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Original Article

SIMULTANEOUS ESTIMATION OF THIOCOLCHICOSIDE AND ACECLOFENAC BY HPTLC

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ABSTRACT

Objective: Therefore the aim of the present work was to develop simple, precise and accurate HPTLC method for simultaneous determination of THIO and ACE in pharmaceutical dosage form and application of the method for dissolution study with help of HPTLC method.

Methods: The TLC procedure was optimized in view to develop a simultaneous assay method for THIO and ACE. HPTLC Pre-coated plates silica gel 60 F_{254} 20×10 cm, layer thickness 0.2 mm (Merck, Germany). The samples were spotted in the form of bands (8 mm) with a Camag 100 microliter sample (Hamilton) syringe on silica gel precoated aluminium 60F_{254} plates. The mobile phase consisted of methanol: chloroform: water (9.6: 0.2: 0.2 v/v/v) and 10 ml of mobile phase were used per chromatography run. Plates were scanned over the range of 200-400 nm and the spectra were overlain.

Results: The detector response was found to be linear in the concentration range of 0.03-0.180 µg/band and 0.75-4.5 µg/band for THIO and ACE and noting the peak areas. Accuracy of the assay method was evaluated with the recovery of the standards from excipients. The mean percentage recoveries obtained for THIO and ACE were 99.34% and 99.08%, respectively, reported. The peak purity of both drugs was assessed by comparing the respective spectra of standard drugs and samples at peak start, peak apex and peak end positions of the spot.

Conclusion: HPTLC, with its advantage of low operating cost, high sample thought and minimum sample preparation need is now days preferred as routine analytical techniques for control and assurance. The validated HPTLC method employed here proved to be simple, fast, accurate, precise and sensitive, thus can be used for routine analysis of Thiocolchicoside and Aceclofenac in tablet dosage form.

Keywords: HPTLC, Thiocolchioside, THIO and ACE, ICH guidelines, Standard stock solutions

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INTRODUCTION

HPTLC [10-16]

HPTLC is the most simple separation technique today available to the analyst.

It can simultaneously handle several samples even of different nature and composition supporting several analysis at a given time.

High-performance thin layer chromatography, also known under the synonym planer chromatography, is a modern, powerful analytical technique with separation power, performance and reproducibility superior to classic TLC. It involves the same theoretical principle of thin layer chromatography wherein substances are separated on the basis of their differential migration in the system of two phases on a special type of plates. This technique is mostly used in many fields both for qualitative and quantitative (identification and estimation) of constituents mixture. This can easily be validated and fully compliant with GMP.

Advantages of HPTLC

1) Short development time.
2) Wide choice of stationary phase.
3) Early recovery of separated components.
4) Superior separation effects.
5) Easy visualization of separated compounds.

The main and important steps involved in this are as follows:

Sample preparation

F normal phase chromatography using silica gel/Alumina percolated plates, solvent generally should be non-polar and volatile type. Since polar solvent tend to give circular shape at the origin. For reverse phase chromatography usually, polar solvents are used for dissolving the sample.

Selection of chromatographic layer

Selection of layer depends on the nature of the material to be separated.

Commonly used materials are Silica gel 60F, Allumina, Cellulose, PEI, impregnated cellulose etc.

Plates

Standard size plates for HPTLC are manufactured by various companies which are most satisfactory. Handmade plates can be prepared and used which are economical. Generally, plates of 20 X 20 cm or 5 X 7.5 cm size having 100–250 mm adsorbent thickness are used for quantitative analysis. Silica gel 60F254 having a pore size 6 mm with a fluorescent indicator is a coat material. The basic difference in TLC and HPTLC plate is particle size of coated material which is 5-20 µm for TLC and 4-8 µm for HPTLC.

Pre-washing

Plates need to be pre-washed to remove water vapors or other volatile impurities, which might get trapped in the plates. These gives dirty zones and spots on the plates. To avoid this, plates are cleaned by using methanol as a solvent by ascending or descending or by dipping continuous mode.

Conditioning

The pre-washed plates exposed to humidity and surroundings are needed to be activated by placing them in the oven at 120 °C for 15 to 20 min. This process is known as conditioning. This allows the active centers of coating materials attenuated for better separation of sample material.

Sample application

It is a most important step for obtaining good resolution and results. Application of 1.0-5 µl is most satisfactory, for HPTLC, application of the sample and standard as a band gives better separation, equal Rf values and less spot broadening. This sample application is carried out by Linomat type applicator on the plates which give a uniform, safe and standard results.

Preconditioning (Chamber saturation)

This has a profound influence on the effective separation of the sample. For low polarity mobile phase there is no need of saturation, however, saturation is desirable in the case of highly polar mobile phases. Partial saturation is recommended for mobile phase composition leading to phase separation. For reverse phase chromatography, it is essential to saturate the chamber with methanol or polar solvent.

Mobile phase

The selection of appropriate mobile phase is based on the trial and error in which chemical properties of solute and solvent, the solubility of analyte absorbent layer etc. are considered along with analyst own experience.

Chromatographic development

Various forms of chromatographic development like ascending, descending, and horizontal, continuous, gradient can be tried. For HPTLC plates, migration distance of 5-6 mm is sufficient. After development, plates are removed from the chamber and dried to remove traces of mobile phase. Common problems encountered during chromatographic development are as follows:

(a) Tailing

This may occur due to the presence of traces of impurities or due to the presence of more than one ionic species of substances being chromatographed. This can be reduced by buffering the mobile phase system with acidic (1-2 % acetic acid) or basic (ammonia) solution. It keeps the materials to be separated in non-ionic forms.

b) Diffusion

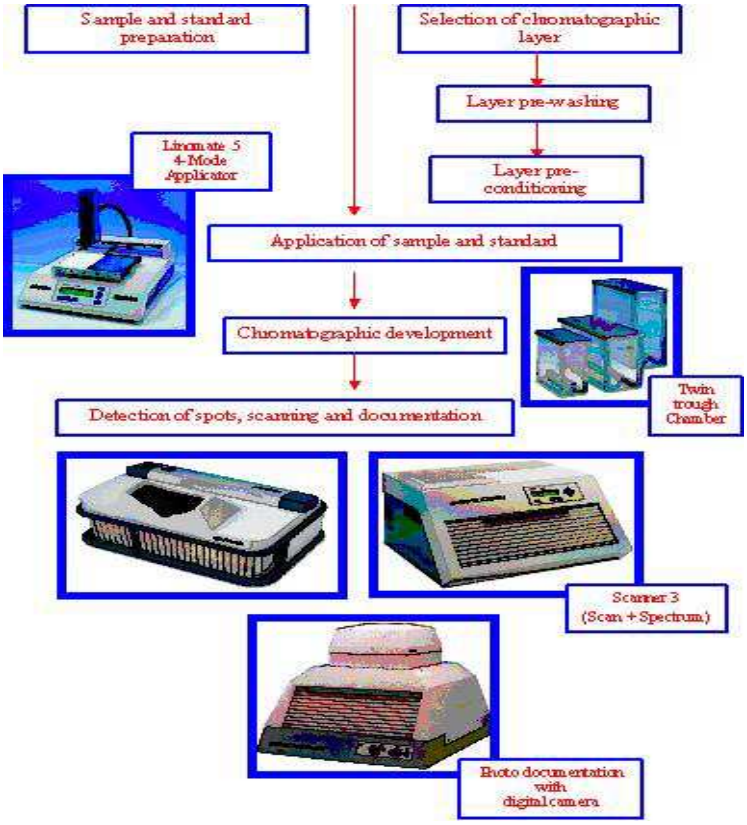
This is seen as zones of chromatographic plates. This may arise due to non-uniformity of mobile phase, longitudinal diffusion between the mobile phase and stationary phase or due to non-equilibrium of stationary phase.

Detection spots

Immediately after the development step is completed. The plates are removed from the chamber and dried to remove traces of mobile phase. Generally, detection can be done by iodine vapour in iodine chamber. Alternatively, detection can be done by visual inspection examination at 254 nm of the ultraviolet region in UV cabinet.

Scanning and documentation

Now day HPTLC equipment are supplied with computer and data recording and staring devices. The development of HPTLC plates are scanned at selected UV region wavelength by the instrument and the detected spots are seen on the computer in the form of peaks. The scanner converts band into peaks and peak height or area is related to the concentration of the substance on the spot.



MATERIALS AND METHODS

Materials and reagents

Tablets used for analysis were ZIX-MR manufactured by Jenburk Pharmaceuticals ltd. Andheri, Mumbai were used for analysis containing THIO 4 mg and ACE 100 mg per tablet. Pure drug sample of THIO, % purity 98.5 and ACE, % purity 99.8 was kindly supplied as a gift sample by Medley Pharmaceuticals Ltd. Baddi, and Curex Pharmaceuticals Jalgaon, India, respectively. These samples were used without further purification. HPTLC precoated plates silica gel 60 F_{254} 20×10 cm, layer thickness 0.2 mm (Merck, Germany). Analytical grade methanol, chloroform was procured by Merck Chemicals (Mumbai, India).

Chromatographic parameters

The mobile phase consisted of methanol: chloroform: water 9.6:0.2:0.2(v/v/v). After developments, the plate was immediately dried with the help of dryer and was observed under CAMAG TLC Visualizer. The well-resolved bands of drugs were scanned at 254 nm with CAMAG TLC scanner III densitometers controlled by WINCAT's software version 4.

Instrumentation

The samples were spotted in the form of bands (8 mm) with a Camag 100 microlitre sample (Hamilton) syringe on silica gel precoated aluminium $60F_{254}$ plates, (10 cm x 10 cm with 250 mm thickness; E. Merck) using a Camag Linomat V sample applicator. A constant application rate of 150 nL s^{-1} was used and the space between two bands was 12 mm. The slit dimension was kept at 6 mm x 0.30 mm and the scanning speed was 20 mm s^{-1}.

The mobile phase consisted of methanol: chloroform: water (9.6: 0.2: 0.2 v/v/v) and 10 ml of mobile phase were used per chromatography run. Linear ascending development was carried out in a 10 cm x 10 cm twin trough glass chamber (Camag) saturated with the mobile phase and pad which is previously soaked in the mobile phase. The optimized chamber saturation time for the mobile phase was 45 min at room temperature (25 °C±2) at a relative humidity of 60%±5. The length of each chromatogram run was 8 cm. Following the development, the TLC plate was dried with the help of hot air drier. The plate was scanned over 85 mm distance. Densitometric scanning was performed using a Camag TLC scanner III in the absorbance mode at 254 nm and operated by win CATS software (V 1.4.4, Camag). The source of radiation used was a deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm.

The retention factors of THIO and ACE were recorded in Densitogram of THIO and ACE are shown in fig. 1.1.

THIO: 0.70±0.05

ACE: 0.83±0.05

Preparation of standard stock solutions

50 mg of each drug THIO and ACE were weighed separately and dissolved in 20 ml of HPLC grade methanol and then the volume was made up to 50 ml so as to get the concentration 1 mg/ml. From each of these solutions, 1 ml of solution was pipette out and transferred to 10 ml volumetric flasks and volume were made up to the mark using methanol so as to get the concentration 100 µg/ml.

Selection of analytical wavelength

From the standard stock solution further dilutions were done using mobile phase and scanned over the range of 200-400 nm and the spectra were overlain. It was observed that both drugs showed considerable absorbance at 254 nm as shown in fig. 1.2

Formulation analysis

Twenty tablets were weighed accurately and a quantity of tablet powder equivalent to 4 mg of THIO and 100 mg of ACE was weighed and dissolved in the 40 ml of methanol with the aid of ultrasonication for 10 min and the solution was filtered through Whatman paper No. 41 into a 50 ml volumetric flask. The filter paper was washed with methanol, adding washings to the volumetric flask and volume was made up to the mark with methanol. From the filtrate, appropriate dilution was prepared in the mobile phase to get a solution of 4 µg/ml of THIO and 100 µg/ml of ACE. These solutions were spotted keeping an appropriate distance between spots. The results obtained are shown in table 2.1.

Brand: ZIX-MR

Contents: Thiocolchicoside-4 mg

Aceclofenac-100 mg

Manufacturer: JENBURK PHARMACEUTICALS Ltd.

Method development

Method validation

As per the ICH guidelines, the method validation parameters checked were linearity, accuracy, precision, limit of detection, limit of quantitation and robustness and specificity.

Linearity

Stock standard solution was prepared by dissolving 50 mg of THIO and 50 mg of ACE in 50 ml methanol (1000µg/ml). Suitable dilutions using mobile phase were made from the standard stock solution containing 4 µg/ml of THIO and 100 µg/ml of ACE. From this stock solution, THIO and ACE were spotted on the TLC plate to obtain final concentration 30-180 ng/band and 750-4500 ng/band for THIO and ACE, respectively. Each concentration was spotted 3 times on the TLC plate. The plate was developed on the mobile phase.

Accuracy

The accuracy of the assay method was evaluated with the recovery of the standards from excipients. Recovery studies were carried out by applying the method to drug content present in tablet dosage form to which known amount of mix standard of THIO and ACE was added at 50 %, 100 % and 150 % levels. The technique involves the addition of standard drug solution to pre-analysed sample solution. From these solutions, appropriate volumes were applied as a band and the area was noted after the development of plate. At each of the levels, three determinations were performed and results were obtained.

Precision

The precision of the method was demonstrated by Intra-day and inter-day variation studies. In the intra-day studies, 3 repeated measurements of standard and sample solutions were made in a day and percentage RSD were calculated. In the inter-day variation studies, 3 repeated measurements of standard and sample solutions were made on 3 consecutive days and percentage RSD were calculated.

Limit of detection and limit of quantification

The Limit of Detection (LOD) is the smallest concentration of the analyte that gives the measurable response and Limit of Quantification (LOQ) is the smallest concentration of the analyte, which gives a response that can be accurately quantified. LOD and LOQ were calculated using the following formula:

$LOD = (3.3 \times \sigma)/b$

$LOQ = (10 \times \sigma)/b$

Where σ = Standard deviation of the response

b = Slope of the calibration curve

Robustness

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition like methanol: chloroform: water (9.6:0.2:0.2 v/v/v), (9.4:0.4:0.2 v/v/v), (9.5:0.2:0.3 v/v/v/v) were tried and chromatograms were run. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20, 40 and 60 min. Robustness of the method was done at three different concentration levels 30, 60, 90 ng per band and 750, 1500, 2250 ng per band for THIO and ACE, respectively. Robustness of the method was determined by carrying out the analysis under conditions during which mobile phase ratio and ambient temperature were altered and the changes on the R_f values were noted.

Specificity

The specificity of the method was ascertained by overlaying UV spectra of spots for standard drug and sample.

Method optimization

The TLC procedure was optimized in view to develop a simultaneous assay method for THIO and ACE. The mixed standard stock solution was spotted onto TLC plates and run in different solvent systems.

Initially, solvents like toluene, chloroform and methanol were tried in different ratios. Based on the results of these initial trials, toluene, chloroform and methanol in the ratio (9.4: 0.4: 0.2) was selected where THIO and ACE were poorly resolved and RF values were also less. To increase the resolution between THIO and ACE, toluene was replaced with water, then methanol, chloroform and water in the ratio (9.6: 0.2: 0.2) were used. In this mobile phase, THIO and ACE were well resolved and RF values were good. Finally, the optimum mobile phase consisted of Methanol: chloroform: water in the ratio of (9.6: 0.2: 0.2 v/v/v) was chosen as the mobile phase for analysis. Other chromatographic conditions like chamber saturation time, run length, sample application rate and volume, sample application positions, the distance between tracks, detection wavelength, were optimized to give reproducible R_f values, better resolution, and symmetrical peak shape for the two drugs. Densitometry scanning was performed at 254 nm for the detection of THIO and ACE with R_f value of 0.70 and 0.83 respectively. Well-defined spots of standards were obtained without chamber saturation.

RESULTS AND DISCUSSION

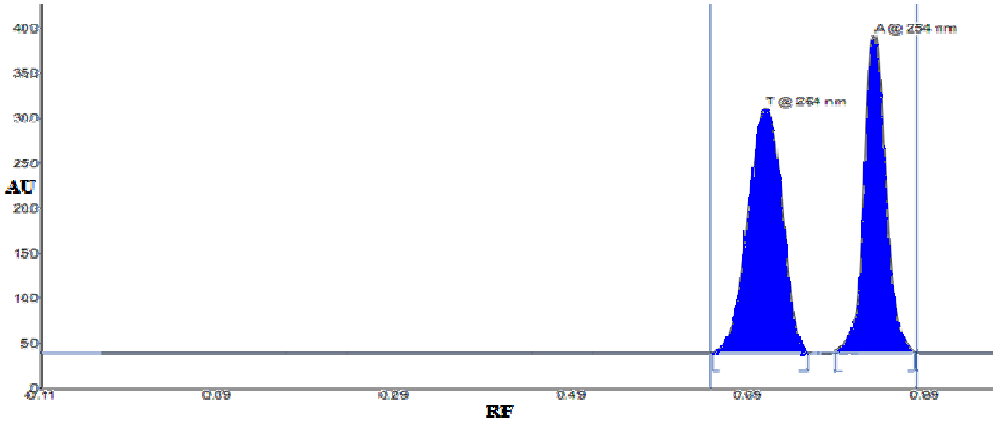


Fig. 1.1: Densitogram of THIO (4 µg/ml) and ACE (100 µg/ml)

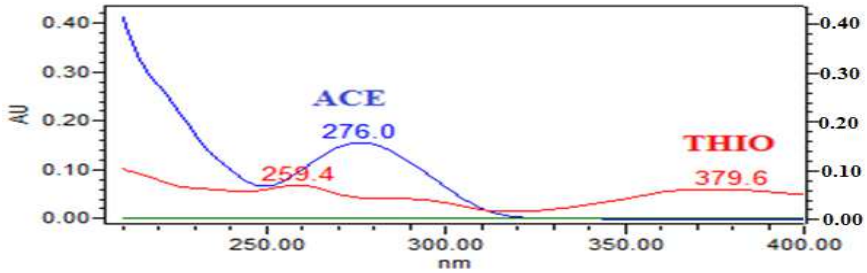


Fig. 1.2: Online overlain spectra of THIO (4 µg/ml) and ACE (100 µg/ml)

Table 2.1: Analysis of tablet formulation

S. No.	Label claim (µg/ml)		Amount found (µg/ml)		% of label claim		Peak purity	
	THIO	ACE	THIO	ACE	THIO	ACE	r(S,M)	r(M,E)
1.	4	100	4.03	99.96	98.87	99.41	0.9991	0.9997
2.	4	100	3.97	98.89	99.55	99.47	0.9996	0.9999
3.	4	100	4.05	99.85	100.02	99.54	0.9992	0.9996
4.	4	100	3.99	100.01	99.92	100.01	0.9995	0.9994
5.	4	100	3.98	99.98	99.65	99.91	0.9994	0.9992
6.	4	100	4.01	98.87	99.78	99.45	0.9998	0.9993
Mean			4.005	99.5933				
SD			0.028	0.506				
%RSD			0.702	0.508				

Method validation

Linearity

Linearity of the method was studied by spotting six concentrations of the drug prepared in the mobile phase in the range of 30-180 ng/band and 750-4500 ng/band for THIO and ACE. The results obtained are shown in table 3.1 and 3.2 the peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

Precision

The intra-day precision of the developed TLC method was determined by preparing the tablet samples of the same batch in nine determinations with three concentrations and three replicate each on the same day. The inter-day precision was also determined by assaying the tablets in triplicate per day for consecutive 3 d. The result obtained for intraday and Inter-day variations are shown for THIO and ACE in table 3.3 and 3.4, respectively.

Accuracy

The accuracy of the assay method was evaluated with the recovery of the standards from excipients. The mean percentage recoveries obtained for THIO and ACE were 99.34% and 99.08%, respectively, reported in table 3.6 and 3.7.

Table 3.1: Linearity of THIO (n=6)

Standard concentrations	60 ng	90 ng	120 ng	150 ng	180 ng	
Replicates	Peak area					
1	1584	3176	4524	5930	7429	9080
2	1582	3178	4628	5929	7418	9087
3	1579	3198	4528	6016	7437	9198
4	1568	3245	4537	5998	7558	9295
5	1588	3179	4578	5986	7587	9195
6	1559	3180	4525	5928	7477	9058
Mean	1576.66	3192.66	4553.33	5964.5	7484.33	9152.16
SD	10.02774	24.51983	38.19977	36.55931	65.47688	84.31966
% RSD	0.636009	0.768005	0.838941	0.612948	0.874853	0.921308

Table 3.2: Linearity of ACE (n=6)

Standard concentrations	750 ng	1500 ng	2250 ng	3000 ng	3750 ng	4500 ng
Replicates	Peak area					
1	4150	8428	12750	16264	20750	25400
2	4251	8429	12876	16397	20855	26549
3	4149	8526	12987	16487	20987	25421
4	4148	8578	12869	16589	21758	25406
5	4157	8527	12967	16499	20765	25408
6	4147	8446	12867	16264	20745	25466
Mean	4167	8489	12886	16416.67	20976.67	25608.33
SD	37.705	57.5963	77.46827	121.3983	359.4536	421.2445
% RSD	0.904847	0.678482	0.601182	0.739482	1.713588	1.644951

Regression equation: Y = 5589x+49.33, r = 0.9993

Table 3.3: Intraday and Inter-day precision of THIO (n=3)

THIO	Measured concentration (ng/spot), % RSD	
Conc. (ng/spot)	Intra day	Inter day
30	30.07, 0.52	30.81, 0.87
60	60.91,0.63	60.04, 0.56
90	89.91, 1.25	29.14, 0.74

Table 3.4: Intraday and Inter day precision of ACE (n=3)

ACE	Measured concentration (ng/spot), % RSD	
Conc. (ng/spot)	Intra day	Inter day
750	750.88, 0.79	749.05, 0.26
1500	1499.86, 1.20	1498.13, 1.41
2250	2245.14, 1.19	2248.01, 0.36

Table 3.5: Recovery studies of THIO

Thiocolchicoside	Densitometric peak area		
	Level of recovery		
	50 %	100 %	150 %
	30ng/spot	60 ng/spot	90 ng/spot
Replicate 1	1584	3176	4524
Replicate 2	1599	3245	4587
Replicate 3	1613	3189	4528
Mean	1598.667	3203.333	4546.333
SD	11.84155	29.93697	28.80201
% RSD	0.740714	0.934557	0.633552
Mean conc. found (ng/ml)	29.12	61.08	90.01
Mean % Recovery	99.58	99.98	99.45

Table 3.6: Recovery studies of ACE

Aceclofenac	Densitometric peak area		
	Level of recovery		
	50 %	100 %	150 %
	750 ng/spot	1500 ng/spot	2250 ng/spot
Replicate 1	4150	8428	12750
Replicate 2	4196	8487	12898
Replicate 3	4199	8567	12748
Mean	4181.667	8494	12798.67
SD	22.42518	56.96198	70.24402
% RSD	0.536274	0.670614	0.548839
Mean conc. found (ng/ml)	750.09	1499.19	2249.51
Mean % recovery	98.84	99.19	100.01

Limit of detection (LOD)

THIO: 10 ng/spot

ACE: 250 ng/spot

Limit of quantification (LOQ)

THIO: 30 ng/spot

ACE: 750 ng/spot

Range

THIO: 30-180 ng/spot

ACE: 750-4500 ng/spot

Specificity

The peak purity of both drugs was assessed by comparing the respective spectra of standard drugs and samples at peak start, peak apex and peak end positions of the spot. A blend of commonly used tablet excipients was treated as per developed procedure and the chromatogram shows no inferring peaks at retention time of the two drugs.

Robustness

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition like methanol: chloroform: water (9.6: 0.2: 0.2 v/v/v), (9.4:0.4:0.2 v/v/v), (9.5:0.2:0.3 v/v/v/v) were tried and chromatograms were run. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20, 40 and 60 min. Robustness of the method was done at three different concentration levels 30, 60, 90 ng per band and 750, 1500, 2250 ng per band for THIO and ACE, respectively. Robustness of the method was determined by carrying out the analysis under conditions during which mobile phase ratio and ambient temperature were altered and the changes on the RF values were noted. The standard deviation of peak areas was calculated for each parameter and % RSD was found to be less than 2 %. Results of robustness study are presented in table 3.7.

Table 3.7: Robustness study of THIO and ACE (n = 3)

Parameter	SD of peak area		% RSD	
	THIO	ACE	THIO	ACE
Mobile phase composition	7.64	6.97	0.86	0.24
Amount of mobile phase	20.87	8.98	1.08	0.83
Time from spotting to chromatography	15.21	18.03	0.57	1.23
Time from chromatography to scanning	6.89	24.11	0.85	0.77
Plate from different lot no.	4.90	8.69	0.46	1.49

CONCLUSION

HPTLC, with its advantage of low operating cost, high sample thought and minimum sample preparation need is now days preferred as routine analytical techniques for control and assurance. The validated HPTLC method employed here proved to be simple, fast, accurate, precise and sensitive, thus can be used for routine analysis of Thiocolchicoside and Aceclofenac in tablet dosage form.

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CONFLICT OF INTERESTS

Declare none

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