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Preformulation characterization of Thiocolchicoside

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Abstract

Introduction: Thiocolchicoside is semi-synthetic sulfur derivative of colchicine, which is a naturally occurring glucoside in the Colchicine plant. It is potent muscle relaxant with anti-inflammatory and analgesic activities. Thiocolchicoside is biopharmaceutical classification system (BCS-III) drug.

Aim & objectives: The present research work was aimed to study the pre-formulation parameters of Thiocolchicoside to develop suitable and stable subcutaneous formulation. Objective of the study was to develop promising subcutaneous dosage form to overcome the existing limitations of the current route of delivery and to prepare a way by observing the physical and chemical properties of drug substance for transforming into drug product in such a way that drug product can be administered in a right way.

Methods: Organoleptic characteristics of Thiocolchicoside were analyzed. Centrifugation method was used to analyze the solubility. Partition coefficient was determined. Melting point was analyzed by capillary method. Infra red spectroscopy was performed. X-ray diffraction was conducted to confirm the inside arrangement. Nature of peak was analyzed through differential scanning calorimetry. Calibration plot of drug with HPLC with acetonitrile: water (70:30) was carried out for LOD and LOQ determination.

Results: Thiocolchicoside found with pale yellow color and mild odour. Solubility found 12.65 mg/ml. Partition coefficient found 0.378. Melting point found in the range of 163-169.33 º C. IR spectra had given peaks for primary and secondary amide at 1603 cm^{-1}& 3291.2 cm^{-1}, ketone moiety of tropane nucleus at 1649.7 cm^{-1}, thioether at 2360.8 cm^{-1} and hydroxyl group at 3299.1 cm^{-1}. Single exothermic peak observed at 161.83 º C temperature with differential scanning calorimetry. Drug was subjected for X-ray diffraction analysis from temperature range at 10 º C to 90 º C, 79.4% crystallinity & 20.6% amorphousness of drug was found. Calibration plot with HPLC has given regression coefficient of 0.998. Mean retention time for peaks was observed 2.655 minutes. LOD was 1.092µg/ml and LOQ was 3.311µg/ml.

Conclusion: Drug found stable under all analyzed parameters. The target dosage form with Thiocolchicoside is promising to overcome the existed limitations with current route of delivery.

Keywords: Centrifuge; Compatibility; DSC; HPLC; IR; XRD

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1. Introduction

Thiocolchicoside is a semi-synthetic sulfur derivative of the colchicine, a natural anti-inflammatory glycoside which originates from the flower seeds of *Superba gloriosa.* It is national flower of Zimbabwe and state flower of Tamilnadu, India. It is muscle relaxant with anti-inflammatory and analgesic effects. Thiocolchicoside is a skeletal muscle-relaxant drug used in the treatment of orthopedic, traumatic and rheumatologic disorders. It is indicated as an adjuvant drug in the treatment of painful muscle contractures and is indicated in acute spinal pathology, for adults and adolescents 16 years of age and older (13). Recent studies have examined its effect on muscle tone, stiffness, contractures, and soreness experienced by athletes during sporting competitions. Thiocolchicoside also claimed to possess GABA mimetic and glycinergic actions. It binds to GABA-A and strychnine sensitive glycine receptors and acts as GABA-A antagonist (8).Thiocolchicoside is micromolecule with molecular weight of 563.62 D. IUPAC name for Thiocolchicoside is N-[(7S)-3-(β-D-Glucopyranosyloxy)-1,2-dimethoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl] acetamide. Chemical formula for Thiocolchicoside is $C_{27}H_{33}NO_{10}S$ (17). Pre-formulation study is multidisciplinary approach and uses various aspects of pharmacology, toxicology, physical and chemical characteristics of drug substance (10). Before starting pre-formulation, studies researcher should be aware about physical, chemical and biological properties of drug substance. Validated and optimized methods used to be planned to develop dosage form (5). Crystalline nature of drug substance can affect the physico-chemical properties of drug substance from the flow ability to stability. Polymorphic compounds may affect the melting point and solubility of drug substance. Approximate 1/3 organic compounds are polymorphic in nature (16).Particle size of drug substance is very crucial parameter. It not only affects the physical properties of drug substance while affect the biopharmaceutical behavior of developed dosage form (7). Pre-formulation characteristics of carrier systems are evaluated to improve some crucial parameters which will furnish the biological characteristics of drug substance viz. micro particles of poor water drugs, drugs with partition coefficient outside the range of 1.5-4. (6). Pre-formulation studies are conducted to ensure the quantitative assessment of chemical stability of drug substance and newly developed dosage form (17, 18). The use of inactive components which will furnish the physico-chemical and biological properties of developed dosage form will pay their significant distribution among whole of the process i.e., use of various co-solvents and other type of excipients (1).

2. Material and methods

Thiocolchicoside was kindly provided by Oyster Labs, Ambala Cantt. All the solvents for pre-formulation characterization were used of analytical grade. Distilled water was collected from research lab, n-octane was procured from Fischer Scientific and Acetonitrile of HPLC grade was procured from Merck life sciences Pvt. Ltd. And HPLC water was procured from Rankem.

2.1. Methods for pre-formulation studies

2.1.1. Organoleptic characteristics of Thiocolchicoside

The need of organoleptic characteristics of drug is to observe the basic parameters viz. color, odor and taste of drug.

Organoleptic characteristics were observed macroscopically. The organoleptic characteristics of Thiocolchicoside as shown in below given table (14).

Table 1 Organoleptic characteristics of Thiocolchicoside

Drug name	Color	Odor	Taste
Thiocolchicoside	Pale yellow to yellow	Characteristic	Unpleasant

2.1.2. Determination of solubility of Thiocolchicoside in distilled water

Solubility of drug is important characteristic which confirms the compatibility of drug at tissue site and fluid present at that site.

Solubility of THC in distilled water was determined by bath shaker method (Labman Scientific Pvt. Ltd.). 10 ml distilled water was poured in 25 ml conical flask. Excess and un-weighed amount of Thiocolchicoside was poured in conical flask to prepare a supersaturated solution of drug. Conical flask was placed in bath shaker for 24 hours at 25ºC and collected flask from bath shaker then content was poured in centrifuge tubes and operated at 1500 rpm for 20 minutes, then

supernatant from the centrifuge tube was collected and diluted as per requirement of sample and observed through UV-Visible spectrophotometer (19).

2.1.3. Calibration plots of Thiocolchicoside

Drug was treated with various solvents to evaluate the linearity or distribution of drug in solvent/s uniformly.

Stock solution of drug in various solvents viz. distilled water, 0.01M PBS, n-octanol and methanol were prepared and observed for wavelength (λ_{max}), regression coefficient (r^2) and slope value (m) (2). The dilutions of stock solutions of Thiocolchicoside for all solvents were prepared in triplicate (n=3) to construct calibration plot and to validate the data for reproducibility.

Table 2 Calibration plots of drug in various solvents

Approach	Double beam UV-Visible spectrophotometer (Shimadzu UV-1800).
Solvent/s	Distilled water, 0.01M PBS, n-octanol and methanol.
Drug	Thiocolchicoside.
Stock concentration	10 µg/ml - 40 µg/ml.
Scanning for λ_{max}	200 nm – 400 nm.

UV-Visible spectrophotometric method was validated to ensure the reproducibility of data. Method was validated for linearity, precision, accuracy, LOD and LOQ. All parameters were validated for calibration plots of Thiocolchicoside in all above mentioned solvents. To validate the linearity the regression coefficient (r^2) of dilutions were evaluated, to validate precision the absorbance value of 40 µg/ml dilution was evaluated, to validate accuracy the %age recovery of drug for 40 µg/ml dilution was evaluated. Limit of detection (LOD) and limit of quantification (LOQ) was validated for dilutions. All parameters are validated in triplicate (n=3).

2.1.4. Determination of partition coefficient of Thiocolchicoside in water/n-octanol system

Partition coefficient of drug was evaluated to confirm the affinity of drug either for aqueous phase or for organic phase. Ideal value of partition coefficient is 1.5 to 4. Drug possesses value in between this range having good absorption characteristics through biological membrane.

Partition coefficient was observed through separating funnel. A separating funnel of 200 ml was taken. 5 mg Thiocolchicoside was weighted and poured in 50 ml aqueous phase and similar for organic phase. 50 ml distilled water containing 5 mg drug and 50 ml n-octanol containing 5 mg drug was poured in separating funnel and kept the funnel in tripod stand for 48 hours. Both the phases were separated carefully after 48 hours. Dilutions of both phases were prepared and absorbance of aqueous phase and organic phase observed at pre-determined λ_{max}. The partition coefficient of drug in both phases observed by following equation i.e. concentration of drug in organic phase/concentration of drug in aqueous phase. If partition coefficient value is < 2 then drug having more affinity towards aqueous phase i.e. drug is hydrophilic and if partition coefficient value is ≥ 2 then drug having more affinity towards organic phase i.e. drug is lipophilic (19).

2.1.5. Determination of melting point of Thiocolchicoside

Melting point (Univolt) of drug confirms about the purity of substance. If melting point changes i.e. depress or broaden then drug is impure means any component is present in the drug as impurity which is changing the melting point every time, if this phenomenon does not occur then purity of drug confirms.

Thiocolchicoside was taken in sealed capillary tube and tube was placed in melting point apparatus. Temperature was noted down at which drug melted. Melting point of drug was observed in hexaplicate (n=6) to ensure the reproducibility (12).

2.1.6. Infra-red (IR) spectroscopy of Thiocolchicoside

Infra-red (Perkin Elmer) spectroscopy was performed to confirm the structure and purity of drug. Various functional groups were confirmed with the peaks at stretching and bending levels at particular wavelength at infra-red region. Wavelength was measured in cm^{-1} for infra-red region.

Infra-red spectrophotometer was prepared for calibration at least 40 minutes before placing the sample. After preparation of instrument Thiocolchicoside was placed on the surface of IR spectrophotometer and instrument had taken 24 scans of drug and peaks were observed at stretching and bending levels according to the functional groups present in drug and correlated the observed peaks with reported IR spectra of Thiocolchicoside and confirmed the identification of drug which also confirms the purity of drug (12).

2.1.7. DSC of Thiocolchicoside

DSC (TA-DSC-25) measures the heat capacity of drug substance as a function of temperature. Melting point, crystallinity, enthalpy and compatibility of drug substance with other excipients also confirmed. Drug substance and other compounds give either endothermic or exothermic peak through differential scanning calorimetry.

Thiocolchicoside was poured in aluminium hermetic pan. Gross weight of Thiocolchicoside was approximate 10 mg in pan and placed in DSC chamber. Drug scanned up to temperature range 50ºC to 250 ºC at interval of rise in temperature 10 ºC/minute. At a particular range of temperature (ºC) peak of drug was observed and nature of drug peak was identified (12).

2.1.8. XRD of Thiocolchicoside

Drug was confirmed for its crystalline or amorphous behavior through X-ray differactor (XRD). Diffraction pattern of drug substance is identified and XRD also provides information how actual structure deviates from original one.

Thiocolchicoside was placed in powder form on the sample cell. Sample cell was exposed in XRD chamber and drug sample was irradiated with incident X-rays and intensities and scattering angles of X-rays were measured with respect to the powder form of Thiocolchicoside(3).

2.1.9. Standard plot of Thiocolchicoside in acetonitrile: water (70:30) through HPLC

HPLC method was conducted to establish the solvent system for the determination of drug in the blood plasma. Both the solvents were taken of HPLC grade.

700 ml of acetonitrile HPLC grade was mixed with 300 ml of water HPLC grade. Mixture was degassed with bath sonicator for 25 minutes. Stock solution and dilutions of specific concentration were prepared and filtered through micro pore filter paper. Calibration plot was constructed on the behalf of concentration (x-axis) and area (y-axis) of chromatogram. Straight line is obtained, through which slope value and intercept value was obtained. Limit of detection (LOD) and limit of quantification (LOQ) of the method was calculated, all the calculations were performed on an excel sheet and regression coefficient, standard deviation of intercept and standard error were calculated. The details of Thiocolchicoside chromatogram has given in below table (14).

Table 3 HPLC chromatogram of Thiocolchicoside

Approach	**Agilent Technologies**
Stock solution concentration	50 µg/ml
Dilution range	2 µg/ml-10 µg/ml
Filter paper size	0.22 µ
Column type	C-18
Column dimensions	250 mm X 4.6 mm
Column size	5 µ
Column temperature	32°C
Flow rate	1 ml/min
Run time	6 minutes
Retention time	2.655 minutes
Injection volume	20 µl
Wavelength (λ_{max})	286 nm

HPLC method was validated to ensure the reproducibility of data. Method was validated for linearity, precision, accuracy, LOD and LOQ. To validate the linearity the regression coefficient (r^2) of dilutions were evaluated, to validate precision the area of 10 µg/ml dilution was evaluated, to validate accuracy the %age recovery of drug for 10 µg/ml dilution was evaluated. Limit of detection (LOD) and limit of quantification (LOQ) was validated for dilutions. All parameters are validated in triplicate (n=3).

3. Results and discussion

3.1. Organoleptic Characteristics of Thiocolchicoside

- Organoleptic characteristics of Thiocolchicoside to be observed for ensure the purity of drug.
- Color and odor of drug were observed macroscopically (14). Taste of Thiocolchicoside was not confirmed.
- Results: The sample of Thiocolchicoside was observed as pale yellow in color with mild odor.
- Inference: The observed organoleptic characteristics indicated the purity of Thiocolchicoside.

3.2. Determination of solubility of Thiocolchicoside in distilled water:

Solubility of drug is an important parameter for the absorption of drug at membrane site and to achieve the desired concentration of drug in systemic circulation for achieving required pharmacological response. Solubility of Thiocolchicoside is to be determined to observe the better absorption of drug.

Solubility of Thiocolchicoside in distilled water was determined by bath shaker method (19).Centrifuged sample was diluted up to 1000X and absorbance of diluted sample was 0.253. Observed slope value (m) of Thiocolchicoside in distilled water was 0.020 at λ_{max} of 292 nm. The solubility of given sample of Thiocolchicoside in distilled water is as given in table.

Table 4 Solubility of Thiocolchicoside in distilled water

Drug	Observed solubility	Reported solubility
Thiocolchicoside	12.65 mg/ml	10 mg/ml

- Result: The Thiocolchicoside was sparingly soluble in distilled water.
- Inference: This range of Thiocolchicoside solubility indicated that drug should be absorbed easily through biological membrane to provide desired therapeutic response at required site.

3.3. Calibration plots of Thiocolchicoside

The calibration plots of Thiocolchicoside were prepared to find out their peaks (λ_{max}) in various solvents and to observe the slope (m) value and regression coefficient (r^2) of Thiocolchicoside in the respective solvents for the analysis of drug.

Calibration plots of Thiocolchicoside in different solvents were plotted by using double beam UV-Visible spectrophotometer (Shimadzu UV-1800) (4).

3.4. Determination of λ_{max} in different solvents:

Thiocolchicoside to be scanned for observe the peak of drug in particular solvent to ensure the identity of drug.

Thiocolchicoside was scanned in the range of 200-400 nm wavelengths with different solvents. Thiocolchicoside had given peak at different wavelength in different solvents. The observed $\lambda_{max}$of Thiocolchicoside given in below mentioned table.

Table 5 Observed λ_{max} of Thiocolchicoside in different solvents

Sr. No.	Solvent	Observed λ_{max} (nm)	Reported λ_{max}(nm)
1	Distilled water	292	287.5 (11).
2	Methanol	248	251 (4).

3	n-Octanol	280	-
4	0.01M PBS 7.4	302	298 (11).

- Results: The observed λmax of Thiocolchicoside was closer to the reported data as indicated in above mentioned table.
- Inference: The observed λmax ensured about the identity of drug i.e. drug was Thiocolchicoside.

3.5. Calibration plot of Thiocolchicoside in different solvents:

Calibration plots of Thiocolchicoside in various solvents to be plotted to ensure uniform distribution of drug in solvent. The calibration plot s of Thiocolchicoside is to be required to obtain slope value (m) and regression coefficient (r^2) value of drug in taken solvents. Slope value of Thiocolchicoside to be required to calculate the concentration of drug in sample and regression coefficient value to be required to ensure the linearity of plot in respective solvent.

For calibration plots of Thiocolchicoside the dilutions were prepared in the range of 10-40 µg/ml. in each solvent. The absorbance of every dilution in each solvent was taken and plots were plotted to calculate the slope and regression coefficient values. Calibration plot data of Thiocolchicoside in various solvents mentioned in below given table.

Table 6 Calibration plot data of Thiocolchicoside for their slope and regression coefficients in various solvents

Sr. No.	Solvent	Conc. range (µg/ml)	Absorbance range	r^2	m (slope)
1	Distilled water	10-40	0.212-0.829	0.999	0.020
2	Methanol	10-40	0.182-0.719	0.999	0.018
3	n-octanol	10-40	0.200-0.815	0.998	0.020
4	0.01M PBS 7.4	10-40	0.212-0.837	0.995	0.021

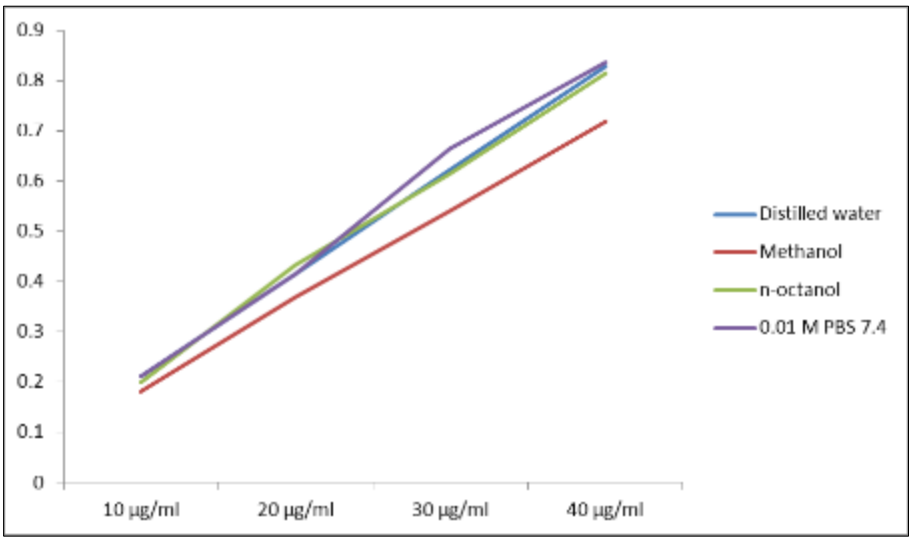

Figure 1 Calibration plots of Thiocolchicoside in various solvents

- Results: Slope and regression coefficient of Thiocolchicoside in distilled water, methanol, n-octanol and 0.01 M PBS 7.4 were observed with linearity in dilution range of 10-40 µg/ml.
- Inference: The observed linearity in the range of 10-40 µg/ml. among the calibration plots of Thiocolchicoside indicated that drug was uniformly distributed in various solvents under study.

3.6. Thiocolchicoside %age recovery in various solvents

The %age drug recovery is to be calculated to observe the accuracy of experimental work.

The %age drug recovery was calculated for dilutions with concentration range from 10-40 µg/ml. in each solvent. Concentration of drug in each dilution was found with the equation: Absorbance/slope* dilution factor. An observed value was divided with theoretical concentration and multiplied with 100 to observe the %age drug recovery. The % age recovery data of Thiocolchicoside is mentioned in below given table.

Table 7 Practical conc. and % age drug recovery of Thiocolchicoside in various solvents

Sr. no.	Solvent	Theoretical conc. range (µg/ml)	Practical conc. range (µg/ml)	Drug recovery (%)
1	Distilled water	10 – 40	10.6 - 41.45	103.83 – 106.01
2	Methanol	10 – 40	10.11 - 39.94	99.86 – 101.04
3	n-octanol	10 – 40	10 – 40.75	100 – 108.25
4	0.01 M PBS 7.4	10 – 40	10.09 – 39.85	99.04 – 105.55

- Results: Practical concentration of drug was in the range of 10 – 41.45 µg/ml. and drug recovery was in the range of 100 – 108.25%.
- Inference: The observed data indicated the accuracy of various dilutions for calibration plot of Thiocolchicoside.

3.7. Validation of UV spectrophotometric method:

Validation of UV spectrophotometric method was to be performed to ensure the reproducibility of data by analyzing the number of similar set of samples.

Linearity was evaluated to observe the ability to obtain the test results which are directly proportional to the concentration of analyte in the samples. Regression values (r^2) of standard plots were evaluated for linearity. Samples were prepared in triplicate (n=3) in each solvent for linearity.

Table 8 UV-Visible spectrophotometric validation for linearity

Linearity of standard plot of Thiocolchicoside in DW				
Sr. no.	**Regression value**	**Arithmetic mean**	**Standard deviation**	**% RSD**
1	0.999	0.999	0	0
2	0.999			
3	0.999			
Linearity of standard plot of Thiocolchicoside in methanol				
Sr. no.	**Regression value**	**Arithmetic mean**	**Standard deviation**	**% RSD**
1	0.999	0.999	0	0
2	0.999			
3	0.999			
Linearity of standard plot of Thiocolchicoside in n-octanol				
Sr. no.	**Regression value**	**Arithmetic mean**	**Standard deviation**	**% RSD**
1	0.997	0.998	0.001	0.1
2	0.999			
3	0.998			
Linearity of standard plot of Thiocolchicoside in 0.01 M PBS 7.4				

Sr. no.	Regression value	Arithmetic mean	Standard deviation	% RSD
1	0.995	0.995	0.001	0.1
2	0.996			
3	0.994			

- Results: Standard deviation and % RSD (relative standard deviation) of Thiocolchicoside in distilled water and methanol were observed zero and 0.001 and 0.1 in n-octanol and 0.01 M PBS 7.4
- Inference: The observed data indicated the concentration of dilutions were directly associated with the absorbance of dilutions.

Precision was evaluated to observe the degree of agreement among individual test results when the procedure is applied repeatedly to the multiple samplings. Absorbance values of 40µg/ml were evaluated for precision. Samples were prepared in triplicate (n=3) in each solvent for precision.

Table 9 U.V.-Visible spectrophotometric validation for precision

Precision of standard plot of Thiocolchicoside in DW				
Sr. no.	**Absorbance of 40 µg/ml dilution sample**	**Arithmetic mean**	**Standard deviation**	**% RSD**
1	0.829	0.825	0.004	0.48
2	0.821			
3	0.825			
Precision of standard plot of Thiocolchicoside in methanol				
Sr. no.	**Absorbance of 40 µg/ml dilution sample**	**Arithmetic mean**	**Standard deviation**	**% RSD**
1	0.719	0.717	0.001732051	0.24
2	0.716			
3	0.716			
Precision of standard plot of Thiocolchicoside in n-octanol				
Sr. no.	**Absorbance of 40 µg/ml dilution sample**	**Arithmetic mean**	**Standard deviation**	**% RSD**
1	0.815	0.812	0.002645751	0.32
2	0.811			
3	0.81			
Precision of standard plot of Thiocolchicoside in 0.01 M PBS 7.4				
Sr. no.	**Absorbance of 40 µg/ml dilution sample**	**Arithmetic mean**	**Standard deviation**	**% RSD**
1	0.837	0.835666667	0.001154701	0.14
2	0.835			
3	0.835			

- Results: Standard deviation values of absorbance values for the dilution samples (40µg/ml) in all solvents were in the range of 0.001-0.004 and %RSD values were in the range of 0.14-0.48.
- Inference: The observed data indicated the strong agreement among the test results of repeatedly multiple samplings.

Accuracy was evaluated to observe the accurate quantification of drug in the prepared samples. Samples were prepared in triplicate (n=3) in each solvent for accuracy.

Table 10 UV-Visible spectrophotometric validation for accuracy

Accuracy of standard plot of Thiocolchicoside in DW						
Sr. no.	**Conc. (µg/ml)**	**Practical conc. of drug (µg)**	**% recovery**	**Arithmetic mean**	**Standard deviation**	**% RSD**
1	40	41.45	103.625	103.125	0.5	0.48
2	40	41.05	102.625			
3	40	41.25	103.125			
Accuracy of standard plot of Thiocolchicoside in methanol						
Sr. no.	**Conc. (µg/ml)**	**Practical conc. of drug (µg)**	**% recovery**	**Arithmetic mean**	**Standard deviation**	**% RSD**
1	40	39.94	99.85	99.583	0.230940108	0.23
2	40	39.78	99.45			
3	40	39.78	99.45			
Accuracy of standard plot of Thiocolchicoside in n-octanol						
Sr. no.	Conc. (µg/ml)	Practical conc. of drug (µg)	% recovery	Arithmetic mean	Standard deviation	% RSD
1	40	40.75	101.875	101.5	0.330718914	0.32
2	40	40.55	101.375			
3	40	40.5	101.25			
Accuracy of standard plot of Thiocolchicoside in 0.01 M PBS 7.4						
Sr. no.	**Conc. (µg/ml)**	**Practical conc. of drug (µg)**	**% recovery**	**Arithmetic mean**	**Standard deviation**	**% RSD**
1	40	39.86	99.625	99.475	0.129903811	0.13
2	40	39.76	99.4			
3	40	39.76	99.4			

- Results: Standard deviation values of %age drug recovery for the dilution samples in all solvents were in the range of 0.13-0.5 and %RSD values were in the range of 0.13-0.48. The %RSD value for each set of sample was less than 2%, which consider as acceptable value for the data.
- Inference: The observed data indicated the accurate quantification of the drug in each set of dilution samples.

Limit of detection (LOD) was evaluated to observe the lowest concentration of drug in sample dilution. Samples were prepared in triplicate (n=3) in each solvent for LOD.

Table 11 UV-Visible spectrophotometric validation for limit of detection (LOD)

LOD of standard plot of Thiocolchicoside in DW				
Conc. (µg/ml)	**Absorbance**	**Std Deviation**	**Slope**	**LOD (µg/ml)**
10	0.212	0.265818704	0.02	43.89
20	0.415			
30	0.623			
40	0.829			

LOD of standard plot of Thiocolchicoside in methanol				
Conc. (µg/ml)	**Absorbance**	**Std Deviation**	**Slope**	**LOD (µg/ml)**
10	0.182	0.230214936	0.018	42.17
20	0.369			
30	0.541			
40	0.719			
LOD of standard plot of Thiocolchicoside in n-octanol				
Conc. (µg/ml)	**Absorbance**	**Std Deviation**	**Slope**	**LOD (µg/ml)**
10	0.2	0.260597391	0.02	42.9
20	0.422			
30	0.593			
40	0.815			
LOD of standard plot of Thiocolchicoside in 0.01 M PBS 7.4				
Conc. (µg/ml)	**Absorbance**	**Std Deviation**	**Slope**	**LOD (µg/ml)**
10	0.212	0.274814483	0.021	43.18
20	0.416			
30	0.665			
40	0.837			

- Results: Standard deviation values of LOD for the dilution samples in all solvents were in the range of 0.23-0.27 and LOD were in the range of 42.1-43.89µg/ml.
- Inference: The observed data indicated the LOD of the dilution samples were close with each other.

Limit of quantification (LOQ) was evaluated for the lowest possible concentration of the drug that can be quantified by the method in a reliable way.

Table 12 UV-Visible spectrophotometric validation for limit of quantification (LOQ)

LOQ of standard plot of Thiocolchicoside in DW				
Conc. (µg/ml)	**Absorbance**	**Std Deviation**	**Slope**	**LOQ (µg/ml)**
10	0.212	0.265818704	0.02	133
20	0.415			
30	0.623			
40	0.829			
LOQ of standard plot of Thiocolchicoside in methanol				
Conc. (µg/ml)	**Absorbance**	**Std Deviation**	**Slope**	**LOQ (µg/ml)**
10	0.182	0.230214936	0.018	127.77

20	0.369			
30	0.541			
40	0.719			
LOQ of standard plot of Thiocolchicoside in n-octanol				
Conc. (µg/ml)	**Absorbance**	**Std Deviation**	**Slope**	**LOQ (µg/ml)**
10	0.2	0.260597391	0.02	130
20	0.422			
30	0.593			
40	0.815			
LOD of standard plot of Thiocolchicoside in 0.01 M PBS 7.4				
Conc. (µg/ml)	**Absorbance**	**Std Deviation**	**Slope**	**LOQ (µg/ml)**
10	0.212	0.274814483	0.021	130.86
20	0.416			
30	0.665			
40	0.837			

- Results: Standard deviation values of LOQ for the dilution samples in all solvents were in the range of 0.23-0.27 and LOQ were in the range of 127.77-133µg/ml.
- Inference: The observed data indicated the LOQ of the dilution samples were close with each other.

Table 13 Validation summary of UV-Visible spectrophotometer

Solvent	**Validated parameter**	**Arithmetic mean**	**Standard deviation**	**%RSD**	
Distilled water	Linearity (regression value)	0.999	0	0	
	Precision (absorbance of dilution of 40 µg/ml conc.)	0.825	0.004	0.48	
	Accuracy (% drug recovery for 40 µg/ml conc.)	103.125	0.5	0.48	
	LOD (µg/ml)				43.89
	LOQ (µg/ml)				133
Methanol	Linearity (regression value)	0.999	0	0	
	Precision (absorbance of dilution of 40 µg/ml conc.)	0.717	0.001732051	0.24	
	Accuracy (% drug recovery for 40 µg/ml conc.)	99.583	0.230940108	0.23	
	LOD (µg/ml)				42.17
	LOQ (µg/ml)				127.77
n-Octanol	Linearity (regression value)	0.998	0.001	0.1	
	Precision (absorbance of dilution of 40 µg/ml conc.)	0.812	0.002645751	0.32	
	Accuracy (% drug recovery for 40 µg/ml conc.)	101.5	0.330718914	0.32	
	LOD (µg/ml)				42.9
	LOQ (µg/ml)				130

0.01 M PBS 7.4	Linearity (regression value)	0.995	0.001	0.1	
	Precision (absorbance of dilution of 40 µg/ml conc.)	0.835	0.001154701	0.14	
	Accuracy (% drug recovery for 40 µg/ml conc.)	99.475	0.129903811	0.13	
	LOD (µg/ml)				43.18
	LOQ (µg/ml)				130.86

3.8. Determination of partition coefficient of Thiocolchicoside in water/n-octanol system:

Partition coefficient of drug is to be determined to observe the affinity of drug either for aqueous phase or organic phase.

Partition coefficient was determined by using separating funnel (19). Concentration of drug in an aqueous phase and organic phase was calculated by applying equation: Absorbance*dilution factor / slope. Partition coefficient was calculated by using equation: concentration of drug in an organic phase / concentration of drug in an aqueous phase.

Table 14 Drug conc. in an aqueous phase

Absorbance	Drug slope in DW	Dilution factor	Conc.(µg/ml)	Conc.(mg/ml)
1.044	0.020	100 X	5220	5.220

Table 15 Drug conc. in an organic phase

Absorbance	Drug slope in n-octanol	Dilution factor	Conc.(µg/ml)	Conc.(mg/ml)
0.989	0.020	40 X	1978	1.978

Table 16 Observed value of partition coefficient of Thiocolchicoside

Sr. No.	Observed partition coefficient	Reported partition coefficient
1	0.378	0.340

- Results: The observed partition coefficient was 0.378.
- Inference: Drug was having more affinity towards an aqueous phase, which indicated that drug will be miscible with the biological fluid and will be absorbed easily.

3.9. Determination of melting point of Thiocolchicoside

The melting point of drug is to be observed for the purity of drug substance.

Melting point of Thiocolchicoside was observed with melting point apparatus (Univolt) in hexaplicate (19). Lower and upper range of melting point of drug mentioned in below given table.

Table 17 Observed value of melting point of Thiocolchicoside

Sr. No.	Observed m. p.(ºC; n=6)	Observed m .p. (ºC)	Reported m. p. (ºC)
1	162-167	163-169.33	161–168 (9).
2	164-172		
3	162-169		
4	163-169		

5	164-170		
6	163-169		

- Results: The observed melting point of Thiocolchicoside was 163-169.33ºC.
- Inference: Melting point indicated the Thiocolchicoside was lacking of impurities.

3.10. IR spectroscopy of Thiocolchicoside

Infra-red spectroscopy is to be performed for confirming the structure of Thiocolchicoside.

IR spectroscopy of Thiocolchicoside was determined through spectrophotometer (Perkin Elmer). Various peaks of Thiocolchicoside were observed through IR spectroscopy as shown in below given table (12).

Table 18 Observed IR spectra of Thiocolchicoside

Sr. No.	Functional group	Reported Wavelength (cm^{-1})	Observed Wavelength (cm^{-1})
1	Carbonyl (1˚ amide)	1525.5	1603.1
2	C=O str (Tropane ring)	1627.8	1649.7
3	N-H str (2˚ amide)	3325.9	3291.2
4	Thioether	2360.7	2360.8
5	-OH str	3336.6	3299.1

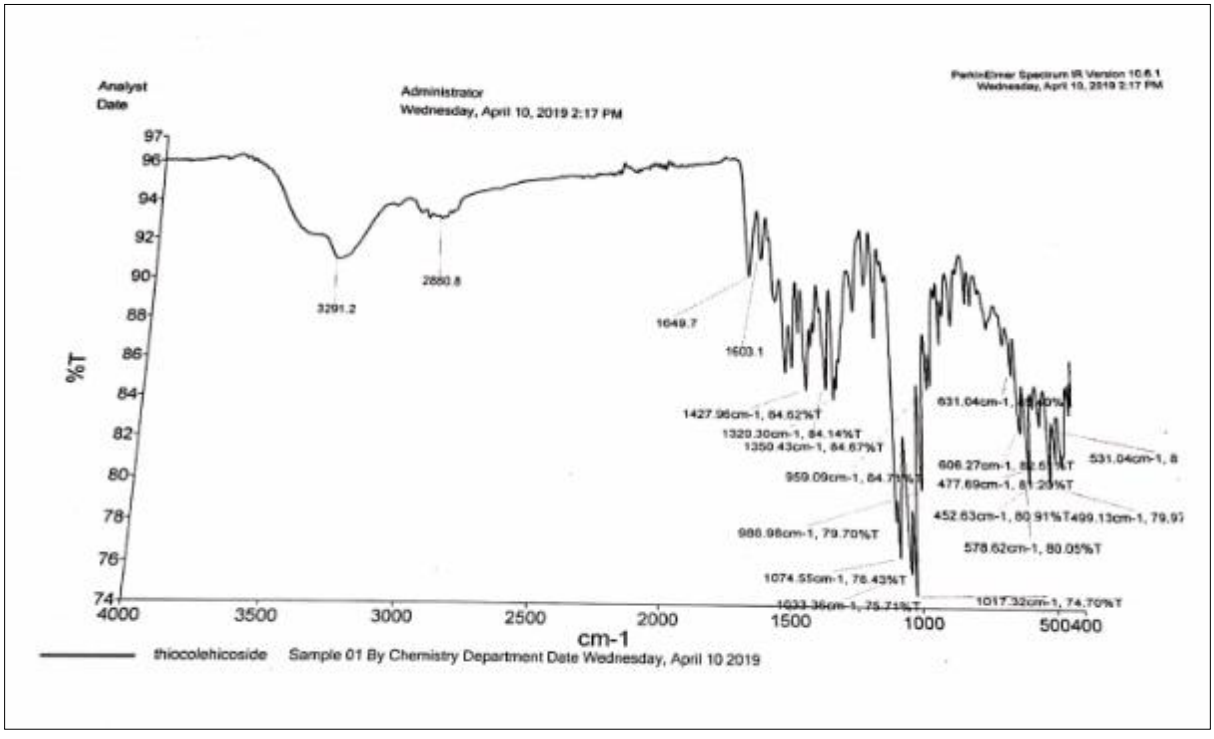

Figure 2 IR spectroscopy of Thiocolchicoside

- Results: Peaks were observed for primary amide, stretching peak for ketone group of tropane ring, stretching peak for secondary amide, thioether and stretching peak for hydroxyl group. Observed stretching peaks were almost closer to reported peaks for Thiocolchicoside
- Inference: Infra red spectroscopy confirmed that the sample substance was Thiocolchicoside.

3.11. DSC of Thiocolchicoside

Differential scanning calorimetry is to be performed to ensure the nature of peak, either endothermic or exothermic and to ensure melting point of drug substance.

Peak of Thiocolchicoside was observed through differential scanning calorimetry (TA-DSC-25). Observed peak of drug through DSC mentioned in below given table (12).

Table 19 DSC peak of Thiocolchicoside

Sr. No.	Nature of peak
1	Exothermic peak at 161.83ºC

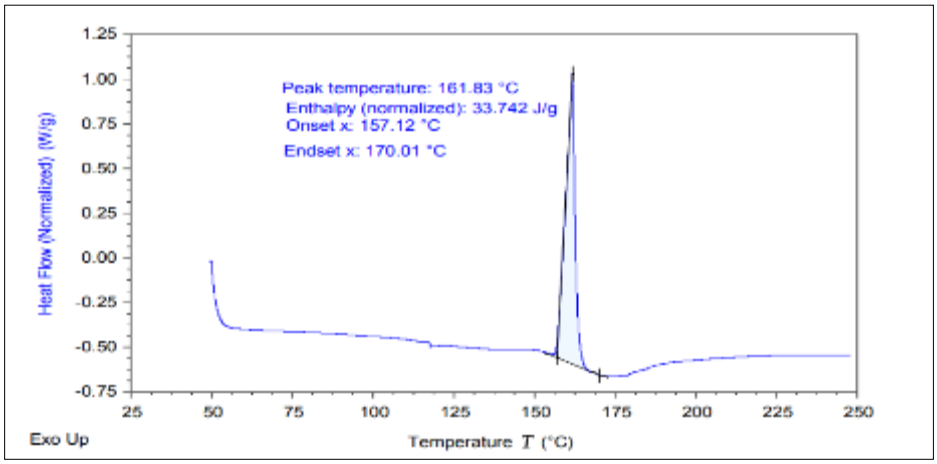

Figure 3 DSC plot of Thiocolchicoside

- Results: Exothermic peak of Thiocolchicoside was observed at temperature of 161.83ºC with enthalpy of 33.742 J/g.
- Inference: It indicated that Thiocolchicoside showed exothermic peak and observed melting point through DSC indicated the purity of Thiocolchicoside.

3.12. XRD of Thiocolchicoside

The X-ray diffraction is to be performed for ensuring the crystalline and amorphous pattern of Thiocolchicoside.

Thiocolchicoside was subjected to X-ray differactor (Bruker D8 ADVANCE ECO) for its internal arrangement of particles (12). Arrangement details at temperature ranges from 10ºC to 90 ºC and 10ºC to 50 ºC mentioned in below given figures.

Figure 4 Confirmation of crystalline pattern at temperature range from 10˚C to 90˚C

Figure 5 Confirmation of crystalline pattern at temperature range from 10˚C to 50˚C

Table 20 XRD pattern of Thiocolchicoside

Sr. No.	Observed Peak of Thiocolchicoside	d (Å)	Reported peak of Thiocolchicoside
1	10.138˚	8.69984	10.46
2	11.868˚	7.45580	11.38
3	12.640˚	6.98792	13.34
4	14.300˚	6.18656	14.48
5	17.373˚	5.09606	17.32
6	18.757˚	4.72387	18.06
7	20.271˚	4.38008	20.94

d= Particle-Particle distance.

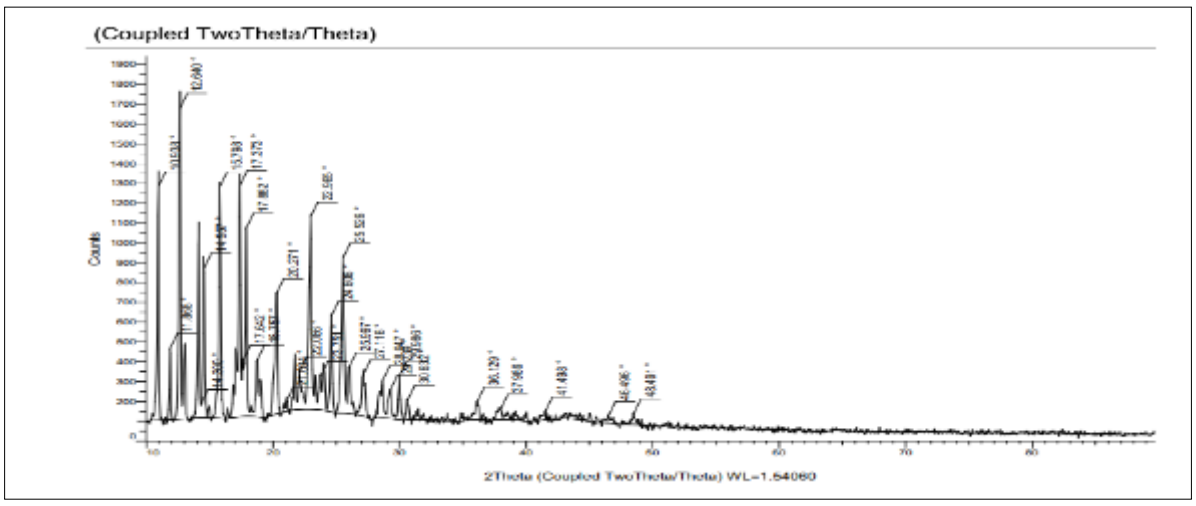

Figure 6 XRD plot of Thiocolchicoside

- Results: At temperature range of 10ºC to 90ºC the crystalline pattern of Thiocolchicoside was 79.4% and amorphous pattern was 20.6%. At temperature range of 10ºC to 50ºC the crystalline pattern of Thiocolchicoside was 82.9% and amorphous pattern was 17.1%.
- Inference: XRD indicated that Thiocolchicoside was crystalline in nature.

3.13. Calibration plot of Thiocolchicoside in acetonitrile: water (70:30) through HPLC:

3.13.1. Calibration plot

Calibration plot of Thiocolchicoside by HPLC is to be plotted for calculating the limit of detection and limit of quantification of drug in taken solvent.

Limit of detection requires for observing the lowest concentration of drug in sample and limit of quantification requires for observing the lowest concentration of sample at which some predefined goal for imprecision is met.

Calibration plot of Thiocolchicoside in acetonitrile: water (70:30) was prepared (11, 15). The detail for selected parameters for HPLC has given in section 5.1.9. Chromatogram area was obtained with linearity with the concentration range 2-10 µg/ml at wavelength of 286 nm as mentioned in below given table.

Table 21 Conc.-area data for Thiocolchicoside in acetonitrile: water (70:30)

Concentration (µg/ml)	Area	λ_{max} (nm)
2	62810	286
4	215213	
6	412397	
8	601040	
10	769469	

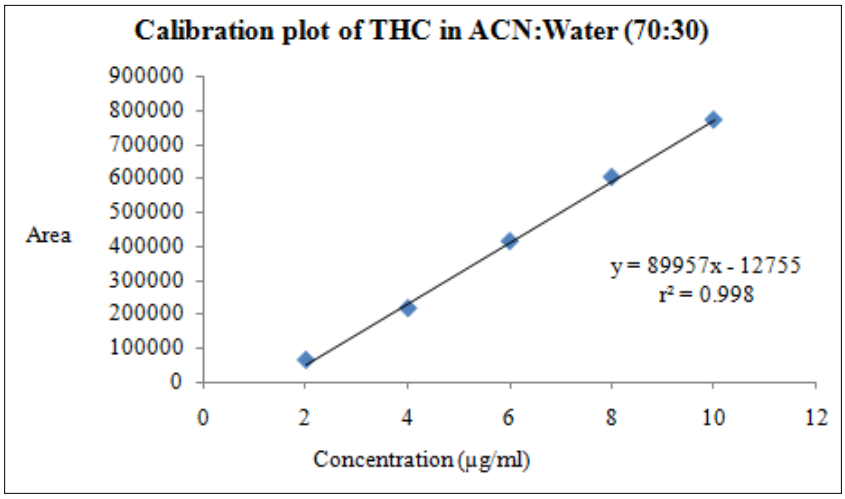

Figure 7 Calibration plot of Thiocolchicoside in acetonitrile: water (70:30)

Observed chromatogram peaks of Thiocolchicoside in acetonitrile: water (70:30) in the range of 2µg/ml to 10µg/ml given below:

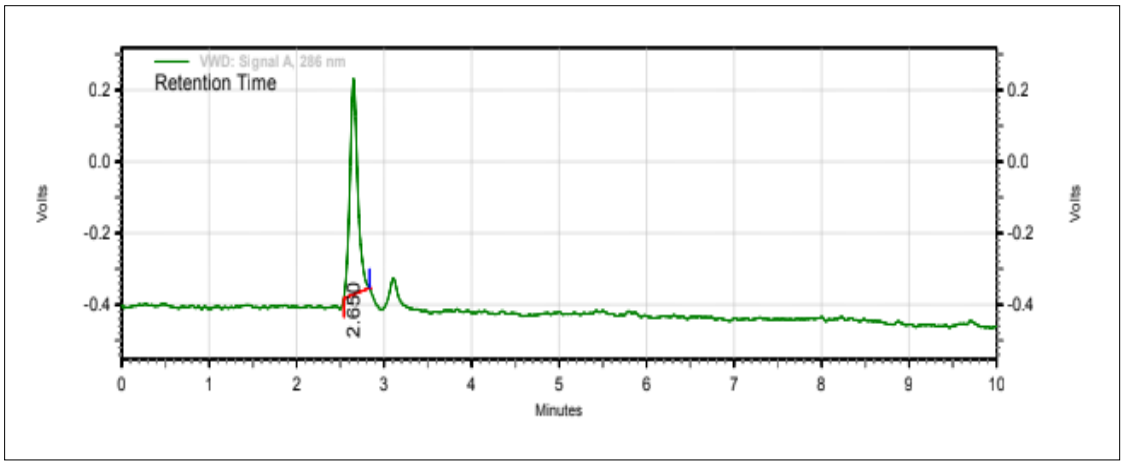

Figure 8 Chromatogram peak for 2µg/ml

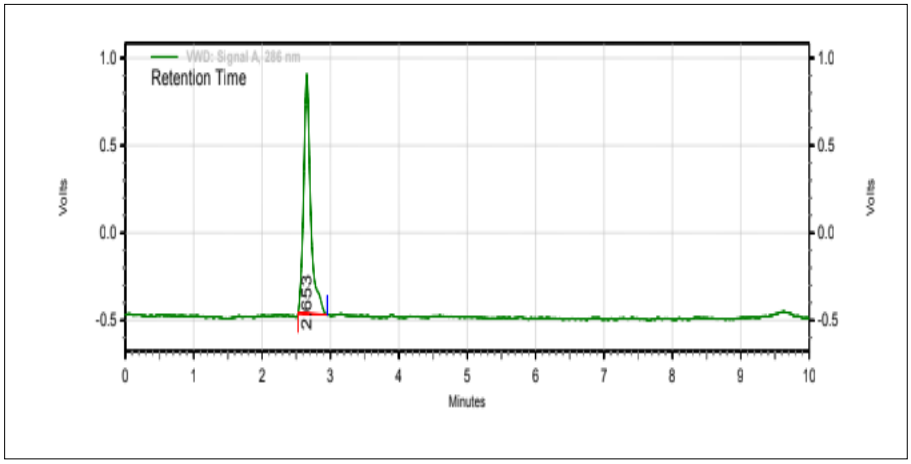

Figure 9 Chromatogram peak for 4µg/ml

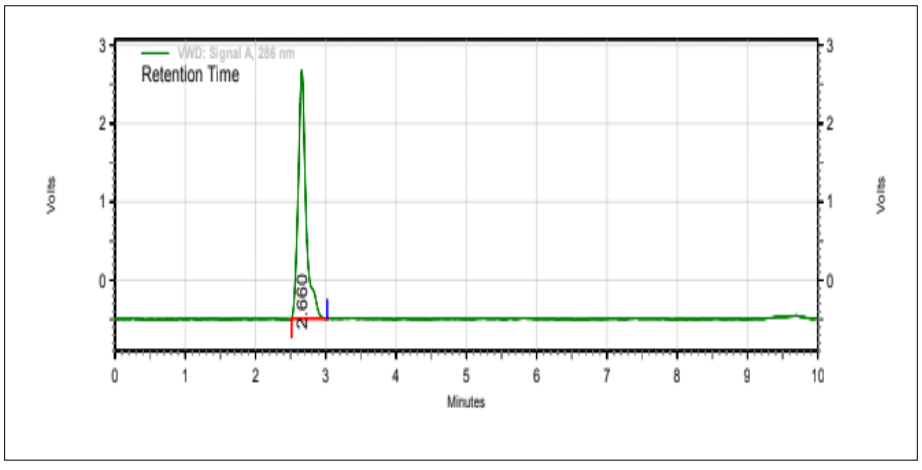

Figure 10 Chromatogram peak for 6µg/ml

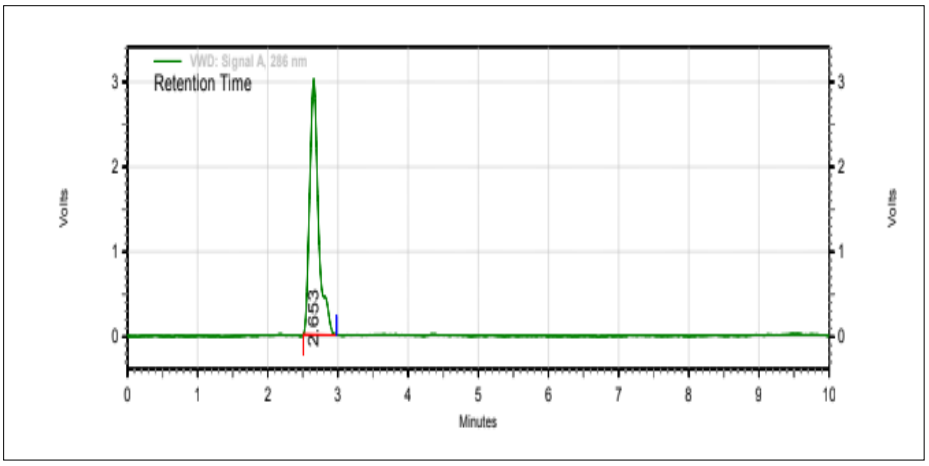

Figure 11 Chromatogram peak for 8µg/ml

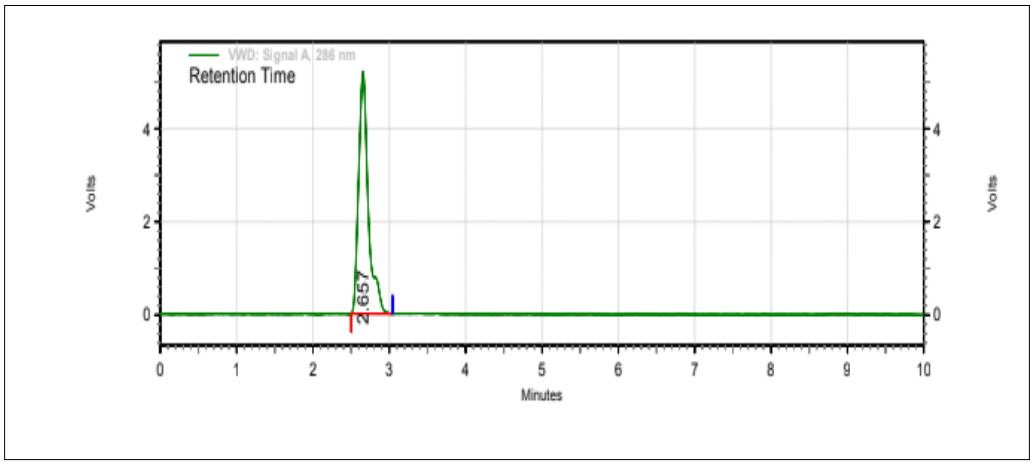

Figure 12 Chromatogram peak for10 µg/ml

Table 22 Calibration plot data of Thiocolchicoside in acetonitrile: water (70:30)

Solvent	Regression coefficient (r^2)	Slope (m)	LOD (µg/ml)	LOQ (µg/ml)
Acetonitrile :water (70:30)	0.9985	89957	1.092	3.311

LOD: Limit of detection; LOQ: Limit of quantification.

- Results: Linearity obtained with the dilution concentration range of 2 µg/ml-10 µg/ml. The observed regression coefficient (r^2) was 0.9985 with slope value (m) of 89957. Retention time for peaks was observed in between 2.650 to 2.660 minutes. Limit of detection (LOD) was1.092 µg/ml and limit of quantification (LOQ) was 3.311µg/ml.
- Inference: Linear plot of drug in acetonitrile: water (70:30) indicated that distribution of drug in solvent was directly related with the concentration of Thiocolchicoside in the sample across the specific range. LOD was calculated to observe the lowest concentration of Thiocolchicoside in the test sample while LOQ was calculated to ensure the precision of samples.
- Validation of HPLC method: Validation of HPLC method was to be performed to ensure the reproducibility of data by analyzing the number of similar set of samples.

Linearity was evaluated to observe the ability to obtain the test results which are directly proportional to the concentration of analyte in the samples. Regression value (r^2) of standard plot was evaluated for linearity. Samples were prepared in triplicate (n=3) in ACN: DW (70:30) for linearity.

Table 23 HPLC validation for linearity

Linearity of standard plot of Thiocolchicoside in Acetonitrile :DW (70:30)				
Sr. no.	**Regression value**	**Arithmetic mean**	**Standard deviation**	**% RSD**
1	0.998	0.998	0	0
2	0.998			
3	0.998			

- Results: Results: Standard deviation and % RSD (relative standard deviation) of Thiocolchicoside in acetonitrile: DW (70:30) was zero.
- Inference: The observed data indicated the concentration of dilutions were directly associated with the observed area of sample dilutions.

Precision was evaluated to observe the degree of agreement among individual test results when the procedure is applied repeatedly to the multiple samplings. Area for10µg/ml was evaluated for precision. Samples were prepared in triplicate (n=3) in ACN: DW (70:30) for precision.

Table 24 HPLC validation for precision

Precision of standard plot of Thiocolchicoside in Acetonitrile :DW (70:30)				
Sr. no.	**Area of 10 µg/ml dilution sample**	**Arithmetic mean**	**Standard deviation**	**% RSD**
1	769469	769439.3333	31.08590314	0.004
2	769407			
3	769442			

- Results: Standard deviation values of absorbance values for the dilution samples (10µg/ml) in ACN: DW (70:30) was 31.08and %RSD values was 0.004.
- Inference: The observed data indicated the strong agreement among the test results of repeatedly multiple samplings.

Accuracy was evaluated to observe the accurate quantification of drug in the prepared samples. Samples were prepared in triplicate (n=3) in ACN: DW (70:30) for accuracy.

Table 25 HPLC validation for accuracy

Accuracy of standard plot of Thiocolchicoside in Acetonitrile :DW (70:30)						
Sr. no.	**Conc. (µg/ml)**	**Practical conc. of drug (µg)**	**% recovery**	**Arithmetic mean**	**Standard deviation**	**% RSD**
1	10	8.55	85.5	85.5	0	0
2	10	8.55	85.5			
3	10	8.55	85.5			

- Results: Standard deviation and %RSD values of %age drug recovery for the dilution samples in ACN: DW (70:30) observed zero. The %RSD value for each set of sample was less than 2%, which consider as acceptable value for the data.
- Inference: The observed data indicated the accurate quantification of the drug in prepared set of dilution samples.

Limit of detection (LOD) was evaluated to observe the lowest concentration of drug in sample dilution. Samples were prepared in triplicate (n=3) in ACN: DW (70:30) for LOD.

Table 26 HPLC validation for limit of detection (LOD)

LOD of standard plot of Thiocolchicoside in Acetonitrile: DW (70:30).				
Conc. (µg/ml)	**Area**	**Std Deviation**	**Slope**	**LOD (µg/ml)**
2	62810	284683.584	89957	1.092
4	215213			
6	412397			
8	601040			
10	769469			

- Results: Standard deviation values of LOD for the dilution samples in ACN: DW (70:30) was 284683.584and LOD was 1.092µg/ml.
- Inference: The observed value of LOD indicated the potency of the method to detect the drug in sample.

Limit of quantification (LOQ) was evaluated for the lowest possible concentration of the drug that can be quantified by the method in a reliable way.

Table 27 HPLC validation for limit of quantification (LOQ)

LOQ of standard plot of Thiocolchicoside in Acetonitrile :DW (70:30)				
Conc. (µg/ml)	**Area**	**Std Deviation**	**Slope**	**LOQ (µg/ml)**
2	62810	284683.584	89957	3.311
4	215213			
6	412397			
8	601040			
10	769469			

- Results: Standard deviation values of LOQ for the dilution samples in ACN: DW (70:30) was 284683.584and LOD was 3.311µg/ml.
- Inference: The observed value of LOQ indicated the potency of the method to quantify the drug in sample.

Table 28 Validation summary of HPLC method

Solvent	**Validated parameter**	**Arithmetic mean**	**Standard deviation**	**%RSD**	
Acetonitrile : Distilled water (70:30)	Linearity (regression value)	0.998	0	0	
	Precision (area of dilution of 10 µg/ml conc.)	769439.333	31.8590314	0.004	
	Accuracy (% drug recovery for 10 µg/ml conc.)	85.5	0	0	
	LOD (µg/ml)				1.092
	LOQ (µg/ml)				3.311

4. Conclusion

Thiocolchicoside was found to be sparingly soluble in distilled water. Observed p value was less than 2, which indicates the drug was hydrophilic in nature. The drug does not possess sharp melting point. An infra-red spectrum of drug confirmed that drug was stable, not degraded. Observed peaks and reported peaks for crystallinity of drug were almost

similar. The drug had given exothermic peak during differential scanning calorimetry. Linearity of standard plot was observed with limit of detection less than 2µg/ml.

Compliance with ethical standards

Disclosure of conflict of interest

The authors have no conflicts of interest regarding this investigation**.**

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