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Stability indicating thin-layer chromatographic determination of chlorzoxazone, diclofenac sodium and paracetamol as bulk drug: Application to forced degradation study

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ABSTRACT

A simple, sensitive and precise thin-layer chromatographic method for determination of chlorzoxazone, diclofenac sodium and paracetamol both as bulk drug and in formulations was developed and validated. The separation was achieved on silica gel 60F-254 thin-layer chromatographic (TLC) plate as the stationary phase and the mobile phase consisted of toluene-ethyl acetate-glacial acetic acid (6.5:3.5:0.02 v/v/v). The solvent system was found to give compact spot for chlorzoxazone (R_f values of 0.23 ± 0.021), diclofenac sodium (R_f values of 0.51 ± 0.035) and paracetamol (R_f values of 0.64 ± 0.028). Densitometric analysis was carried out in the absorbance mode at 274 nm. The linear regression analysis data for the calibration plots showed good linear relationship with respect to peak area in the concentration range 200–800 ng spot^{-1} of chlorzoxazone (with r = 0.9974), 40–160 ng spot^{-1} of diclofenac sodium (with r = 0.9914) and 260–1040 ng spot^{-1} of paracetamol (with r = 0.9965). The method was validated for limit of detection, limit of quantitation, accuracy, precision, robustness and recovery. Chlorzoxazone, diclofenac sodium and paracetamol were subjected to acid and alkali hydrolysis, oxidative, heat treatment and photodegradation. The drug undergoes degradation under acidic and oxidative conditions and upon UV light treatment. The degraded products were well separated from the pure drugs. The result and statistical analysis proves that the developed method is reproducible and selective for the estimation of said drugs. As the method could effectively separate the drugs from its degradation product, it can be employed as stability indicating one.

Key Words: Chlorzoxazone, Diclofenac sodium, Paracetamol, Thin-layer chromatography, Stability indicating, Validation

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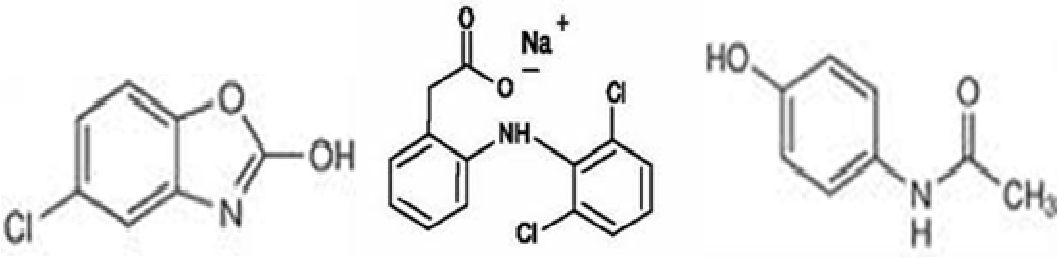
INTRODUCTION

Chlorzoxazone {5-Chlorzoxazoneoro-2-hydroxybenzoxazole}(Fig.1A) is a skeletal muscle relaxant. It acts by inhibiting multi synaptic reflexes involved in producing and maintaining skeletal muscle spasm of varied etiology [1, 2]. Diclofenac Sodium {2-[(2, 6-dichlorophenyl)-amino] phenyl acetate} (Fig.1B)



(A) (B) (C)

Figure 1: Chemical structure of (A) Chlorzoxazone (B) Diclofenac sodium (C) Paracetamol

It is a non-steroidal anti-inflammatory drug. It mainly acts by inhibiting the enzyme cyclo-oxygenase in the pathway of prostaglandin synthesis. It affords quick relief of pain and wound edema [3]. Paracetamol {4-hydroxyacetanilide}(Fig.1C) has antipyretic and mild analgesic actions due to inhibition of prostaglandin synthesis. It is much safer than aspirin in terms of gastric irritation, ulceration and bleeding [4, 5].

Literature survey reveals simultaneous estimation of paracetamol with diclofenac sodium using RP- HPLC [6], paracetamol with chlorzoxazone using RP-HPLC [7], and paracetamol with chlorzoxazone and nimesulide using HPTLC [8]. Various methods have been reported in literature for analysis of chlorzoxazone, diclofenac sodium and paracetamol including spectrophotometric [9] and high-performance liquid chromatography [10-13]. However to our knowledge, no article related to stability indicating high-performance thin-layer chromatography (HPTLC) determination of chlorzoxazone, diclofenac sodium and paracetamol in pharmaceutical dosage form has ever been mentioned in literature.

The International Conference on Harmonization (ICH) guidelines entitled 'stability testing of new drug substances and product requires the stress testing of the drug substance should be carried out to elucidate the inherent stability characteristics of the active substance [14]. An ideal stability indicating method is one that quantifies the drug per se and also resolves its degradation product. HPTLC has become a part of routine analytical techniques in many product development and analytical laboratories due to its advantages [15-17]. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering the analysis time and cost per analysis with high sample throughput [18]. The uniform particle size (7 µm) of precoated HPTLC plates enables achievement of a greater resolution and an easy reproducible separation. The method of detection does not place any restriction on the choice of the mobile phase and unlike HPLC, mobile phases having pH 8 and above can be employed [19,20]. Suspensions, dirty or turbid samples can be directly applied. Additionally, it permits simultaneous assay of several components in a multicomponent formulation or herbal extracts.

The aim of the present work was to develop an accurate, specific, reproducible and stability indicating HPTLC method for determination of chlorzoxazone, diclofenac sodium and paracetamol in presence of its degradation products and related impurities as per the ICH guidelines [21].

MATERIALS AND METHODS

Authentic chlorzoxazone, diclofenac sodium and paracetamol were kindly received as a gift sample from ZIM Laboratories, Nagpur, India. All chemicals and reagents used are analytical grade. Toluene was purchased from Rankem, New Delhi, India, Ethyl acetate and Glacial acetic acid was purchased from Merck, Mumbai, India.

2.2. HPTLC instrumentation

Standard experimental conditions were optimized in view to develop a stability indicating assay method to quantify chlorzoxazone, diclofenac sodium and paracetamol as in bulk drug and in pharmaceutical dosage form. Samples were spotted in the form of band of 6 mm with Camag microlitre syringe on precoated silica gel aluminium plate 60F-254 (20 cm x 10 cm, 200 µm thickness, E. Merck, Germany) using a Camag Linomat IV (Switzerland) sample applicator. The plates were pre-washed by methanol and activated at 60°C for 10 min prior to chromatography. A constant application rate of 0.16 µL s^{-1} was employed and space in between two bands was 4 mm. The slit dimension

was kept 5 mm x 0.45 mm and 10 mm s^{-1} scanning speed was employed. The mobile phase consisted of toluene-ethyl acetate-glacial acetic acid (6.5:3.5:0.02 v/v/v) was optimized for good resolution with compact spots.The detection of wavelength was selected from *in-situ* overlain spectra of the drugs(Fig.2)

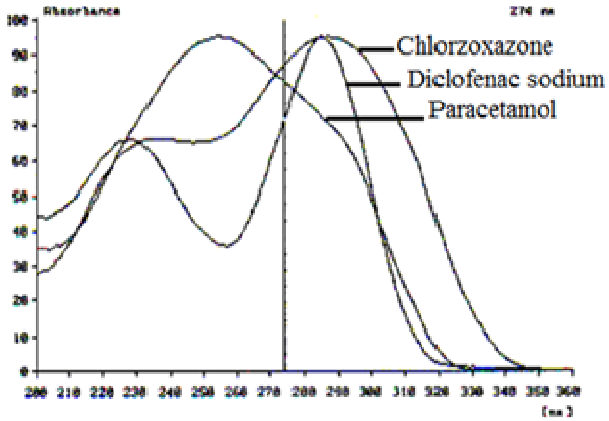


Figure 2 Overlain spectra for selection of wavelength (274nm)

Linear ascending development was carried out in twin-trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 10 min at temperature of 20 ± 5ºC and at relative humidity of 60 ± 5%. The length of chromatogram run was 80 mm. Subsequent to the development; TLC plate was dried in a current of air with the help of an air-dryer. Densitometric scanning was performed on Camag TLC scanner III in the absorption mode at 274 nm. The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum in the range of 190-400 nm. Evaluation was done using linear regression analysis by peak areas.

2.3. Calibration curves

A mixed stock solution of chlorzoxazone (250 µg mL^{-1}), diclofenac sodium (50 µg mL^{-1}) and paracetamol (325 µg mL^{-1}) was prepared in methanol. Different volumes of mixed stock solution 2, 3, 4, 5, 6, 7 and 8 mL were transferred to 10 mL volumetric flask and made to volume with methanol. Four microlitres from each diluted stock solution was spotted on TLC plate to obtain final concentration range of 200 – 800 ng $spot^{-1}$ of chlorzoxazone, 40 – 160 ng $spot^{-1}$ of diclofenac sodium and 260 – 1040 ng $spot^{-1}$ of paracetamol. Each concentration was spotted three times on TLC plate. The data of peak area plotted against corresponding concentration were treated by linear least-square regression analysis.

2.4. Method validation

2.4.1. Precision

Precision of an analytical method was expressed in terms of percent relative standard deviation (% R.S.D.) and standard error (S.E.) of the series of measurement. For system precision, repeatability of the sample application and measurement of peak areas were carried out using six replicates of the same concentration (500 ng $spot^{-1}$ of chlorzoxazone,100 ng $spot^{-1}$ of diclofenac sodium and 650 ng $spot^{-1}$ of paracetamol). For method precision, the Intra-day and Inter-day variations were carried out at three different concentration level (300, 500 and 700 ng $spot^{-1}$ of chlorzoxazone, 60, 100 and 140 ng $spot^{-1}$ of diclofenac sodium, and of 390, 650 and 910 ng $spot^{-1}$ of paracetamol).

2.4.2. Robustness of the method

By introducing small changes in optimized chromatographic conditions, the effects on the results were examined. Robustness of the method was studied in triplicate at a concentration level of 500 ng $spot^{-1}$ of chlorzoxazone, 100 ng $spot^{-1}$ of diclofenac sodium, and 650 ng $spot^{-1}$ of paracetamol. Mobile phases having different compositions of toluene-ethyl acetate- glacial acetic acid (6.4:3.5:0.02 v/v/v), (6.6:3.5:0.02 v/v/v), (6.5:3.6:0.02 v/v/v), (6.5:3.4:0.02 v/v/v), and (6.4:3.6:0.02 v/v/v) were tried and chromatograms were run. Mobile phase volume was varied at 10 ± 1 mL (9, 10 and 11 mL) and duration of saturation time was varied at 10 ± 5 min (5, 10 and 15 min). The plates were prewashed by methanol and activated at 60°C for 8, 10 and 12 min prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 10 and 20 min.

2.4.3. Limit of detection and limit of quantitation
In order to estimate limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six time on TLC plate following the same method as describe in section 2.2 and standard deviation of the magnitude of analytical response was determined on the basis of signal to noise ratio.

2.4.4. Specificity
The specificity of the method was ascertained by analyzing the standard drug and sample with respect to R_f value and spectra. The peak purity of chlorzoxazone, diclofenac sodium and paracetamol were assessed by comparing the two spectra at three different levels, viz. peak start (S), peak apex (M) and peak end (E) positions of the spot.

2.4.5. Recovery study
Recovery study was carried out by applying the method to drug samples to which known amount of chlorzoxazone, diclofenac sodium and paracetamol corresponding to 80%, 100% and 120% of label claim had been added. At each level of the amount, six determinations were performed. This was done to check recovery of the drugs at different levels in the formulations.

2.5. Analysis of the marketed formulation
To determine the content of commercial formulation, 20 tablets (chlorzoxazone 250 mg : diclofenac sodium 50 mg : paracetamol 325 mg) were weighed, their mean weight determined and they were finely powdered. An accurately weighed quantity of tablet powder equivalent to 10 mg of diclofenac sodium was shaken with 5 mL of methanol, sonicated for 10 min and diluted to 10 mL with methanol. The solution was centrifuged at 2000 rpm for 5 min and 500 µL of supernatant was diluted to 10 mL with methanol. Two microlitres (500 ng spot^{-1} of chlorzoxazone, 100 ng spot^{-1} of diclofenac sodium, and 650 ng spot^{-1} of paracetamol) of filtered solution was applied on TLC plate followed by development and scanning as described in section 2.2. The analysis was repeated in triplicate and the possibility of excipients interference in the analysis was studied.

2.6. Forced degradation study
Forced degradation study was carried out by attempting deliberate exposing the drugs to different stress conditions.

A Mixed stock solution of chlorzoxazone (50 mg), diclofenac sodium (10 mg) and paracetamol (65 mg) was prepared in 100 mL methanol. This mixed stock solution was used for forced degradation to provide an indication of the stability indicating property and specificity of the proposed method.

2.6.1. Acid and base induced degradation product
To 10 mL of methanolic mixed stock solution, 10 mL of 1N HCl and 10 mL of 1N NaOH were added separately. These mixtures were reflux separately for 3 h at 80°C. The forced degradation study in acidic and basic media was performed in the dark in order to leave out the possible degradative effect of light. Two microlitres (500 ng spot^{-1} of chlorzoxazone, 100 ng spot^{-1} of diclofenac sodium and 650 ng spot^{-1} of paracetamol) of resultant solutions were applied on TLC plate and developed as described in section 2.2.

2.6.2. Hydrogen peroxide induced degradation product
To 10 mL of methanolic mixed stock solution, 10 mL of hydrogen peroxide (H_2O_2) (30% v/v) was added. This solution was heated in boiling water bath for 10 min to remove completely the excess of hydrogen peroxide and reflux for 3 h at 80°C. Two microlitres (500 ng spot^{-1} of chlorzoxazone, 100 ng spot^{-1} of diclofenac sodium and 650 ng spot^{-1} of paracetamol) of resultant solutions were applied on TLC plate and developed as described in section 2.2.

2.6.3. Dry heat induced degradation product
Dry heat degradation was performed by exposing the powdered chlorzoxazone (50 mg), diclofenac sodium (10 mg) and paracetamol (65 mg) to 60°C for 24 h under dry heat condition to study the inherent stability of the drugs. Dry heat exposed drugs was dissolved in 100 mL methanol. 10 mL of this solution was diluted to 20 mL with methanol and two microlitres (500 ng spot^{-1} of chlorzoxazone, 100 ng spot^{-1} of diclofenac sodium and 650 ng spot^{-1} of paracetamol) of resultant solution was applied on TLC plate and developed as described in section 2.2.

2.6.4. Wet heat induced degradation product
Ten microlitres of methanolic mixed stock solution was diluted to 20 mL with methanol and reflux for 3 h at 80°C. Two microlitres (500 ng spot^{-1} of chlorzoxazone, 100 ng spot^{-1} of diclofenac sodium and 650 ng spot^{-1} of paracetamol) was applied on TLC plate and developed as described in section 2.2.

2.6.5. Photolytic induced degradation product
Ten microlitres of methanolic mixed stock solution was diluted to 20 mL with methanol. For photochemical stability study, 10 mL of resultant diluted stock solution was exposed to direct sunlight for 8 days on a wooden plank and kept on terrace. For UV radiation degradation study 10 mL of resultant diluted stock solution was exposed to UV radiations at 265 nm for 24 h in UV chamber.

Two microlitres (500 ng spot^{-1} of chlorzoxazone, 100 ng spot^{-1} of diclofenac sodium and 650 ng spot^{-1} of paracetamol) of each resultant exposed solution was applied on TLC plate and developed as described in section 2.2.

2.6.6. Neutral hydrolysis
Ten microlitres of methanolic mixed stock solution was diluted to 20 mL with distilled water and was reflux for 8 h. Two microlitres (500 ng spot^{-1} of chlorzoxazone, 100 ng spot^{-1} of diclofenac sodium and 650 ng spot^{-1} of paracetamol) was applied on TLC plate and developed as described in section 2.2.

RESULTS AND DISCUSSION

3.1. Optimization of chromatographic conditions
The chromatographic conditions were optimized with a view to develop a stability indicating assay method to quantify the chlorzoxazone, diclofenac sodium and paracetamol from pharmaceutical dosage form. Initially, variety of individual solvents and solvent mixtures in varying ratios were tried. The mobile phase toluene-ethyl acetate-glacial acetic acid (6.5:3.5:0.02 v/v/v) gave good resolution with R_f = 0.23 ± 0.021 for chlorzoxazone, R_f = 0.51 ± 0.035 for diclofenac sodium and R_f = 0.64 ± 0.028 for paracetamol (Fig. 3).

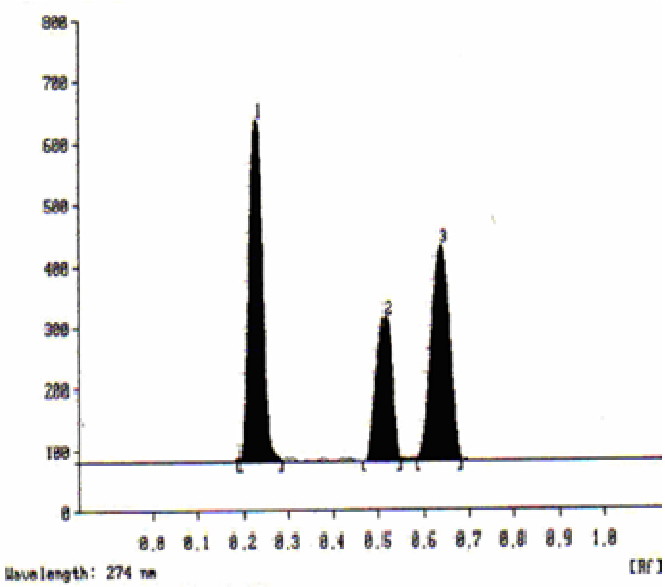


Figure 3: Chromatogram of (1) chlorzoxazone [R_f = 0.23], (2) diclofenac sodium [R_f = 0.51] and (3) paracetamol [R_f = 0.64]

The developed plates were evaluated densitometrically in absorbance mode at 274 nm with slit dimensions at 5 x 0.45 mm. Well defined spot were obtained when chamber was saturated with mobile phase for 10 min at temperature 20 ± 5ºC and relative humidity 60 ± 5%.

3.2. Calibration curves

Linearity was established by least-squares linear regression analysis to calculate the calibration equation and correlation coefficient. The regression data (Table 1) showed a good linear response over the concentration range 200 – 800 ng spot^{-1} of chlorzoxazone, 40 – 160 ng spot^{-1} of diclofenac sodium and 260 – 1040 ng spot^{-1} of paracetamol with respect to peak area. The linearity of calibration graphs and adherence of the system to Beer's law was validated by correlation coefficient. No significant difference was observed in the slopes and standard curves (ANOVA, $p<0.05$).

Table 1 Summary of linear regression and validation data

Data	Chlorzoxazone	Diclofenac Sodium	Paracetamol
Linear Range (ng spot^{-1})	200-800	40 – 160	260-1040
Coefficient of Correlation ($\mathbf{r^2}$) (± SD)	0.9974 ± 0.0018	0.9914 ± 0.0025	0.9965 ± 0.0021
Slope (± SD)	2525.14 ± 0.0489	11980.8 ± 0.0732	2314.48 ± 0.0788
Y-Intercept (± SD)	3752.68 ± 0.1229	736.40 ± 0.9827	5199.19 ± 0.1.029
LOD (ng spot^{-1})	61.25	12.25	76.56
LOQ (ng spot^{-1})	153.8	30.76	192.25
Specificity	Specific	Specific	Specific
Ruggedness	Rugged	Rugged	Rugged

SD = Standard Deviation

3.3. Validation of the method

3.3.1.Precision

The repeatability of sample application and measurement of peak areas at 500 ng spot^{-1} of chlorzoxazone, 100 ng spot^{-1} of diclofenac sodium and 650 ng spot^{-1} of paracetamol were expressed in terms of % R.S.D. and S.E. and was found to be <2. The measurement of the peak areas at three different concentration levels showed low value of % R.S.D. (<2) and low value of S. E. (<2) for intra- and inter-day variation, which suggested an excellent precision of the method (Table 2).

Table 2 Precision study (n = 6)

Parameters	Chlorzoxazone				Diclofenac sodium				Paracetamol			
	Theoretical amount (ng)	Amount estimated (ng)	% RSD	SE	Theoretical amount (ng)	Amount estimated (ng)	% RSD	SE	Theoretical amount (ng)	Amount estimated (ng)	% RSD	SE
Repeatability	500	492.83	0.8144	0.3435	100	99.63	1.0245	0.4327	650	637.28	1.3615	.5437
Intra-day precision	300	308.28	1.0311	0.4138	60	59.19	0.8214	0.3445	390	393.19	0.9562	0.3728
	500	491.24	0.9229	0.3827	100	97.31	0.8955	0.3577	650	646.52	0.2821	0.1337
	700	696.69	1.4587	0.5922	140	142.17	0.6252	0.2652	910	903.19	0.6842	0.2831
Iner-day precision	300	305.22	0.8714	0.3483	60	58.98	1.2656	0.5178	390	379.71	1.0248	0.4372
	500	495.27	0.8148	0.3413	100	101.19	0.9825	0.3927	650	643.21	1.2652	0.5262
	700	693.39	1.0245	0.4079	140	136.27	0.7222	0.3081	910	905.29	0.9826	0.3828

Table 3 Robustness of the HPTLC method (n = 3; 500 ng spot^{-1} of chlorzoxazone, 100 ng spot^{-1} of diclofenac sodium and 650 ng spot^{-1} of paracetamol)

Parameter	Chlorzoxazone			Diclofenac Sodium			Paracetamol		
	SD	% RSD	SE	SD	% RSD	SE	SD	% RSD	SE
Mobile phase composition; toluene : ethyl acetate : glacial acetic acid (6.5 ± 0.1:3.5 ± 0.1:0.02 v/v/v)	1.26	1.2115	0.7274	1.62	1.4832	0.9353	1.26	1.1284	0.7275
Mobile phase volume (9, 10 and 11 mL)	1.38	1.2889	0.7967	1.29	1.1128	0.7448	1.19	0.9821	0.6871
Duration of saturation time (5, 10 and 15 min)	1.3	1.2836	0.7506	0.92	0.8349	0.5312	0.95	0.9127	0.5485
Activation of prewashed TLC plate at 60°C (8, 10 and 12 min)	1.69	1.5326	0.9758	0.35	0.3125	0.2021	0.88	0.8135	0.5081
Time from spotting to chromatography (0, 10 and 20 min)	0.89	0.7263	0.5139	0.59	0.4826	0.3406	0.56	0.4867	0.3233
Time from chromatography to scanning (0, 10 and 20 min)	0.78	0.6386	0.4503	0.73	0.5839	0.4215	0.58	0.5249	0.3349

3.3.2.Robustness of the method

The % R.S.D. and S.E. of the peak areas were calculated for change in mobile phase composition, mobile phase volume, duration of saturation time, duration of activation of prewashed TLC plate, time from spotting to chromatography and time from chromatography to scanning in triplicate at the concentration level of 500 ng spot^{-1} of chlorzoxazone, 100 ng spot^{-1} of diclofenac sodium and 650 ng spot^{-1} of paracetamol. The low values of % R.S.D.

(<2) and S.E. (<2) obtained after introducing small deliberate changes in the developed HPTLC method indicated the robustness of the method (Table 3).

3.3.3.LOD and LOQ

Detection limit and quantitation limit was calculated as described in section 2.4.3. The signal to noise ration of 3:1 and 10:1 were considered for LOD and LOQ respectively. LOD was found to be 61.25, 12.25 and 76.56 ng spot^{-1} where as LOQ was found to be 153.80, 30.76 and 192.25 ng spot^{-1} for chlorzoxazone, diclofenac sodium and paracetamol respectively, which indicates the adequate sensitivity of the method (Table 1).

3.3.4.Specificity

The peak purity of chlorzoxazone, diclofenac sodium and paracetamol were assessed by comparing the spectra at peak start, peak apex and peak end position of the spot. Good correlation (r=0.9997) was obtained between standard and sample spectra indicated specificity of the method.

3.3.5.Recovery studies

The proposed method when used for extraction and subsequent estimation of chlorzoxazone, diclofenac sodium and paracetamol from pharmaceutical dosage form after spiking with additional drug afforded recovery of 99.81-101.01% of chlorzoxazone, 99.54-100.26 % of diclofenac sodium and 100.15-101.24% of paracetamol as listed in Table 4. The low values of % R.S.D. (<2) indicated non interference of the sample matrix.

Table 4 Recovery study (n = 6)

% of label claim	Chlorzoxazone			Diclofenac Sodium			Paracetamol		
	% Recovery	% RSD	SE	% Recovery	% RSD	SE	% Recovery	% RSD	SE
80	99.81	0.7828	0.3038	99.54	1.0514	0.4237	100.92	1.1718	0.4772
100	100.25	0.3871	0.1523	99.83	0.6825	0.2809	100.15	0.8199	0.3317
120	101.01	0.8922	0.3615	100.26	1.0226	0.4217	101.24	1.2629	0.5205

3.4. Analysis of marketed formulations

A single spot at R_f values 0.23 of chlorzoxazone, 0.51 of diclofenac sodium and 0.64 of paracetamol were observed in the chromatogram of sample extracted from tablet. There was no interference from the excipients commonly present in the tablets. The drug content was found to be 99.62%, 99.34% and 99.52% with percent relative standard deviation (% R.S.D.) of 0.3728, 0.6285 and 0.7929 and standard error (S.E.) of 0.1668, 0.2931 and 0.3418 of chlorzoxazone, diclofenac sodium and paracetamol respectively. It may inferred that the degradation of drugs had not occurred in the marketed formulations that were analyzed by this method. The low % R.S.D. value indicated the suitability of this method for routine analysis of chlorzoxazone, diclofenac sodium and paracetamol in pharmaceutical dosage form.

3.5. Stability indicating property

The number of degradation products with their R_f values and percentage recovery of chlorzoxazone, diclofenac sodium and paracetamol were calculated and listed in Table 5**.**

Table 5 Summary of forced degradation study

Sr. No.	Exposed stress conditions	Chlorzoxazone	Diclofenac Sodium	Paracetamol	R_f value of degradation product	Figure
1	Acid, 10 mL (1N HCl, reflux for 3 h at 80^{O}C)	97.28 ± 2.89	79.19 ± 5.10	99.35 ± 2.81	0.01	Fig. 4
2	Base, 10 mL (1N NaOH, reflux for 3 h at 80^{O}C)	98.54 ± 3.56	98.96 ± 3.67	92.10 ± 3.85	Not detected	-------
3	Oxide,10 mL,30%v/v H_2O_2 (reflux for 3 h at 80^{O}C)	74.44 ± 2.84	72.15 ± 3.59	97.57 ± 4.15	0.015, 0.56	Fig. 5
4	Dry heat (24 h at 60°C)	108.87 ± 4.19	97.84 ± 2.84	99.15 ± 3.59	Not detected	-------
5	Wet heat (reflux for 3 h at 80^{O}C)	103.65 ± 3.58	96.48 ± 2.81	97.68 ± 3.72	Not detected	-------
6	Photo stability (Daylight, 8 days)	96.12 ± 4.52	97.14 ± 3.86	95.29 ± 3.49	Not detected	-------
7	UV (254 nm for 24 h)	99.18 ± 3.87	69.17 ± 2.46	98.22 ± 4.16	0.38	Fig. 6
8	Neutral hydrolysis (reflux for 8 h)	99.21 ± 2.79	99.35 ± 1.98	98.81 ± 3.19	Not detected	-------

(% Estimated ± Standard Deviation)

3.5.1.Acid induced degradation product
The chromatogram of the acid degraded sample showed additional peak at R_f value *0.01*. The percent drug recovery of diclofenac sodium at the level of 79.19% suggested that diclofenac sodium undergoes mild degradation under acidic condition(Fig.4)

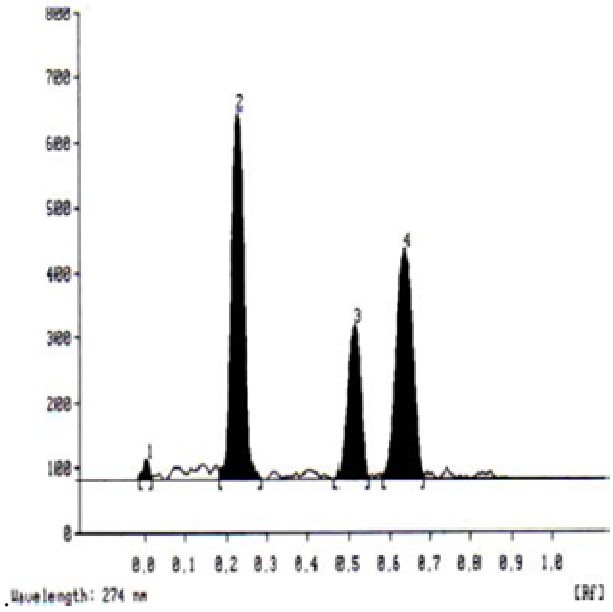


Figure 4
Chromatograms of acid [1N HCl (reflux for 3 h at 80°C)] treated sample.
Peak 1, degradant [Rf = 0.01];
Peak 2, chlorzoxazone [Rf = 0.23];
Peak 3, diclofenac sodium Rf = 0.51]; and
peak4,paracetamol[Rf=0.64]

The chromatogram of sample treated with 1N NaOH showed no additional peak other than standard drugs indicating the stability of chlorzoxazone, diclofenac sodium and paracetamol toward the basic induced degradation.

3.5.2. Hydrogen peroxide induced degradation product
The sample degraded under hydrogen peroxide showed additional peak at R_f values 0.015 and 0.56 (Fig. 5).

The areas of H_2O_2 induced degradation product were found to be small and the % drug recoveries at the level of 74.44% of chlorzoxazone and 72.15% diclofenac sodium suggests mild degradation of these drugs under oxidative condition.

3.5.3.Dry heat and wet heat induced degradation product
The sample degraded under dry heat and wet heat showed no additional peak other than the standard peaks of chlorzoxazone, diclofenac sodium and paracetamol, suggesting the stability of the drugs under heat conditions.

3.5.4.Photochemical degradation product
The chromatogram of the sample exposed to photochemical degradation showed no additional peak other than the standard peaks of chlorzoxazone, diclofenac sodium and paracetamol. The chromatogram of the sample exposed to ultraviolet (UV) light at 254 nm showed additional peak at R_f value 0.38 (Fig. 6).

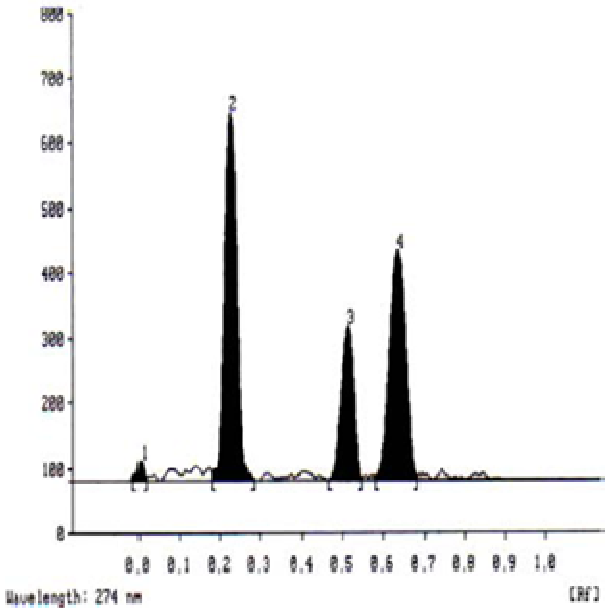


Figure 5 **Chromatograms of hydrogen peroxide (30%) [(reflux for 3 h at 80°C)] treated sample.**
Peak 1, degradant [Rf = 0.015];
Peak 2, chlorzoxazone [Rf = 0.23];
Peak 3, diclofenac sodium Rf = 0.51];
Peak 4, degradant [Rf = 0.56]; and
Peak 5, paracetamol [Rf = 0.64]

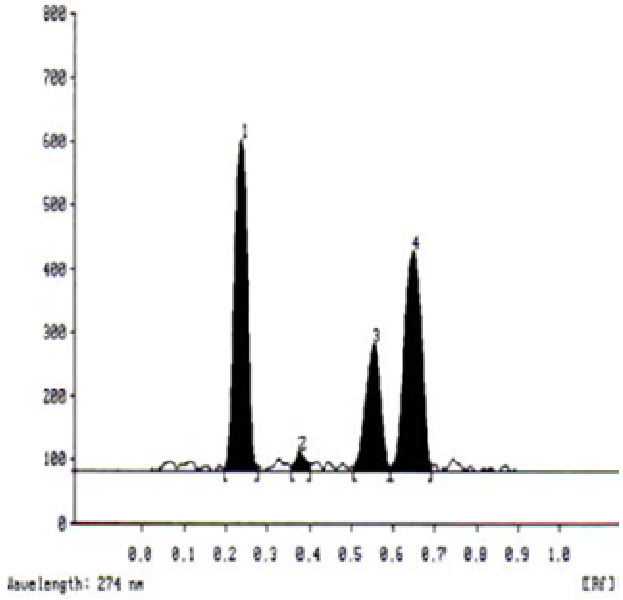


Figure 6 **Chromatograms of UV (254 nm) light [24 h] treated sample.**
Peak 1, chlorzoxazone [Rf = 0.23];
Peak 2, degradant [Rf = 0.38];];
Peak 3, diclofenac sodium Rf = 0.54]; and
Paek 4, paracetamol [Rf = 0.64]

The percent drug recovery of diclofenac sodium at the level of 69.17% suggests significant degradation of diclofenac sodium towards UV irradiation for the exposure period under study.

CONCLUSION

The developed HPTLC method enables accurate, precise, specific and stability indicating for determination of chlorzoxazone, diclofenac sodium and paracetamol. Statistical analysis proves that the method is reproducible and selective for routine analysis of chlorzoxazone, diclofenac sodium and paracetamol in pharmaceutical dosage form without interference from excipients. It may further be extended to study the degradation kinetics and also for determination of these drugs in plasma and other biological fluids. As the method separated the drugs from degradation products, it can be employed as a stability indicating one.

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