Bulletin of Pharmaceutical Research 2014;4(2):94-9
An Official Publication of Association of Pharmacy Professionals
ISSN: 2249-6041 (Print); ISSN: 2249-9245 (Online)

RESEARCH ARTICLE

HPTLC METHOD FOR SIMULTANEOUS QUANTITATION OF THIOCOLCHICOSIDE, PARACETAMOL AND ACECLOFENAC IN BULK DRUG AND FORMULATION

Jyoti Shrivastava[1]*, K. R. Mahadik[2] and Sunil Dhaneshwar[2]

[1]*Department of Pharmaceutical Chemistry, Sagar Institute of Research and Technology - Pharmacy, Bhopal, Madhya Pradesh, India*
[2]*Department of Pharmaceutical Chemistry, Poona College of Pharmacy, Bharati Vidyapeeth University, Pune, Maharashtra, India.*

**E-mail*: jshrivastava20@gmail.com
Tel.: +91 9584358258.

Received: December 24, 2013 / Revised: August 10, 2014 / Accepted: August 11, 2014

A simple, precise and accurate HPTLC method was developed for the simultaneous estimation of thiocolchicoside (THIO), paracetamol (PAR), and aceclofenac (ACF) for the bulk drugs and their combined tablet dosage form. The method involved TLC on aluminum plates precoated with silica gel using toluene:acetone:methanol:formic acid 8:2:2:1 *(v/v/v/v)* as mobile phase. Densitometric scanning was performed at 263 nm. The method was validated as per ICH guidelines. R_f values of 0.13±0.03, 0.42±0.04 and 0.57±0.02 were obtained for THIO, PAR and ACF respectively. Precision, accuracy and specificity were in accordance with ICH guidelines.

Key words: HPTLC, Densitometry, Validation, Thiocolchicoside, Paracetamol, Aceclofenac.

INTRODUCTION

A combination of thiocolchicoside (THIO), paracetamol (PAR) and aceclofenac (ACF) is used in the treatment of musculoskeletal disorders. THIO allosterically inhibit strychnine sensitive glycine receptor in brain stem and spinal cord, and this may provide a possible mechanism for the myorelaxant activity. THIO has high affinity for [^{3}H] strychnine binding sites (Balduini *et al* 1999; Cimino *et al* 1996). PAR (*p*-hydroxy acetanilide) has analgesic and antipyretic effects. The mechanism of action of PAR is inhibition of the cyclooxygenase enzyme and the prostaglandin synthesis in the central nervous system (Graham and Schott, 2005) and its direct activity on the centre for the body temperature regulation in the hypothalamus (Dollery, 1999). ACF inhibits the synthesis of inflammatory cytokines interleukin (IL)-1, tumor necrosis factor and prostaglandin E_2 (PGE_2) production which is responsible for its anti-inflammatory and analgesic effects.

Analytical techniques have been remained as reliable methods for estimation of drugs alone or in combination (Shrivastava *et al* 2011; Basaveswara Rao *et al* 2012; Singh *et al* 2013; Singh and Dahiya, 2014). In literature, few analytical methods are described for determination of THIO like HPLC (Rosso and Zuccaro, 1998; Vargas *et al* 2001), LC-MS method for quantitation in human plasma (Ferrari, 2001; Sutherland *et al* 2002) and HPTLC method for quantitation.

To date, there have been no published reports about the simultaneous quantitation of THIO, PAR, and ACF by HPTLC in bulk drug and in pharmaceutical dosage forms. Keeping in mind advantages of HPTLC method in pharmaceutical analysis, an attempt was made to develop and validate simultaneous method for the quantitation of THIO, PAR, and ACF by HPTLC in bulk drug and in pharmaceutical dosage forms

for the first time. The proposed method was validated as per ICH guidelines (ICH, Q2 (R1)).

EXPERIMENTAL

Materials

Working standards of pharmaceutical grade THIO (Batch no. 077101805008), ACF (Batch no. 18060131) and PAR (Batch no. 260738) were obtained as generous gift samples from Alkem International Limited (Haryana, India), Suyash Labs (Thane, Maharashtra, India) and Bal Pharmaceuticals Limited (Pune, Maharashtra, India) respectively. They were used without further purification and certified to contain 99.9% (*w/w*), 99-101% (*w/w*) and 99.80% (*w/w*) on dry weight basis of THIO, ACF and PAR respectively. Fixed dose combination tablets (ACENAC-MR, Batch no. 90AT 109) containing 4 mg of THIO, 500 mg of PAR and 100 mg of ACF were procured from Medley Pharmaceutical Limited, Mumbai, India. All chemicals and reagents of analytical grade were purchased from Merck Chemicals, Mumbai, India.

Chromatographic conditions

The samples were spotted in the form of bands of width 6 mm with a Camag 100 μl sample syringe (Hamilton, Bonaduz, Switzerland) on silica gel precoated aluminum plate 60 F_{254} plates, [10 cm × 10 cm with 250 μm thickness; E. Merck, Darmstadt, Germany] using a Camag Linomat V (Switzerland) sample applicator. The plates were prewashed with methanol and activated at 110^{o}C for 5 min prior to chromatography. A constant application rate of 0.1 μl s^{-1} was used and the space between two bands was 6 mm. The slit dimension was kept at 5 mm × 0.45 mm and the scanning speed was 10 mm s^{-1}. The monochromator bandwidth was set at 20 nm, each track was scanned three times and baseline correction was used. The mobile phase consisted of toluene:acetone:methanol: formic acid (8:2:2:1, *v/v/v/v*) and 15 ml of mobile phase was used per chromatographic run. Linear ascending development was carried out in a 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25^{o}C ± 2) at relative humidity of 60% ± 5. The length of each chromatogram run was 8 cm. Following the development, the TLC plates were dried in a current of air with the help of an air dryer in a wooden chamber with adequate ventilation. The flow of air in laboratory was maintained unidirectional (laminar flow, towards the exhaust). Densitometric scanning was performed using a Camag TLC scanner III in the reflectance-absorbance mode at 263 nm and operated by CATS software (V 3.15, Camag). The source of radiation used was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compound chromatographed were determined from the intensity of the diffused light. Evaluation was by peak areas with linear regression.

Preparation of standard stock solutions

Standard solution was prepared by dissolving 2 mg of THIO in 50 ml methanol, 50 mg of ACF in 50 ml methanol and 250 mg of PAR in 50 ml methanol separately in different volumetric flask. From the standard stock solution, the mixed standard solution was prepared using the methanol to contain 40 ng μl^{-1} of THIO, 100 ng μl^{-1} of ACF and 500 ng μl^{-1} of PAR. The stock solution was stored at 2-8 °C protected from light.

Optimization of the HPTLC method

The development of simultaneous assay method for the combination of THIO, ACF and PAR was very critical because of different polarities of these drugs. THIO was polar in nature whereas ACF and PAR were non-polar in nature and their polarity index was closely similar. Hence, the mixed standard stock solution (40 ng μl^{-1} of THIO, 100 ng μl^{-1} of ACF and 500 ng μl^{-1} of PAR) was spotted on to HPTLC plates and run in different solvent systems. Out of various solvent systems tried, the mobile phase consisting of toluene:acetone:methanol:formic acid in the ratio of 8:2:1:1, *v/v/v/v* was found to be optimum. The densitometric scanning was done at wavelength 263 nm and graphical data is represented in **Figure 1**.

Validation of the method

Validation of the optimized HPTLC method was carried out with respect to the following parameters:

Linearity range

The mixed standard stock solution was further diluted to get 40 ng μl^{-1} of THIO, 100 ng μl^{-1} of ACF and 500 ng μl^{-1} of PAR. From diluted mixed standard stock solution, 2 to 10 μl solutions were spotted on HPTLC plate to obtain final concentration of 80-400 ng spot^{-1} for THIO,

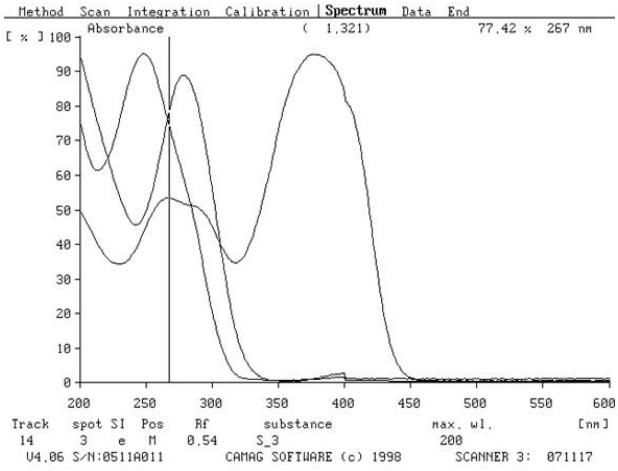

Fig. 1. Densitometric scanning graph using various mobile phases

1000-5000 ng spot^{-1} for PAR and 200-1000 ng spot^{-1} for ACF. Each concentration was applied six times on the HPTLC plate. The plate was then developed using the previously described mobile phase and the peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations (80, 240, 400 ng spot^{-1} for THIO, 1000, 3000, 5000 ng spot^{-1} for PAR and 200, 600 and 1000 ng spot^{-1} for ACF) six times on the same day. The intermediate precision of the method was checked by repeating studies on three different days.

Limit of detection and quantitation

Limits of detection (LOD) and limit of quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratio of 3 for LOD and 10 for LOQ, respectively. LOD and LOQ were determined by measuring the magnitude of analytical background by spotting a blank and calculating the signal-to-noise ratio for THIO, PAR and ACF by spotting a series of solutions until the signal-to-noise ratio of 3 for LOD and 10 for LOQ. To determine the LOD and LOQ, serial dilutions of mixed standard solution of THIO, PAR and ACF was made from the standard stock solution in the range of 10–100 ng spot^{-1}. The samples were applied to HPTLC plate and the chromatograms were run and measured signal from the samples was compared with those of blank samples.

Robustness

Following the introduction of small changes in the mobile phase composition (±0.1 ml for each component), the effects on the results was examined. The amount of mobile phase was varied over the range of ±5%. The plates were prewashed with methanol and activated at 110°C for 2, 5, and 7 min respectively prior to chromatography. The time from spotting to chromatography and from chromatography to scanning was varied by ±10 min. The robustness of the method was determined at three different concentration levels 80, 240, 400 ng spot^{-1} for THIO, 1000, 3000, 5000 ng spot^{-1} for PAR and 200, 600 and 1000 ng spot^{-1} for ACF.

Specificity

The specificity of the method was determined by analyzing standard and drug samples. The spot for THIO, PAR and ACF in the samples was confirmed by comparing the R_f and spectrum of the spot with that of standard. The peak purity of THIO, PAR and ACF was determined by comparing the spectrum at three different regions of the spot *i.e.* peak start (S), peak apex (M) and peak end (E).

Accuracy

Accuracy of the method was carried out by adding known amount of THIO, PAR and ACF standard drug corresponding to 50, 100 and 150% of label claim to the powdered formulation. The mixture was sonicated for 30 min, filtered and analyzed by running chromatogram in optimized mobile phase.

Analysis of marketed formulation

To determine the content of THIO, PAR and ACF in conventional tablet (Brand name: Acenac MR, Label claim: 4 mg of THIO, 100 mg of ACF and 500 mg of PAR per tablet), twenty tablets were weighed, their mean weight determined and finely powdered. The weight of the tablet triturate equivalent to 4 mg of THIO, 100 mg of ACF and 500 mg of PAR was transferred into a 50 ml volumetric flask containing 5 ml methanol, sonicated for 30 min and diluted to 50 ml with methanol. The resulting solution was centrifuged at 3000 rpm for 5 min and the drug content of the supernatant was determined. Then 1 µl of this solution was applied to a HPTLC plate which was developed in optimized mobile phase. The analysis was repeated in triplicate. The possibility of excipients interference with the analysis was examined.

RESULTS AND DISCUSSION

The results of validation studies on simultaneous estimation method developed for THIO, PAR and ACF in the current study involving toluene:acetone:methanol:formic acid (8:2:2:1, *v/v/v/v*) as the mobile phase for HPTLC are given below.

Linearity

The Linear regression data (n=6) showed a good linear relationship over a concentration range of 80-400 ng spot^{-1} (r^2 ± S.D. = 0.9999 ± 0.966), 1000-5000 ng spot^{-1} (r^2 ± S.D. = 0.9975 ± 0.671) and 200-1000 ng spot^{-1} (r^2 ± S.D. = 0.999 ± 0.693) for THIO, PAR and ACF respectively (**Table1**).

Precision

The results of the repeatability and intermediate precision experiments are shown in **Table 2**. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were < 2 %, respectively as recommended by ICH guidelines.

Table 1. Linear regression data for calibration curves

Parameters	THIO	PAR	ACF
Linearity range (ng spot^{-1})	80-400	1000-5000	200-1000
r^2 ± S.D.	0.999 ± 0.966	0.997 ± 0.671	0.999 ± 0.693
Slope ± S.D.	8.483 ± 0.427	2.359 ± 0.899	4.486 ± 1.421
Intercept ± S.D	684.296 ± 0.528	8025.501± 0.994	767.521 ± 0.539

* *n= 6*

Table 2. Determination of precision studies

Concentration (ng spot^{-1})	Repeatability		Intermediate precision	
	Measured	RSD (%)	Measured	RSD (%)
	Concentration ± SD		Concentration ± SD	
For THIO				
160	79.531 ± 0.491	0.617	80.670 ± 0.028	0.034
480	242.080 ± 0.758	0.313	237.866 ± 1.127	0.473
800	397.881 ± 0.944	0.237	406.141 ± 0.791	0.194
For PAR				
1000	1010.011 ± 1.035	0.102	980.890 ± 0.934	0.095
3000	3010.021 ± 1.120	0.037	2997.233 ± 0.495	0.016
5000	4963.199 ± 0.993	0.020	4954.681±16.893	0.340
For ACF				
200	199.661 ± 0.468	0.234	197.433 ± 0.979	0.495
600	594.302 ± 0.521	0.087	607.927 ± 1.004	0.165
1000	989.171 ± 0.773	0.078	990.066 ± 1.014	0.102

* *n= 6*

LOD and LOQ

The signal to noise ratios of 3:1 and 10:1 were considered as LOD and LOQ respectively. The limit of detection (LOD) and the limit of quantitation (LOQ) were found to be 5 ng spot^{-1} and 16.66 ng spot^{-1} for THIO, 375.53 ng spot^{-1} and 1251.78 ng spot^{-1} for PAR and 58.70 ng spot^{-1} and 195.68 ng spot^{-1} for ACF respectively.

Robustness

The standard deviation of peak areas was calculated for each parameter and the % RSD was found to be less than 2 %. The low values of the % RSD, as shown in **Table 3**, indicated robustness of the method.

Specificity

The peak purity of THIO, PAR and ACF was assessed by comparing their respective spectra at the peak start, apex and peak end positions of the spot *i.e.* r (S, M) = 0.9998, r (M, E) = 0.9962 for THIO, r (S, M) = 0.9984, r (M, E) = 0.9995 for PAR, r (S, M) = 0.9984, r (M, E) = 0.9974 for ACF. A good correlation (r^2 = 0.9999, r^2 = 0.9975 and

Table 3. Results of robustness

Parameter	SD of peak area	% RSD	SD of peak area	% RSD	SD of peak area	% RSD
	for ACF		**for THIO**		**for PAR**	
Mobile phase composition (formic acid ± 0.1 ml)	1.370	0.481	2.560	0.394	1.873	0.213
Amount of mobile phase (± 5%)	1.531	0.722	2.522	0.447	1.990	0.596
Time from spotting to chromatography (± 10 min)	2.960	0.185	2.211	0.201	1.431	0.117
Time from chromatography to scanning (± 10 min)	2.751	0.174	3.170	0.285	2.851	0.163

*$n = 6$

r^2 =0.999) was also obtained between standard and sample spectra of THIO, PAR and ACF.

Accuracy

Recovery studies of the drugs were carried out at three levels *i.e.* multiple level. Sample solution from tablet formulation was prepared. To the sample solutions, 50%, 100% and 150% of the standard drug solutions were added. Dilutions were made and the recovery studies were performed (**Table 4**).

Analysis of formulation

Experimental results of the amount of THIO, PAR, and ACF in tablets, expressed as a percentage of label claim were in good agreement with the label claims thereby suggesting that there is no interference from any of the excipients which are normally present in tablets (**Figure 2**).

Table 4. Results of recovery studies

Drugs	Label claim (mg tab^{-1})	Amount added (%)	Total amount (mg)	Actual concentration taken (ng spot^{-1})	Calculated conc. ± SD	RSD (%)	Recovery (%)
THIO	4	50	6	160	158.801 ± 1.740	1.095	98.751
		100	8	480	477.331 ± 0.751	0.157	99.433
		150	10	800	797.541 ± 1.222	0.152	99.682
PARA	500	50	750	1000	993.700 ± 1.999	0.195	99.374
		100	1000	3000	2885.699 ± 1.599	0.055	96.181
		150	1250	5000	4884.371 ± 2.121	0.043	97.688
ACF	100	50	150	200	196.444 ± 0.249	0.126	98.225
		100	200	600	588.233 ± 0.751	0.127	98.030
		150	250	1000	992.110 ± 1.333	0.134	99.211

*$n = 6$

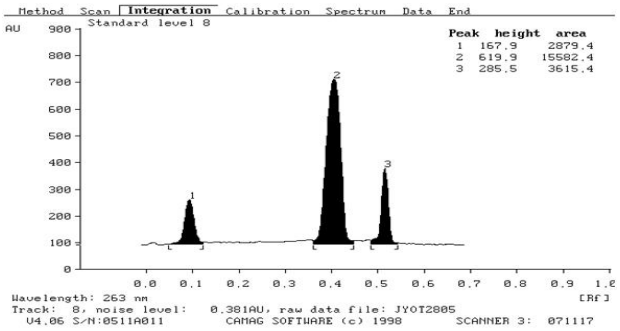

Figure 2. Analysis of Formulation

The drug content was found to be 99.4 % for THIO, 102.0% for PAR and 100.01% for ACF. Two different lots of THIO, PAR, and ACF combination tablets were analyzed using the proposed procedures.

CONCLUSION

The proposed HPTLC technique is simple, precise, specific and accurate. Statistical analysis proved that the method is suitable for the analysis of THIO, PAR, and ACF as bulk drug and

in pharmaceutical formulation without any interference from the excipients. It may be extended to study the degradation kinetics of THIO, PAR, and ACF and also for its estimation in plasma and other biological fluids. The proposed HPTLC method is less expensive, simpler, rapid, and more flexible than HPLC.

ACKNOWLEDGEMENT

The authors would like to thank the Principal of Poona College of Pharmacy, Pune, India for providing necessary facilities, and Mr L. Sathiyanarayanan for his help during the work. The authors also extend thank to AICTE for providing financial assistance.

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