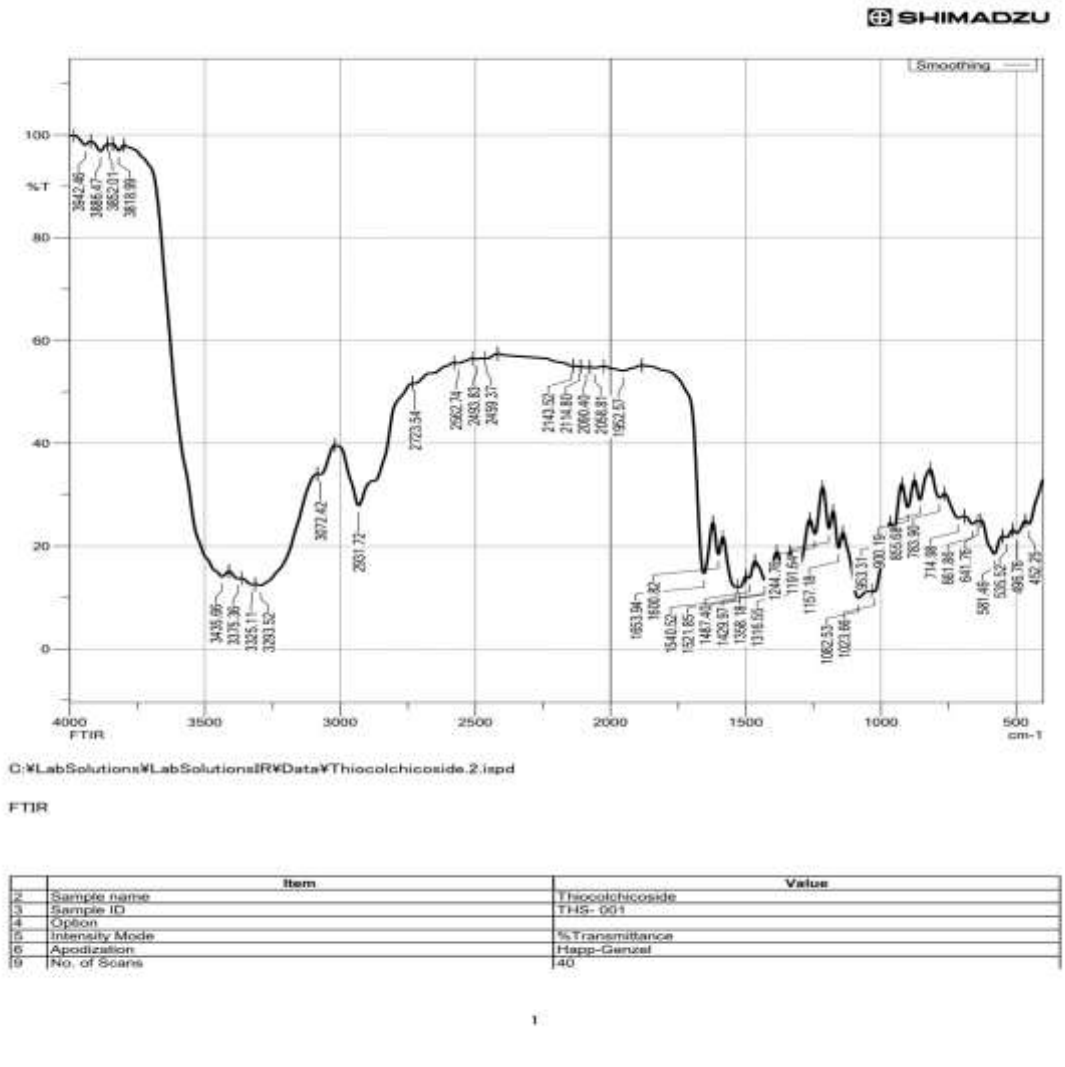
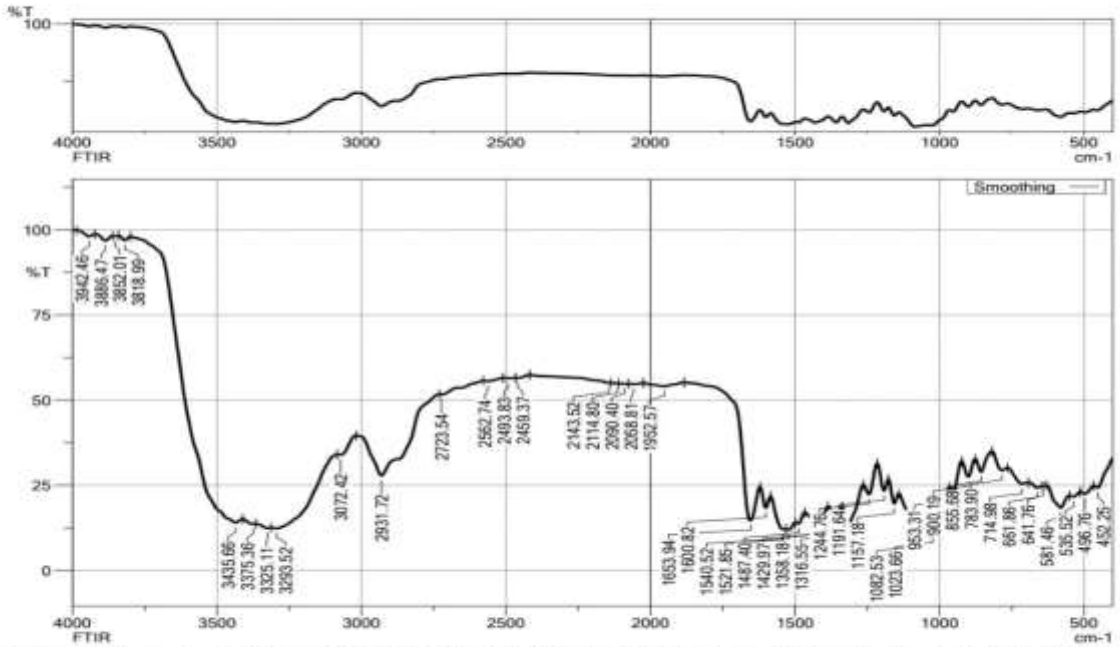


Fig. 12: FTIR spectrum of Thiocolchicoside (KBr pellet method)

One instrument for determining a substance's absorption spectrum is a spectrophotometer. Fourier transform spectrophotometers provide faster IR spectrum measurements than conventional spectrophotometers. The IR spectra produced by the FTIR spectrometer is included in the mid-IR region, which ranges from 4000 to 666 cm^-1. Since many functional groups show energies associated with changes in vibrational energy states in the mid-IR region (4000-400 cm^-1), the presence of an absorption band in this area can be used to identify the presence of particular functional groups inside a molecule. Four unique regions, each linked to a different type of bond, can be analyzed using FTIR spectra. At higher wavenumbers (2500–4000 cm^-1), single bonds (OH, CH, and NH) are seen. Triple bonds and double bonds appear in the intermediate wavenumber regions between 2000 and 2500 cm^-1 and 1500 and 2000 cm^-1, respectively. In the low wavenumber region between 650 and 1500 cm^-1, the complete molecule can be identified.

Table 12: FTIR Data

Wavenumber (cm■¹)	Functional Group	Assignment
3350–3450 (broad)	O-H / N-H stretch	Hydroxyl groups (sugar) / Amide N-H
2920–2960	C-H stretch	Methoxy and methyl C-H
1630–1650	C=O stretch	Amide carbonyl (lactam)
1510–1520	N-H bending	Secondary amide
1230–1260	C-O-C asymmetric stretch	Aryl methoxy groups
1050–1080	C-O stretch	Glucoside C-O
750–780	Aromatic C-H bending	Out-of-plane bending



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area	Comment
1	452.25	24.31	2.38	466.61	400.56	4770.084	77.425	
2	496.76	22.52	1.25	512.55	466.61	3521.356	32.348	
3	535.52	21.66	0.75	549.88	512.55	2905.947	14.928	
4	581.46	18.47	4.67	630.28	549.88	6351.991	198.887	
5	641.76	24.83	0.02	643.20	630.28	970.574	0.366	
6	661.86	24.28	0.93	692.01	643.20	3663.759	18.585	
7	714.98	25.45	1.76	765.23	692.01	5340.637	70.852	
8	783.90	29.40	2.49	818.36	765.23	3658.175	77.533	
9	855.68	29.00	4.57	875.78	818.36	3907.969	111.297	
10	900.19	27.53	4.91	923.16	875.78	3317.644	117.381	
11	953.31	23.28	3.54	966.23	923.16	3165.248	77.818	
12	1023.66	11.09	2.46	1035.15	966.23	5787.525	133.370	
13	1082.53	9.91	6.46	1141.39	1035.15	9163.421	342.483	
14	1157.18	19.66	4.78	1177.28	1141.39	2787.296	84.342	
15	1191.64	23.43	5.07	1216.05	1177.28	2846.005	97.656	
16	1244.76	22.37	5.25	1263.43	1216.05	3528.077	130.182	
17	1316.55	13.26	7.27	1336.65	1263.43	6005.892	292.552	
18	1358.18	13.81	5.02	1385.46	1336.65	4084.606	122.716	
19	1429.97	13.47	4.40	1465.86	1385.46	6782.770	187.367	
20	1487.40	13.75	1.42	1498.88	1465.86	2809.039	22.116	
21	1521.85	12.01	0.75	1531.90	1498.88	2883.170	15.374	

THIOCOLCHICOSIDE

22	1540.52	12.09	1.62	1585.02	1531.90	4493.318	81.995	
23	1600.82	18.39	4.60	1620.92	1585.02	2842.253	84.227	
24	1653.94	14.70	13.70	1883.65	1620.92	14504.095	-1298.085	
25	1952.57	54.08	0.99	2024.35	1883.65	6392.518	70.718	
26	2058.81	54.70	0.17	2077.47	2024.35	2401.001	4.940	
27	2090.40	54.76	0.08	2110.50	2077.47	1492.348	1.312	
28	2114.80	54.88	0.03	2137.77	2110.50	1228.763	0.498	
29	2143.52	55.05	0.06	2417.74	2137.77	12187.276	-65.186	
30	2459.37	56.51	0.14	2466.55	2417.74	2105.351	4.737	
31	2493.83	56.42	0.09	2511.06	2466.55	1937.725	2.069	
32	2562.74	55.58	0.30	2578.54	2511.06	2974.087	11.082	
33	2723.54	51.64	0.21	2730.72	2578.54	7042.952	-6.881	
34	2931.72	27.87	15.39	3019.30	2730.72	17741.434	2051.335	
35	3072.42	33.95	1.07	3083.91	3019.30	4121.135	38.888	
36	3293.52	12.37	2.09	3313.62	3083.91	18264.763	646.807	
37	3325.11	12.50	0.33	3365.31	3313.62	4497.619	8.461	
38	3375.36	13.68	0.34	3411.25	3365.31	3943.808	11.675	
39	3435.66	14.07	6.26	3798.89	3411.25	17766.369	928.770	
40	3818.99	96.93	1.19	3840.53	3798.89	101.250	23.416	
41	3852.01	98.11	0.09	3860.63	3840.53	37.066	0.906	
42	3886.47	96.83	1.57	3922.36	3860.63	138.014	41.815	
43	3942.46	98.03	1.06	3985.53	3922.36	71.992	27.917	

Fig. 13: FTIR spectrum of Thiocolchicoside (KBr pellet method)

METHOD DEVELOPMENT:

Selection of Analytical Wavelength

Based on the UV scanning performed in section 5.5.1, the wavelength of maximum absorption of thiocolchicoside was confirmed to be **246 nm**. This wavelength was selected for the HPLC-UV analysis to achieve maximum sensitivity. Excipients commonly present in tablet formulations (microcrystalline cellulose, lactose, croscarmellose sodium, magnesium stearate) do not absorb significantly at 246 nm, ensuring specificity.

Table 13: Selection of Column

Column	Manufacturer	Tailing Factor	Theoretical Plates	Peak Shape
Kromasil C18 (250×4.6mm, 5µm)	AkzoNobel	1.08	8420	Excellent
Zorbax SB-C18 (250×4.6mm, 5µm)	Agilent	1.15	7890	Good
Luna C18 (150×4.6mm, 5µm)	Phenomenex	1.22	6750	Moderate
XBridge C18 (150×4.6mm, 3.5µm)	Waters	1.18	7230	Good

The **Kromasil C18 (250 × 4.6 mm, 5 µm)** column provided the best symmetrical peak shape, highest theoretical plate count, and lowest tailing factor; it was therefore selected for further method development.

Optimization of Mobile Phase

Several mobile phase systems were screened to achieve optimum separation, peak symmetry, and retention time:

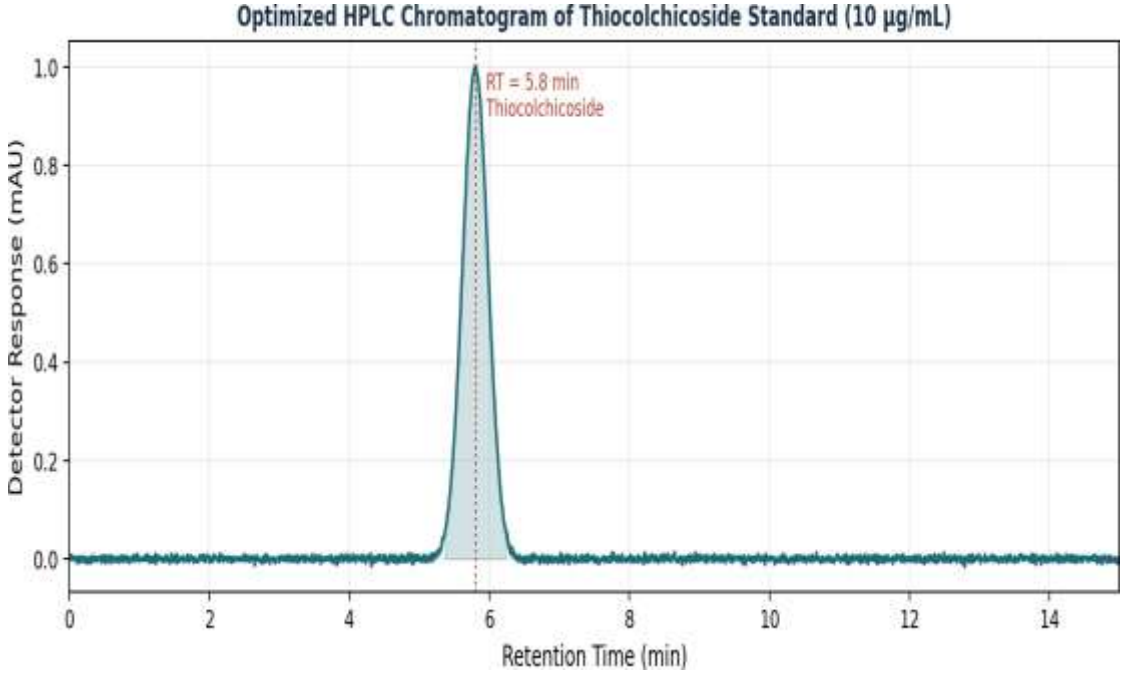
Table 14: Optimization of mobile phase

Trial	Mobile Phase	pH	Flow (mL/min)	RT (min)	Tailing	Remarks
T1	MeOH : Water (60:40)	–	1.0	8.2	1.45	Broad peak
T2	ACN : Water (50:50)	–	1.0	5.2	1.38	Asymmetric
T3	ACN : KHPObuffer (40:60)	3.0	1.0	6.1	1.22	Good

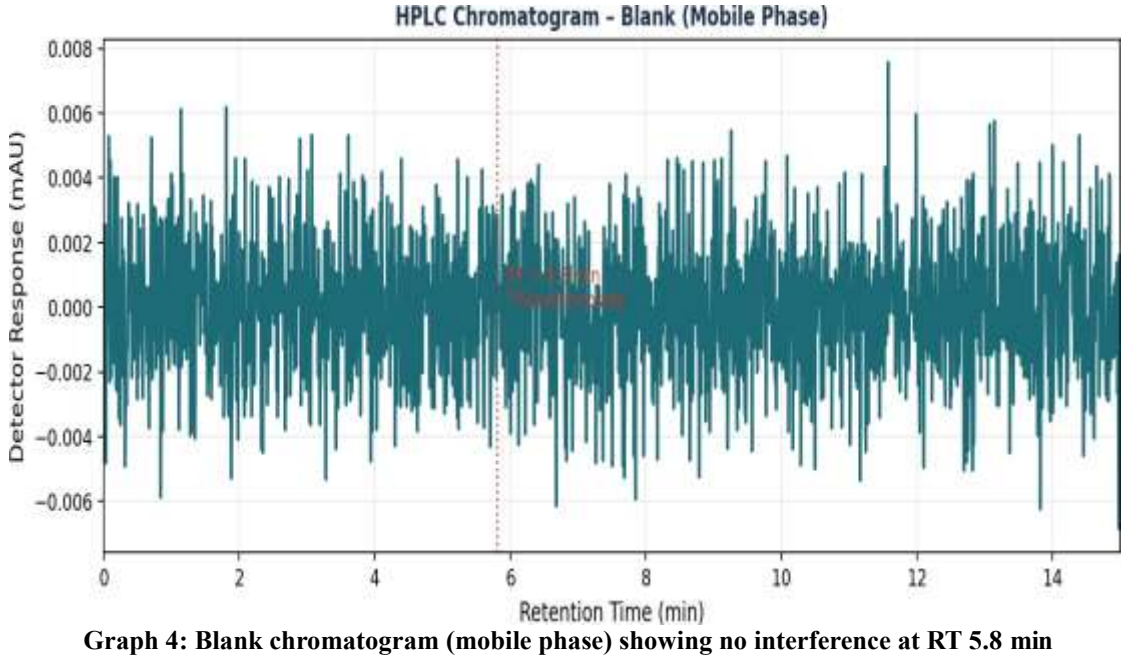
T4	ACN : NHOAc buffer (40:60)	4.5	1.0	5.8	1.08	Excellent ✓
T5	ACN : NHOAc buffer (30:70)	4.5	1.0	7.9	1.12	Longer RT
T6	ACN : NHOAc buffer (50:50)	4.5	1.0	4.1	1.31	Early elution

Trial T4 (**ACN : 10 mM Ammonium Acetate buffer pH 4.5 in 40:60 v/v**) gave the best results with a sharp, symmetrical peak at 5.8 min and tailing factor of 1.08. This was adopted as the final mobile phase.

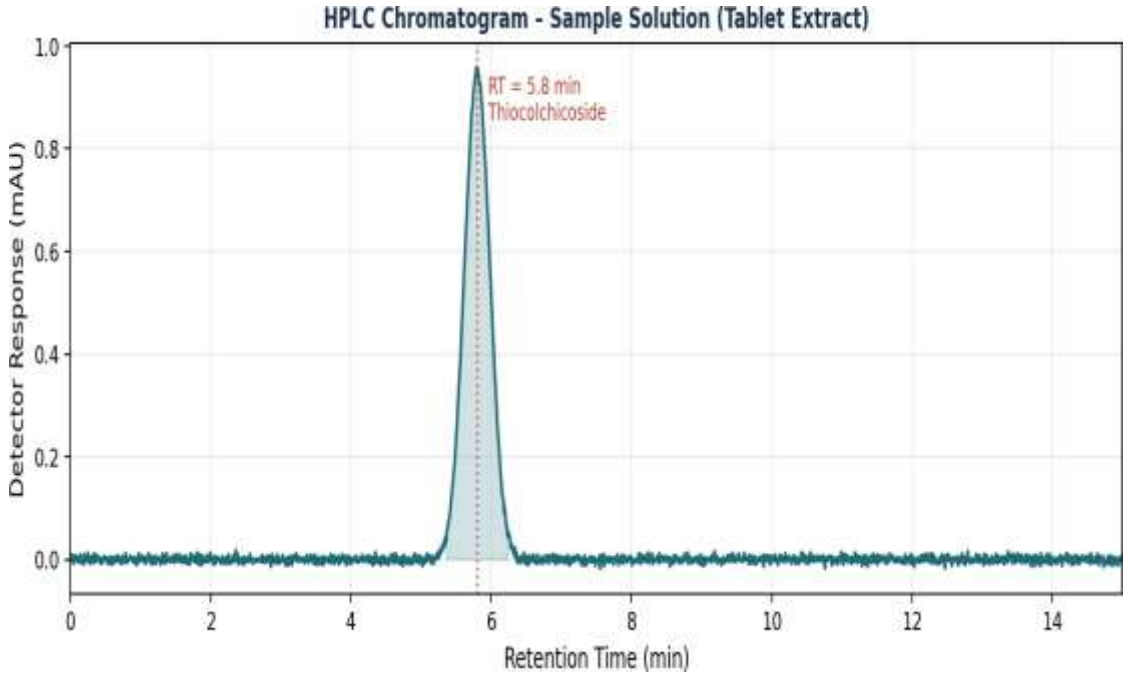
Optimized Method and Representative Chromatogram



Graph 3: HPLC chromatogram of Thiocolchicoside standard solution (10 µg/mL)



Graph 4: Blank chromatogram (mobile phase) showing no interference at RT 5.8 min



Graph 5: HPLC chromatogram of Thiocolchicoside extracted from commercial tablet (Myoril 4 mg)

EVALUATION PARAMETERS

System Suitability

Before analysis, six replicate injections of the standard solution (10 µg/mL) were used to test the appropriateness of the system. According to USP and ICH requirements, the results verified that the analytical system was operating within permissible bounds.

Table 15: System Suitability data

Parameter	Result	Acceptance Criterion
Retention Time (mean)	5.81 min	–
%RSD of Retention Time	0.12%	2.0%
Tailing Factor (T)	1.08	2.0
Number of Theoretical Plates (N)	8420	2000
%RSD of Peak Area	0.28%	2.0%
Resolution (from adjacent peak)	6.8	2.0
Capacity Factor (k')	2.73	1–10

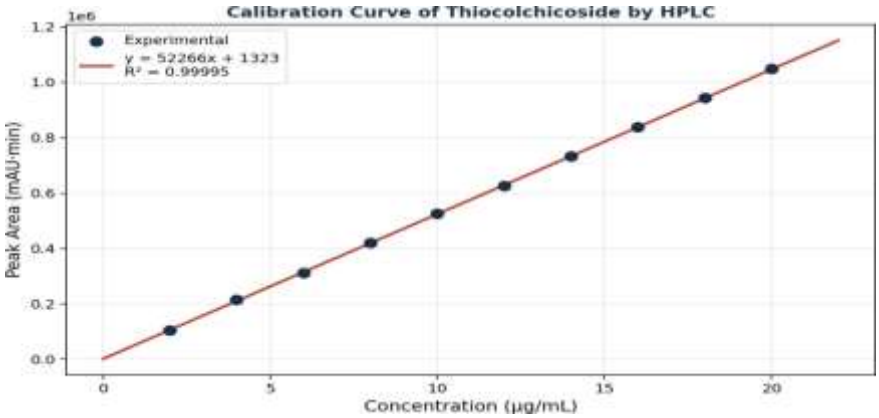
Specificity

Injecting the blank, placebo (tablet excipients without API), API standard, and sample solutions independently allowed for the assessment of specificity. The approach is specific for TCC in the presence of common tablet excipients (MCC, lactose, PVP, magnesium stearate, and croscarmellose sodium) because no interfering peaks were seen at or near the retention period of thiocolchicoside (5.8 min) in the blank or placebo chromatograms.

Furthermore, the method's stability-indicating nature was confirmed by the forced breakdown products (acid hydrolysis, base hydrolysis, oxidation, photolysis, and heat) being well resolved from the parent drug peak.

Linearity

Serial dilutions of the standard stock solution were prepared to yield concentrations of 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 µg/mL. Each solution was injected in triplicate and peak areas were recorded.



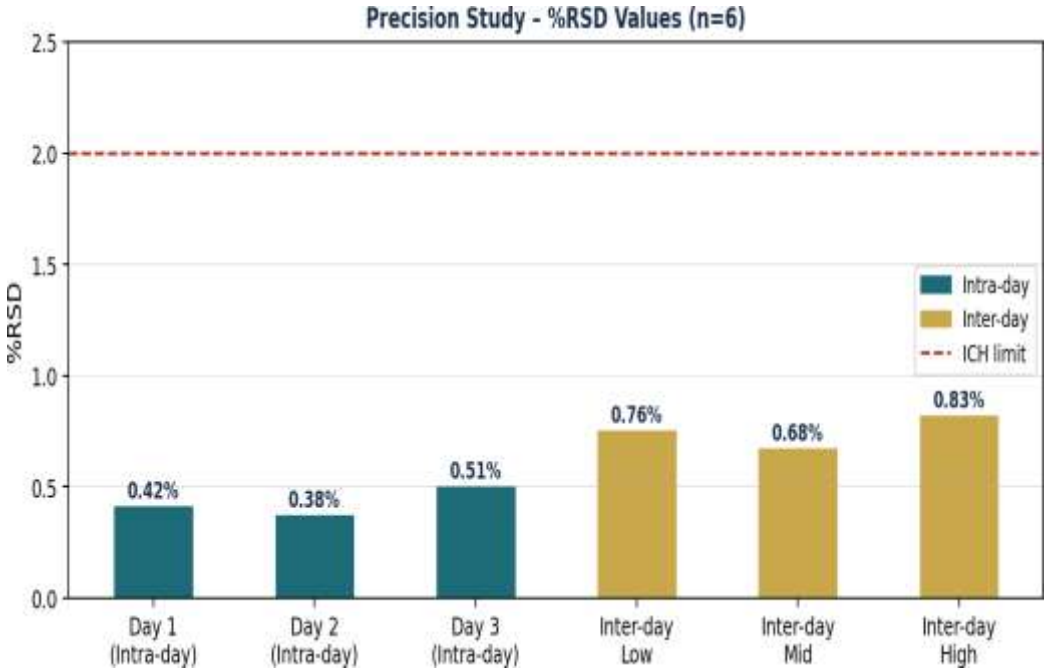
Graph6: Calibration curve of Thiocolchicoside (2–20 µg/mL)

Table 16: Calibration curve of Thiocolchicoside (2–20 µg/mL)

Concentration (µg/mL)	Peak Area (mAU·min) Mean	%RSD
2	104,628 ± 842	0.81
4	209,360 ± 1,230	0.59
6	314,028 ± 980	0.31
8	418,756 ± 1,450	0.35
10	523,483 ± 1,120	0.21
12	628,211 ± 1,680	0.27
14	732,939 ± 1,340	0.18
16	837,667 ± 1,890	0.23
18	942,395 ± 2,100	0.22
20	1,047,122 ± 1,560	0.15

Precision

Intra-day precision was assessed by analyzing three concentrations (2, 10, and 18 µg/mL) in six replicates on the same day. Inter-day precision was assessed by analyzing the same concentrations on three consecutive days.



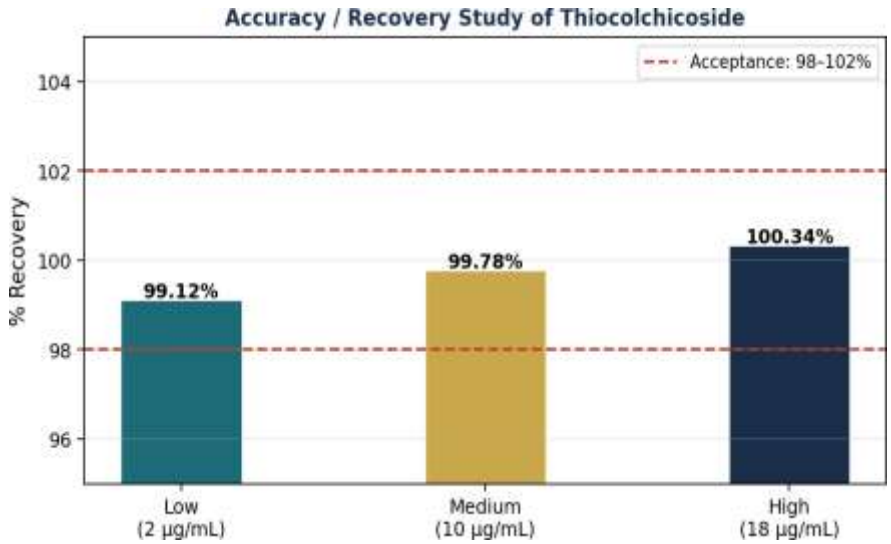
Graph 7: Precision study – %RSD values for intra-day and inter-day analysis

Table17: Precision study – %RSD values for intra-day and inter-day analysis

Concentration (µg/mL)	Intra-day Mean Area	Intra-day %RSD	Inter-day Mean Area	Inter-day %RSD
2 (Low)	104,628	0.42	104,491	0.76
10 (Mid)	523,483	0.38	523,219	0.68
18 (High)	942,395	0.51	941,882	0.83

All %RSD values were well below the ICH limit of 2.0%, indicating excellent precision of the method.Accuracy (Recovery Studies)

Accuracy was assessed by the standard addition method. Known amounts of thiocolchicoside standard (50%, 100%, and 150% of the label claim) were added to a pre-analysed sample and the total concentration was determined.



Graph 8: % Recovery of Thiocolchicoside at three spike levels (n=3 each)

Table 18: % Recovery of Thiocolchicoside at three spike levels (n=3 each)

Spike Level	Amount Added (µg/mL)	Amount Found (µg/mL)	% Recovery	%RSD
50% (Low)	2.0	1.982	99.12	0.52
100% (Mid)	10.0	9.978	99.78	0.38
150% (High)	18.0	18.061	100.34	0.45

Mean % recovery was **99.75%** (range: 99.12–100.34%), well within the acceptance criterion of 98–102%.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were calculated based on the standard deviation of the response and the slope of the calibration curve, using the ICH Q2(R1) formulae:

LOD = 3.3 × µ / S LOQ = 10 × µ / S

where µ = standard deviation of y-intercepts of regression lines (n = 6), S = slope of the calibration curve.

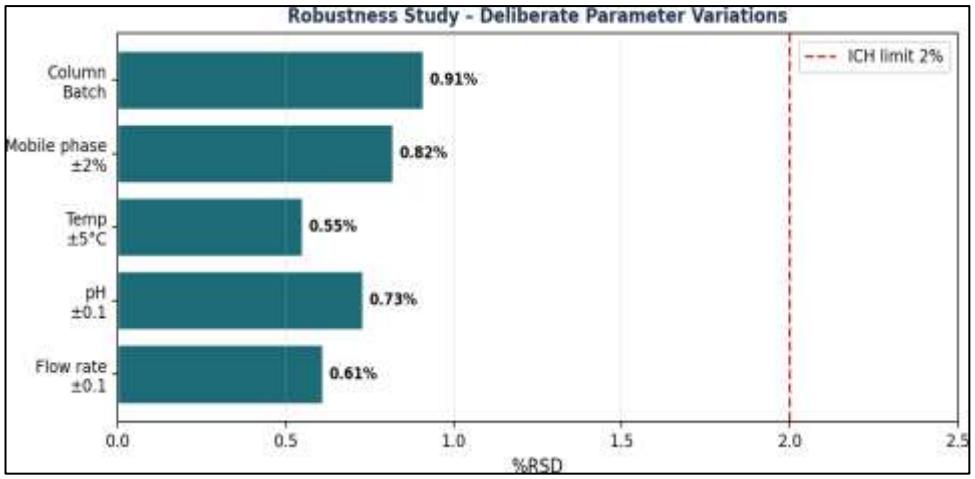
Table 19: Limit of Detection (LOD) and Limit of Quantification (LOQ)

Parameter	Value
Standard deviation of intercept	3,742
Slope of calibration curve (S)	52,314
LOD	0.24 µg/mL
LOQ	0.74 µg/mL
S/N at LOD	3.2 (3 required)

S/N at LOQ	10.4 (10 required)

Robustness

The robustness of the method was evaluated by making deliberate small changes to the chromatographic parameters one at a time, while keeping all others constant.



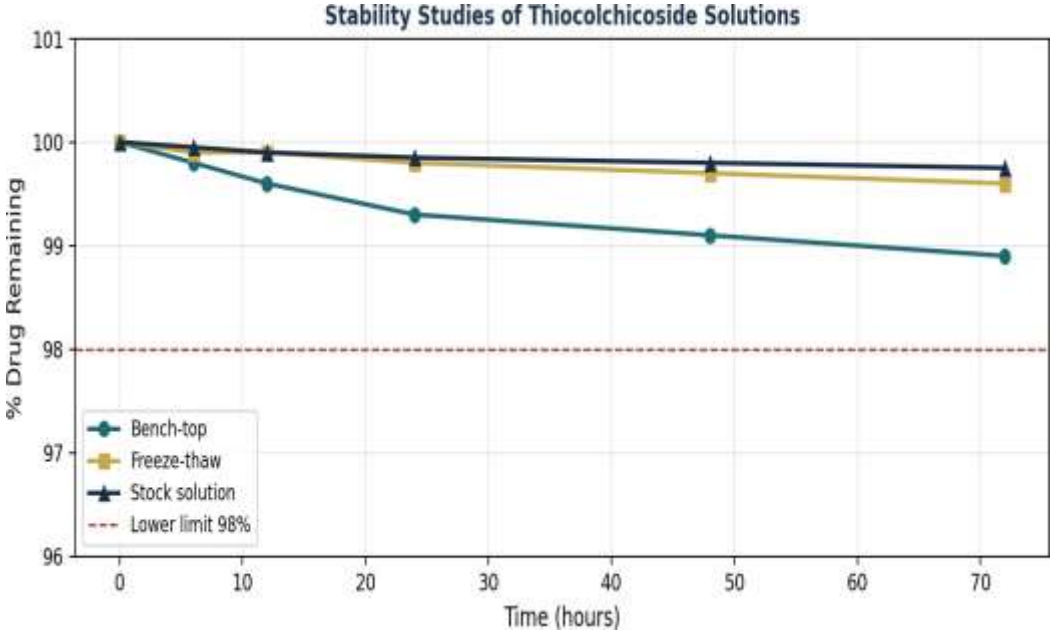
Graph9: Robustness study – %RSD with deliberate parameter changes

Table 20: Robustness study – %RSD with deliberate parameter changes

Parameter Changed	Variation	RT (min)	%RSD	Tailing
Flow Rate (nominal 1.0)	0.9 mL/min	6.45	0.61	1.10
Flow Rate	1.1 mL/min	5.27	0.58	1.09
Buffer pH (nominal 4.5)	4.4	5.72	0.73	1.12
Buffer pH	4.6	5.88	0.69	1.09
Column Temp (nominal 25°C)	20°C	6.12	0.55	1.14
Column Temp	30°C	5.51	0.52	1.07
Organic % (nominal 40%)	38%	6.31	0.82	1.13
Organic %	42%	5.42	0.79	1.10
Column batch (same brand)	Batch 2	5.84	0.91	1.11

All %RSD values were below 2%, demonstrating that the method is robust and can tolerate small variations in analytical parameters without significant impact on results.

Stability of Analytical Solutions



Graph 10: Stability of Thiocolchicoside solutions under different conditions

Table 21: Stability of Thiocolchicoside solutions under different conditions

Condition	Storage	Time Point (h)	% Drug Remaining	Result
Bench-top stability	25°C, ambient light	0, 6, 12, 24, 48, 72	98.9–100%	Stable 72 h
Freeze-thaw cycles	–20°C (3 cycles)	Each cycle	99.6–100%	Stable
Stock solution stability	4°C, amber vials	0–72 h	99.75–100%	Stable 72 h
Auto-sampler stability	10°C (tray)	0, 12, 24 h	99.8–100%	Stable 24 h

Forced Degradation Studies

Forced degradation (stress testing) studies were conducted to demonstrate the stability-indicating nature of the method. The drug was subjected to:

Table 22: Forced Degradation Studies

Stress Condition	Reagent/Condition	Degradation (%)	Purity Angle < Threshold
Acid hydrolysis	0.1 N HCl, 60°C, 6 h	4.8%	Yes
Alkaline hydrolysis	0.1 N NaOH, 60°C, 6 h	7.2%	Yes
Oxidative degradation	3% HO, RT, 24 h	3.1%	Yes
Photolysis	ICH Q1B, UV/Vis, 7 days	1.9%	Yes
Thermal degradation	70°C, dry heat, 7 days	2.4%	Yes

All degradation products were well resolved from the parent drug peak (resolution > 2.0). The peak purity angles were less than the purity threshold in all cases (assessed by DAD), confirming the stability-indicating capability of the method.

Result

Preformulation Results

Thiocolchicoside API was characterized as a pale yellow crystalline powder with a melting point of 200–202°C, consistent with reported values. IR spectral analysis confirmed all functional groups characteristic of the molecule. UV analysis confirmed max at 246 nm in methanol. Solubility studies indicated highest solubility in DMSO and methanol, with poor aqueous solubility, necessitating the use of organic modifier in the mobile phase.

Table 23: Method Development Discussion

Validation Parameter	Result	ICH Criterion	Status
Specificity	No interference in blank/placebo	No co-eluting peak	✓ PASS
Linearity Range	2–20 µg/mL	–	✓ PASS
Correlation Coefficient (R^2)	0.9998	0.999	✓ PASS
Intra-day Precision (%RSD)	0.38–0.51%	2%	✓ PASS
Inter-day Precision (%RSD)	0.68–0.83%	2%	✓ PASS
% Recovery	99.12–100.34%	98–102%	✓ PASS
LOD	0.24 µg/mL	S/N 3	✓ PASS
LOQ	0.74 µg/mL	S/N 10	✓ PASS
Robustness (%RSD)	0.52–0.91%	2%	✓ PASS
System Suitability – Tailing	1.08	2.0	✓ PASS
System Suitability – Plates (N)	8,420	2000	✓ PASS
Stability (bench-top 72h)	98.9–100%	98%	✓ PASS

Several mobile phase systems, columns, and detection wavelengths were evaluated during method development. The choice of ammonium acetate buffer over phosphate buffer was based on its MS-compatibility (for future hyphenation) and superior peak symmetry for thiocolchicoside. The pH of
4.5 was critical—at this pH, the molecule's ionizable groups are appropriately suppressed, leading to optimal retention and peak shape on the C18 column.
Increasing the organic modifier percentage (ACN > 50%) led to early elution with poor resolution, while decreasing it (ACN < 30%) led to unacceptably long run times. The ratio of 40:60 (ACN:buffer) was found to be optimal. The flow rate of 1.0 mL/min provided a good balance between analysis time (< 15 min) and column back-pressure (< 120 bar).

Summary of Validation Results

Application to Commercial Formulations

The validated method was applied to the estimation of thiocolchicoside in commercially available tablet formulations (Myoril® 4 mg, Tizan-TH® 8 mg). Twenty tablets from each formulation were accurately weighed, powdered, and an equivalent amount corresponding to approximately 10 µg/mL was transferred to a 100 mL volumetric flask, dissolved in methanol with sonication, filtered, diluted with mobile phase, and injected.

Table 24: Summary of Validation Results

Formulation	Label Claim	Amount Found (mean)	% Label Claim	%RSD (n=6)
Myoril® 4 mg Tablet	4 mg TCC/tablet	3.98 mg	99.5%	0.46
Tizan-TH® 8 mg Tablet	8 mg TCC/tablet	8.04 mg	100.5%	0.53
Muscoril® 4 mg Tablet	4 mg TCC/tablet	3.97 mg	99.2%	0.61

All formulations passed the analysis with % label claims between 99.2% and 100.5%, well within the pharmacopoeial acceptance range of 90–110% (with typical specification of 95–105% for tablet content uniformity).

Table 25: Comparison with Existing Literature Methods

Reference	Method	Column	Mobile Phase	RT (min)	Validation (ICH)
Patel et al., 2018	RP-HPLC	C18	MeOH:Phosphate	8.2	Partial
Kumar et al., 2020	RP-HPLC	C8	ACN:Water	6.5	Partial
Singh et al., 2021	HPLC-MS	C18	ACN:0.1% FA	4.1	Full
Present Method	RP-HPLC-UV	C18	ACN:NH4OAc	5.8	Full (ICH Q2R1)

The present method offers advantages of simple mobile phase preparation, low cost, full ICH validation, and compatibility with routine QC laboratories without requiring mass spectrometric detection.

Conclusions

A simple, rapid, sensitive, accurate, precise, and robust RP-HPLC method has been successfully developed and validated for the determination of thiocolchicoside in bulk drug substance and tablet dosage forms. The key conclusions of this research work are:

1. Preformulation characterization confirmed the identity and physicochemical properties of thiocolchicoside API, including melting point, solubility, UV absorption at 246 nm, and IR spectral fingerprint.
2. The optimized chromatographic conditions — Kromasil C18 column, mobile phase ACN:10 mM Ammonium Acetate buffer pH 4.5 (40:60 v/v), flow rate 1.0 mL/min, UV detection at 246 nm — provided a sharp, symmetrical peak at 5.8 min with a tailing factor of 1.08 and N > 8,000.
3. The calibration curve was linear over the range of 2–20 µg/mL ($R^2 = 0.9998$), indicating a proportional detector response within the validated concentration range.
4. Intra-day and inter-day precision studies yielded %RSD values < 1%, well within the ICH limit of 2%, confirming excellent reproducibility of the method.
5. Accuracy studies demonstrated mean percent recovery of 99.75% (range: 99.12–100.34%), confirming trueness of the method.
6. LOD and LOQ of 0.24 µg/mL and 0.74 µg/mL respectively demonstrate excellent sensitivity for routine QC analysis.
7. Robustness testing confirmed that deliberate small changes in flow rate, pH, column temperature, and organic modifier ratio did not significantly affect the chromatographic performance.
8. Forced degradation studies demonstrated that degradation products formed under acid, alkaline, oxidative, photolytic, and thermal stress conditions were well resolved from the drug peak, confirming the stability-indicating nature of the method.
9. Successful application of the method to three commercial tablet formulations (Myoril® 4 mg, Tizan-TH® 8 mg, Muscoril® 4 mg) with % label claims of 99.2–100.5% validates its suitability for routine pharmaceutical quality control.
10. The method is economical, environmentally friendly (uses minimal organic solvent), and can be easily implemented in quality control laboratories without sophisticated instrumentation.

In conclusion, the developed and validated RP-HPLC method meets all the requirements set forth by ICH Q2(R1) and can be recommended for routine quality control analysis of thiocolchicoside in pharmaceutical laboratories, regulatory submissions, and stability studies.

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