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SIMULTANEOUS ESTIMATION OF PARACETAMOL, DICLOFENAC SODIUM AND DICYCLOMINE HYDROCHLORIDE IN THEIR TABLET DOSAGE FORM USING DERIVATIZATION METHOD

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Abstract: The study describes method development and subsequent validation of HPTLC method for simultaneous estimation of paracetamol (PCM), diclofenac sodium (DCL) and dicyclomine hydrochloride (DICY) in marketed combined tablet dosage form. The drugs were extracted from marketed tablet dosage form: Spasmodart. The separation was carried out on aluminium sheet precoated with silica gel 60 F_{254} using toluene: ethylacetate: methanol: formic acid (5.5:3.5:2:0.02, v/v/v/v) as a mobile phase. The spots were scanned densitometrically at 266 nm wavelength for paracetamol and diclofenac sodium. The plate was then dipped in p-anisaldehyde reagent and spots of dicyclomine hydrochloride were scanned at 638 nm wavelength. The R_f values of paracetamol, diclofenac sodium and dicyclomine hydrochloride were found to be 0.41, 0.71and 0.24 respectively. Calibration curves were linear in range of 3250-9750ng/spot, 500-1500ng/spot and 200-600ng/spot for PCM, DCL and DICY respectively. The limit of detection (LOD) for paracetamol, diclofenac sodium and dicyclomine hydrochloride were found to be 812.5, 125 and 50ng/spot respectively. The suitability of this method for quantitative determination of compounds was proved by validation in accordance with ICH Guidelines. Therefore this method can be applied for routine analysis of these drugs in tablet formulation.

Keywords: HPTLC method, paracetamol, diclofenac sodium, dicyclomine hydrochloride, p-anisaldehyde

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INTRODUCTION

Acetaminophen (paracetamol), also commonly known as Tylenol. Chemically it is N-(4-hydroxyphenyl) acetamide (figure 1). It is the most commonly taken analgesic worldwide and is recommended as first-line therapy in pain conditions by the World Health Organization (WHO). It is also used for its antipyretic effects, helping to reduce fever. Acetaminophen is often found combined with other drugs in more than 600 over the counter (OTC) allergy medications, cold medications, sleep medications, pain relievers, and other products.[1]

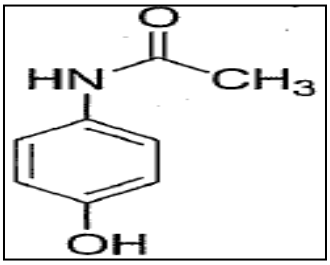

Figure 1. Chemical structure of paracetamol

Diclofenac Sodium is a non-steroidal anti-inflammatory agent (NSAID) with antipyretic and analgesic actions. It is primarily available as the sodium salt. Chemically it is 2-{2-[(2,6-dichlorophenyl)amino]phenyl}acetic acid (figure 2). It is used For the acute and chronic treatment of signs and symptoms of osteoarthritis and rheumatoid arthritis. The antiinflammatory effects of diclofenac are believed to be due to inhibition of both leukocyte migration and the enzyme cylooxygenase (COX-1 and COX-2), leading to the peripheral inhibition of prostaglandin synthesis. As prostaglandins sensitize pain receptors, inhibition of their synthesis is responsible for the analgesic effects of diclofenac. Antipyretic effects may be due to action on the hypothalamus, resulting in peripheral dilation, increased cutaneous blood flow, and subsequent heat dissipation.[2]

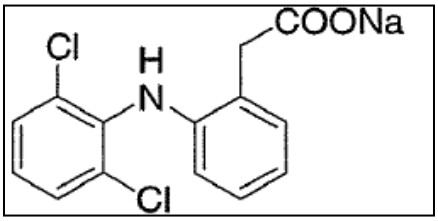

Figure 2. Chemical structure of diclofenac sodium

Dicyclomine Hydrochloride is a muscarinic antagonist used as an antispasmodic and in urinary incontinence. It has little effect on glandular secretion or the cardiovascular system. It does have some local anesthetic properties and is used in gastrointestinal, biliary, and urinary tract spasms. Chemically it ia 2-(diethylamino) ethyl 1-cyclohexylcyclohexane-1-carboxylate (figure

3). Its action is achieved via a dual mechanism: (1) a specific anticholinergic effect (antimuscarinic) at the acetylcholine-receptor sites and (2) a direct effect upon smooth muscle (musculotropic).[3]

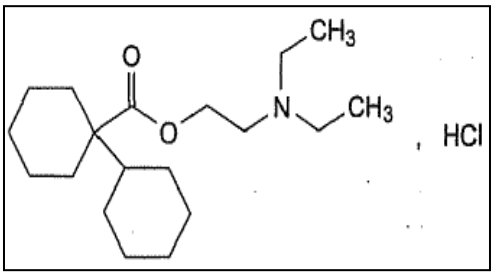

Figure 3. Chemical structure of dicyclomine hydrochloride

The literature review described HPTLC, HPLC and UV-visible spectrophotometric method for determination of paracetamol[6,8-11]; HPTLC, HPLC, UV-visible spectrophotometry, Potentiometric titration method for diclofenac sodium[5,7,9,12-13] and HPTLC, Potentiometric titration and Gas chromatography method for dicyclomine hydrochloride.[5,7,9,14,15] The literature review described HPTLC, HPLC and UV-visible spectrophotometric method for paracetamol, diclofenac sodium and dicyclomine hydrochloride in combination with other drugs or with each other.[16-24]

There was no published analytical method for simultaneous estimation of paracetamol, diclofenac sodium and dicyclomine hydrochloride. So aim of present work is to develop analytical method for simultaneous estimation of paracetamol, diclofenac sodium and dicyclomine hydrochloride. Due to lack of chromophore, dicyclomine hydrochloride has very poor UV absorbance. HPTLC is a well known and versatile separation method which shows a lot of advantages in comparison to other separation techniques. HPTLC has advantage of post-chromatographic derivatization for non UV absorbing compounds. A number of enhancements can be made to the basic method of thin layer chromatography to automate the different steps, to increase the resolution achieved and to allow more accurate quantitative measurements.

MATERIALS AND METHOD

Instrumentation

The HPTLC system (Camag, Switzerland) consisting of a Linomat IV semi automatic spotting device, a glass twin-trough TLC chamber (10cm×10cm, Camag), a TLC scanner (Camag IV) and a data station with Camag Win-CATS software and HPTLC syringe (100µl capacity; Hamilton Company, Reno, Nevada, USA) was used for chromatographic studies.

Chemicals and reagents

Toluene, methanol, concentrated sulphuric acid, ethyl acetate, p-anisaldehyde, glacial acetic acid (s.d.fiNE cHEM Ltd., Mumbai, India), used were of analytical reagent grade. paracetamol and diclofenac hydrochloride were obtained from Yarrow Chem. Products (Mumbai) and dicyclomine hydrochloride from Palam Pharma Pvt. Ltd. (Ahmedabad).

Chromatographic conditions

Separation was performed on 10cm×10cm aluminium backed plates precoated with 0.2mm layers of silica gel $60F_{254}$ (E. Merck, Darmstadt, Germany). The TLC plate was pre-washed with methanol and dried in an oven at 100°C for 5 min. Samples were spotted on the TLC plate 15mm from the bottom edge using Linomat V semi-automatic spotter and analyzed using following parameters: band width, 6mm; track distance, 4mm; volume of mobile phase, 11ml; temperature, 27ºC ± 1ºC; relative humidity, 35–40%; chamber saturation time, 30 min; migration distance, 85 mm; application rate, 150nl/s, slit dimension, 4mm×0.3mm; scanning speed, 20mm/s; detection wavelength, 266nm (for paracetamol and diclofenac sodium) and 638nm (for dicyclomine hydrochloride). Mobile phase consisted of toluene: ethyl acetate: methanol: formic acid (5.5:3.5:2:0.02, v/v/v/v).

Preparation of stock solution of standards

Accurately weighed 325mg, 50mg and 20mg of standard PCM, DCL and DICY respectively were dissolved in 10 ml of methanol to get stock solution of 32.5mg/ml, 5mg/ml and 2mg/ml for PCM, DCL and DICY, respectively.

Preparation of working standard solutions

One milliliter of stock solution of all three standards were diluted to 10ml with methanol to get concentration 3250µg/ml, 500µg/ml and 200µg/ml for PCM, DCL and DICY respectively. Further 2.5ml of above working standard solution were further diluted to 10ml methanol to get final concentration of 812.5µg/ml, 125µg/ml and 50µg/ml for PCM, DCL and DICY respectively.

Preparation of sample solution for analysis of tablet formulation

Twenty tablets were weighed accurately and finely powdered. Tablet powder equivalent to 10mg of DICY was accurately weighed and transferred to 100ml volumetric flask and 70ml of methanol was added. The mixture was sonicated for 10min, diluted to the mark with methanol and filtered through whatman filter paper No. 41. Further 2.5ml filtrate was diluted to 10ml with methanol to get the concentration 812.5µg/ml PCM, 125µg/ml DCL and 50µg/ml DICY.

Preparation of staining reagent

0.5ml of p-anisaldehyde, 10ml of glacial acetic acid, 85ml of methanol and 5ml of concentrated H_2SO_4 dropwise. This reagent was stored at 2-8°C and used within 4-5 days.

Selection of wavelength for detection

Wavelength for determination of PCM and DCL was selected from overlay spectra of both drugs in HPTLC. For DICY, after dipping the plate in dipping reagent, the plate was dried and spectrum was measured and wavelength of detection was noted.

Optimization of mobile phase

Based on literature review, methanol, toluene, ethyl acetate and acetone were tried in different proportions as mobile phase. Modifiers like formic acid, glacial acetic acid and ammonia were used to achieve better resolution and compact spot.

Chromatographic separation

Working standard solution (8µl) and sample solution for analysis of tablet formulation (8µl) were spotted on the pre-washed TLC plate under nitrogen stream using semi-automatic spotter. The plate was dried and developed in a camag twintrough chamber previously saturated for 30min with mobile phase, toluene: ethyl acetate: methanol: formic acid (5.5:3.5:2:0.02, v/v/v/v). After development, the plate was dried in air. Photometric measurements were performed at 266nm for paracetamol and diclofenac sodium in absorbance/reflectance mode with Camag TLC Scanner IV using wincat software incorporating the track optimization option. The plate was then dipped in the p-anisaldehyde reagent solution. After drying at room temperature, plate was heated on hot plate till dark blue colour spots became visible and plate was scanned again at 638nm.

Validation of the proposed method[4]

Linearity

The linear responses for PCM, DCL and DICY in the range of 4-12µl of working standard solution corresponding to 3250-9750ng of PCM/spot, 500-1500ng of DCL/spot and 200-600ng of DICY/spot were assessed in terms of slope, intercept, and correlation coefficient values.

Precision

1) **Repeatability**

Repeatability of measurement of peak area: Working standard solution (8µl, 6500µg/ml of PCM, 1000µg/ml of DCL and 400µg/ml of DICY) was spotted on a TLC plate, developed and dried. The separated spot was scanned for six times without changing plate position and RSD for measurement of peak area was computed.

Repeatability of sample application: Working standard solution 8µl, 6500µg/ml of PCM, 1000µg/ml of DCL and 400µg/ml of DICY) was spotted on a TLC plate six times, developed, dried and photometrically analyzed. The area of six spots was measured and the RSD of peak area was calculated.

2) **Inter-day and Intra-day precision**

The inter-day precision (RSD) was determined by analyzing working standard solution over the entire calibration range for 3 days and %RSD was calculated. The intra-day precision (RSD) was determined by analyzing working standard solution over the entire calibration range for three times on the same day and %RSD was calculated.

Limit of detection and Limit of Quantitation

Detection limit was found by gradually decreasing the concentration untill spot cannot be detected. The quantitation limit was determined by the analysis of drugs with known concentrations and by establishing the minimum level at which the drugs can be quantified with acceptable accuracy and precision.

Specificity

The spots of PCM, DCL and DICY from tablet formulation were confirmed by comparing its R_f and absorbance/reflectance spectrum with that of standard PCM, DCL and DICY. The peak purity of PCM, DCL and DICY was determined by correlating the spectra of PCM, DCL and DICY scanned at peak start, peak apex, and peak end positions of the spot.

Accuracy

The accuracy was determined by standard addition method. To a fixed amount of pre-analyzed sample of PCM, DCL and DICY, predefined amount of standard PCM, DCL and DICY API/solution was added in all the levels (80%, 100% and 120%). The recovery of PCM, DCL and DICY was calculated at each level (n=3).

Detection stability after derivatization

The derivatized HPTLC plate was scanned for Dicyclomine HCl at different interval (0, 10, 20, 30, 40, 50 and 60 min.) after dipping into dipping reagent at 638nm. The stability of derivatized drug was determined.

Analysis of tablet formulation

Working standard solution (8µl) and sample solution (8µl) were spotted on a TLC plate in replicate and analyzed. The amount of PCM, DCL and DICY present in sample solution was determined by fitting area values of corresponding peak into the equation representing calibration curve of PCM, DCL and DICY.

RESULTS AND DISCUSSION

Owing to its versatility, sensitivity, and speed of analysis, the HPTLC method was considered to be suitable, for analysis of paracetamol, diclofenac sodium and dicyclomine hydrochloride in tablet formulation.

Selection of wavelength

Overlay spectra of paracetamol and diclofenac sodium was taken by high performance thin layer chromatography. The wavelength selected for determination of PCM and DCL was 266 nm (figure 4).

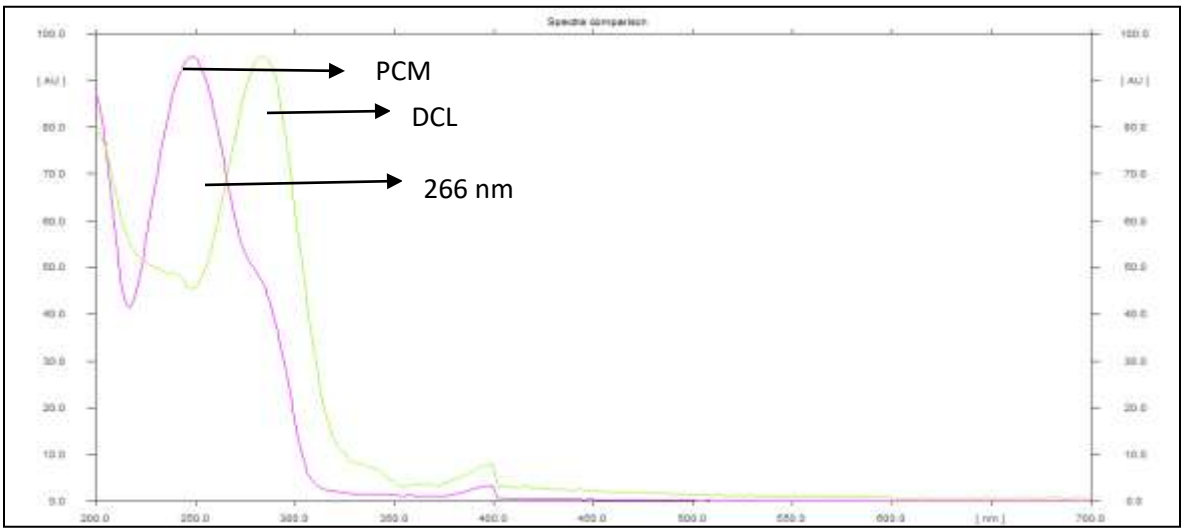

Figure 4. Overlay HPTLC spectra of PCM and DCL showing selection of wavelength for detection

The wavelength was selected for determination of DICY was 638 nm (figure 5).

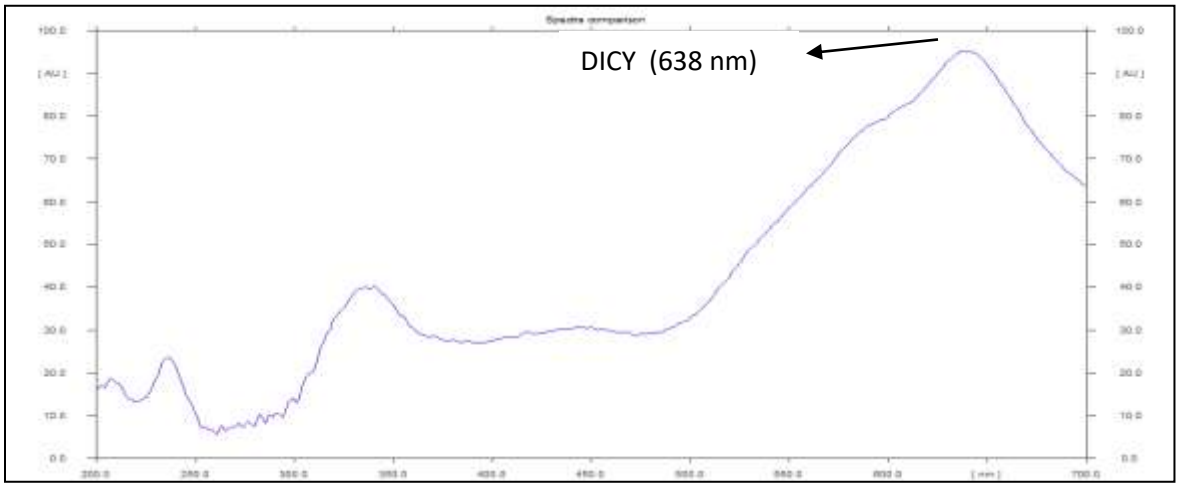

Figure 5. HPTLC spectra of DICY showing selection of wavelength for detection

Selection of staining agent

Many staining agents were tried i.e iodine, dilute potassium iodobismuthate solution, phosphomolybdic acid, vanillin, cobalt thiocyanate, p-anisaldehyde, potassium permanganate solution. Among these staining reagents p-anisaldehyde was found to be better and gave good results.

Mobile phase optimization

Based on reported literature, several mobile phases were tried. The mobile phase toluene: ethyl acetate: methanol: formic acid (5.5:3.5:2:0.02, v/v/v/v) gave good resolution with R_f values of 0.24 ± 0.03, 0.41 ± 0.03 and 0.71 ± 0.03 for DICY, PCM and DCL, respectively as presented in figure 6 and figure 7.

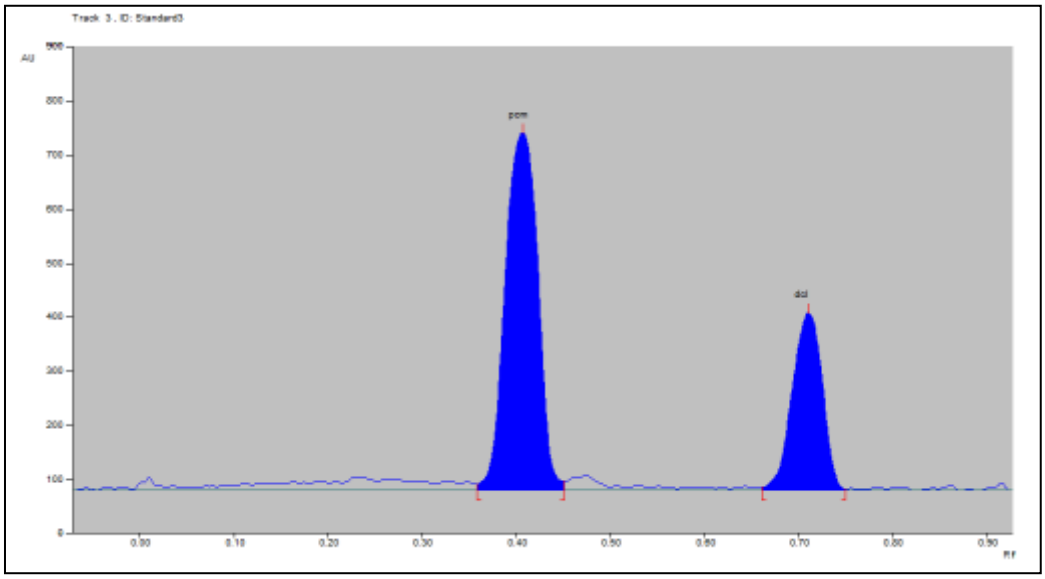

Figure 6. HPTLC Chromatogram of PCM and DCL standards after PCM, DCL and DICY separation

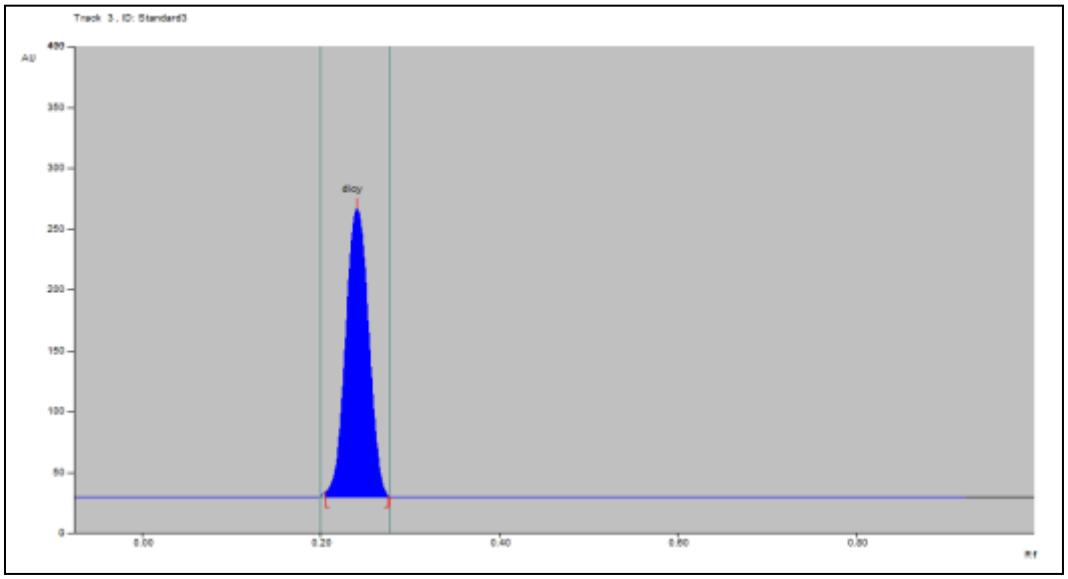

Figure 7. HPTLC Chromatogram of DICY standard after PCM, DCL and DICY separation

Method validation

Linearity

Representative calibration curve of PCM, DCL and DICY was obtained by plotting the mean peak area of PCM, DCL and DICY against concentration over the range of 3250-9750ng/spot (Figure 8), 500–1500ng/spot (Figure 9) and 200–600ng/spot (Figure 10), respectively (n=5).

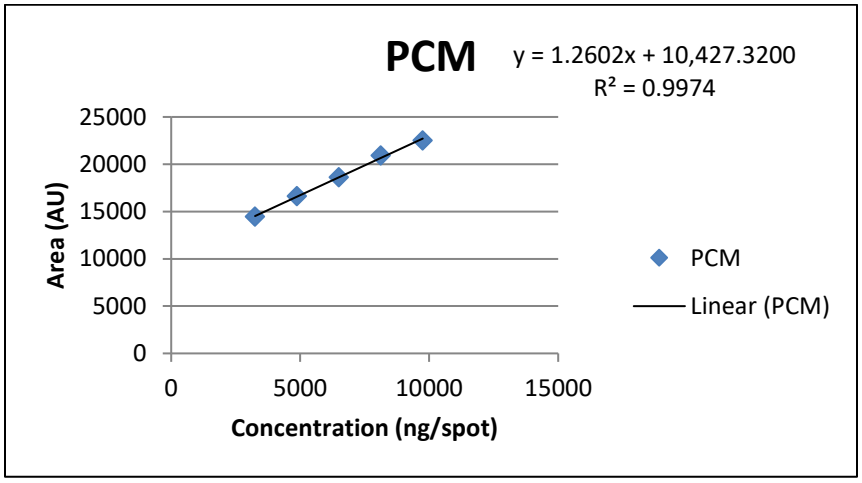

Figure 8. Calibration curve for PCM

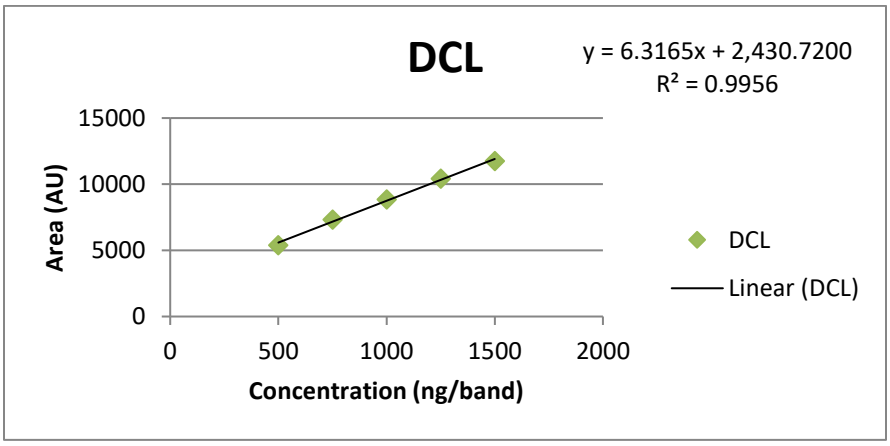

Figure 9. Calibration Curve for DCL

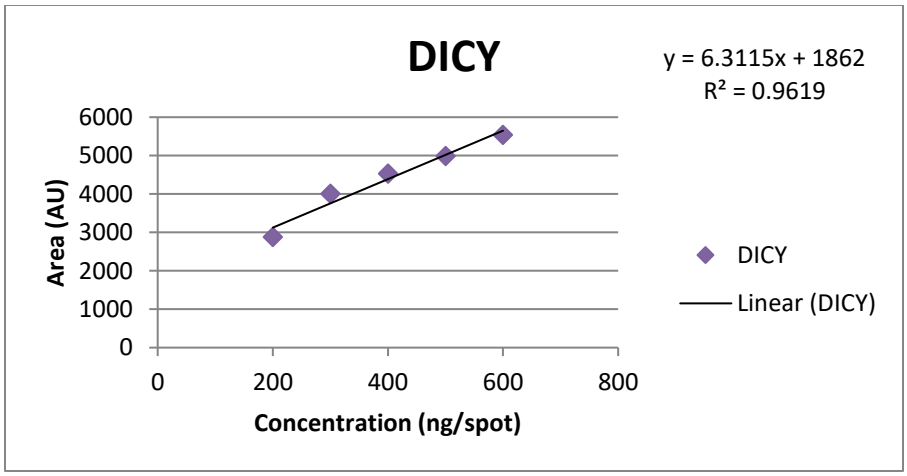

Figure 10. Calibration Curve for DICY

Precision

1) Repeatability

Table 1: Repeatability of sample application (n=6)

	PCM	DCL	DICY
Concentration (ng/band)	6500	1000	400
Area (Mean±SD)	18654.4 ± 171.6	9077.3 ± 99.7	4613.5 ± 66.7
%RSD	0.93	1.09	1.44

Table 2: Repeatability of measurement of peak area (n=6)

	PCM	DCL	DICY
Concentration (ng/band)	6500	1000	400
Area (Mean±SD)	18700.7 ± 67.1	8997.6 ± 61.6	4574.4 ± 44.1
%RSD	0.36	0.68	0.96

2) Inter-day and Intra-day precision

Inter-day precision (RSD, n=5) was found to be 1.35-3.63%, 1.83-3.08 and 1.62-3.76% for PCM, DCL and DICY respectively. Intra-day precision (RSD, n=5) was found to be 1.06-1.92%, 1.04-1.96% and 1.52-2.63% for PCM, DCL and DICY respectively.

Limit of detection and Limit of Quantitation

The results for LOD and LOQ, determined experimentally are presented in Table 3.

Table 3: Results of LOD and LOQ

	PCM	DCL	DICY
LOD (ng/spot)	812	125	50
LOQ (ng/spot)	3250	500	200

Specificity

Comparison of chromatograms of standard and tablet formulation showed identical R_f values for PCM, DCL and DICY, i.e. 0.24 for DICY, 0.41 for PCM and 0.71 for DCL. The excipient and other component present in tablet formulation did not interfere in the separation and resolution of PCM, DCL and DICY. Comparison of the spectra scanned at peak start (s), peak apex (m) and peak end (e) positions of individual spots of PCM, DCL and DICY showed a high degree of correlation (above 0.99), confirmed the purity of the corresponding spots.

Accuracy

The recovery of added sample was 99.7-100.9%, 99.5-100.6% and 99.1-101.7% for PCM, DCL and DICY, respectively (Table 4).

Table 4: Data of recovery study for PCM, DCL and DICY

Drug	Amount taken (ng)	Amount Spiked (ng)	Total amount (ng)	Recovered Concentration (ng) ± SD (n=3)	Mean % Recovery
PCM	3250	-	3227.3	-	-
	3250	2600	5810.7	2583.4±158.1	99.3
	3250	3250	6511.9	3283.6±133.9	101.0
	3250	3900	7143.6	3916.5±137.6	100.4
DCL	500	-	498.6	-	-
	500	400	898.2	399.6±95.9	99.7
	500	500	1001.2	503.2±92.1	100.5
	500	600	1091.8	593.3±74.4	98.9
DICY	200	-	198.5	-	-
	200	160	358.6	158.6±23.4	99.1
	200	200	402.1	203.5±31.4	101.7
	200	240	440.1	241.8±26.7	100.8

Detection stability after derivatization

HPTLC plate was scanned at different interval after derivatizing with dipping agent (p-anisaldehyde) at 638nm. No significant change was observed in area of peak when scanned within 30 min.

Assay of marketed formulation

Tablets were analysed by the proposed HPTLC method. Results were in good agreement with the label claim. The assay results are shown in Table 5.

Table 5: Assay data for tablet formulation

Tablet Formulation	Label Claim (mg)			Assay (%Label Claim) ± SD; (n=3)		
	PCM	DCL	DICY	PCM	DCL	DICY
Spasmodart	325	50	20	100.2±30.0	99.8±84.4	101.1±31.7

CONCLUSION

The developed HPTLC method is precise, specific, and accurate. It can be routinely applied for the analysis of paracetamol, diclofenac sodium and dicyclomine hydrochloride in their combined pharmaceutical formulation.

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