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Research Article

The Development and Validation of HPTLC Method for the Simultaneous Estimation of Thiocolchicoside and Aceclofenac in Pharmaceutical Dosage Form

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The review of the literature revealed that there is no HPTLC method available for determination of Thiocolchicoside and Aceclofenac simultaneously. A simple, specific and accurate reversed phase HPTLC method was developed for this combination. The samples were spotted in the form of bands (8 mm) with a Camag 100 microlitre sample (Hamilton) syringe on silica gel precoated aluminum $60F_{254}$ 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 was used per chromatography run. Linear ascending development was carried out in a 10cm x 10 cm twin trough glass chamber (Camag) saturated with the mobile phase and pad which is previously soaked in mobile phase. The optimized chamber saturation time for the mobile phase was 45 min at room temperature (25 ^{0}C ± 2) at 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 Thiocolchicoside and Aceclofenac were 0.70 ± 0.05 and 0.83 ± 0.05 respectively. 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 Thiocolchicoside and Aceclofenac respectively. The percentage recoveries were found to be in the range of 50-150%. This method can be successfully employed for the quantitative analysis of Thiocolchicoside and Aceclofenac in pharmaceutical dosage form.

Keywords: Thiocolchicoside, Aceclofenac, HPTLC, simultaneously, pharmaceutical dosage form.

INTRODUCTION

Literature survey reveals that few HPLC and UV Spectroscopic methods are reported for the estimation of Thiocolchicoside(THIO) and Aceclofenac (ACE) individually or in combination with other drugs as bulk and in pharmaceutical formulations as described previously in the

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literature survey. Recently to our knowledge optimized RP-HPLC method for the routine quality control analysis of THIO and ACE simultaneously from tabletsare now reported. The review of the literature revealed that there is no HPTLC method available for determination of this combination. Therefore 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. The method was validated according to ICH guidelines

MATERIALS AND METHODS

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 99.78) and ACE (% purity 99.80) was kindly supplied as a gift sample by Bari, Brahmana Pharmaceuticals, Jammu 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 0.25 mm (Merck, Germany). Analytical grade methanol and chloroform were procured from Merck Chemicals (Mumbai, India).

Instrumentation and Chromatographic parameters

The mobile phase consisted of methanol: chloroform: water 9.6:0.2:0.2(v/v/v). After developments, 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 densitometer controlled by WINCAT's software version 4.

The samples were spotted in the form of bands (8 mm) with a Camag 100 microlitre sample (Hamilton) syringe on silica gel precoated aluminum 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 was used per chromatography run. Linear ascending development was carried out in a 10cm x 10 cm twin trough glass chamber (Camag) saturated with the mobile phase and pad which is previously soaked in mobile phase. The optimized chamber saturation time for the mobile phase was 45 min at room temperature (25 ^{0}C ± 2) at 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 Aceclofenac were 0.70 ± 0.05 and 0.83 ± 0.05 respectively. Densitogram of THIO and ACE are shown in Fig.1.

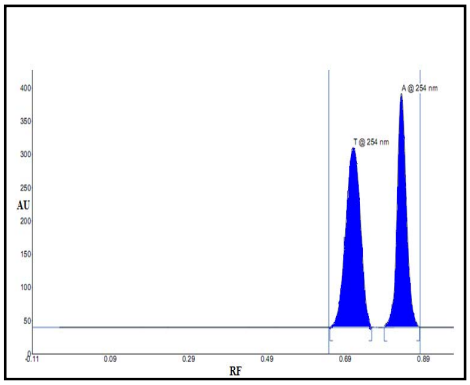

Fig. 1 Densitogram of THIO (4 µg/ml) and ACE (100 µg/ml)

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 volume was made up to 50 ml so as to get the concentration 1 mg/ml. From each of these solutions 1ml of solution was pipette out and transferred to 10 ml volumetric flasks and volume was 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.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 solution was filtered through Whatman paper No. 41 into a 50 ml volumetric flask. 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 mobile phase to get a solution of 4 µg/ml of THIO and 100 µg/ml of ACE. These solutions were spotted keeping appropriate distance between spots. The results obtained are shown in Table 1.

Brand : ZIX-MR

Contents : Thiocolchicoside - 4 mg
Aceclofenac - 100 mg

Manufacturer : Jenburk Pharma. Ltd.

Method validation

As per the ICH guidelines, the method validation parameters checked were linearity, accuracy, precision, LOD and LOQ, robustness and specificity.

Linearity:

Stock standard solution was prepared separately 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 was 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.

Table 1: Analysis of tablet formulation

Sr. 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				

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 addition of standard drug solution to preanalysed sample solution. From these solutions appropriate volumes were applied as band and the area was noted after 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.

Table 2: Linearity of THIO (n=6)

Standard⇨ Concentrations	30 ng	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

Regression equation: Y = 4918x +122.66, r = 0.9995

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 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 minutes. 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.

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.

Table 3: 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

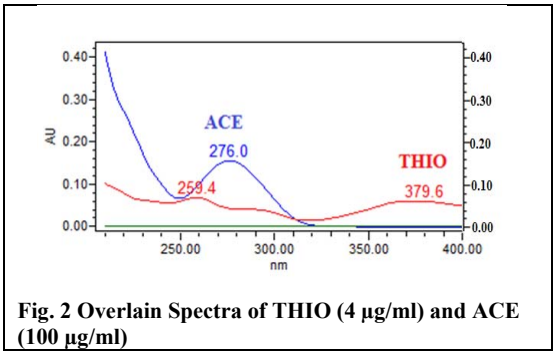

Fig. 2 Overlain Spectra of THIO (4 µg/ml) and ACE (100 µg/ml)

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 R_f values were also less. To increase the resolution between Thiocolchicoside 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 R_f 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, 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

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 correlation coefficient ('r') values were >0.999(n = 6). Typically, the regression equations for the calibration curve were found to be y =4918x +122.66 for THIO, y=5589x + 49.33for ACE. The results are shown in Table 2 and 3.

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 same day. The inter-day precision was also determined by assaying the tablets in triplicate per day for consecutive 3 days. The result obtained for intraday and Inter day variations are shown for THIO and ACE in Table 4 and 5, respectively.

Table 4: Intraday and Inter day precision of THIO (n=3).

THIO	Measured concentration (ng/ spot), % R.S.D	
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 5: Intraday and Inter day precision of ACE (n=3).

ACE	Measured concentration (ng/ spot), % R.S.D	
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

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 99.67% and 99.68%, respectively, reported in Table 6 and 7.

Limit of Detection (LOD)

THIO : 10 ng/ spot

ACE : 250 ng/ spot

Limit of Quantification (LOQ)

THIO : 30 ng/ spot, ACE : 750 ng/ spot

Table 6: 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 7: 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	99.84	99.19	100.01

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 thechromatogram shows no interfering peaks at retention time of the two drugs.

Robustness

Robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatograms, which

Table 8: 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

demonstrated that the RP-HPLC method developed, and System suitability parameters were found to be within acceptable limits. Results were shown in Table 8 indicating that the test method was robust for all variable conditions.

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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