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Validated HPLC method for simultaneous estimation of paracetamol, aceclofenac and thiocolchicoside in bulk drug and formulation

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ABSTRACT

A simple, selective, rapid, precise and economical reverse phase high-pressure liquid chromatographic method has been developed for the simultaneous estimation of Paracetamol, Aceclofenac and Thiocolchicoside from pharmaceutical formulation. The method was carried out on a HiQ Sil C_{18} (250 mm × 4.6 mm, 5.0 µ) from Japan, with a mobile phase consisting of acetonitrile: water (30: 70, v/v) at flow rate of 1.0 ml/min. Detection was carried out at 263 nm. This method has been applied to formulation without interference of excipients of formulation. The linear regression analysis data for the calibration plots showed a good linear relationship over the concentration range of 50-175 µg/mL for Paracetamol, 10-35 µg/mL for Aceclofenac and 0.40-1.4 µg/mL for Thiocolchicoside respectively. The mean values of the correlation coefficient, slope and intercept were 0.9959 ± 0.98, 11389 ± 1.02 and 7020 ± 0.86 for Paracetamol and 0.9975 ± 0.64, 15521 ± 0.32 and 16800 ± 0.86 for Aceclofenac and 0.9984 ± 0.73, 13144 ± 0.74 and 357.9 ± 1.11 for Thiocolchicoside respectively. The method was validated for precision, robustness and recovery. The limit of detection (LOD) and limit of quantitation (LOQ) was 0.25 µg/mL and 0.50 µg/mL for Paracetamol, 1 µg/mL and 2 µg/mL for Aceclofenac and 0.24 µg/mL and 0.4 µg/mL for Thiocolchicoside respectively. Statistical analysis showed that the method is repeatable and selective for the estimation of Paracetamol, Aceclofenac and Thiocolchicoside.

Keywords: Paracetamol, Aceclofenac, Thiocolchicoside, RP-HPLC, Validation.

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INTRODUCTION

Paracetamol is chemically *N*-(4-hydroxyphenyl) acetamide (Figure 1). It is a centrally and peripherally acting non-opioid analgesic and antipyretic. Paracetamol is official in IP, BP and USP [1-3].

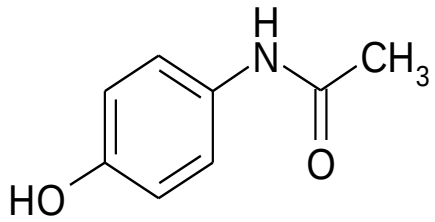

Figure 1 Structure of Paracetamol

Aceclofenac chemically is 2-[[2-[2-[(2, 6-dichlorophenyl) amino] phenyl] acetyl] oxy] acetic acid (Figure 2). It is used as analgesic and anti-inflammatory. Aceclofenac plays an important role in symptomatic management of osteoarthritis, rheumatoid arthritis and ankylosing spondylitis and other acute pain conditions. The mode of action of aceclofenac is largely based on the inhibition of prostaglandin synthesis. Aceclofenac is potent inhibitor of the enzyme cyclooxygenase, which is involved in the production of prostaglandins [4-5].

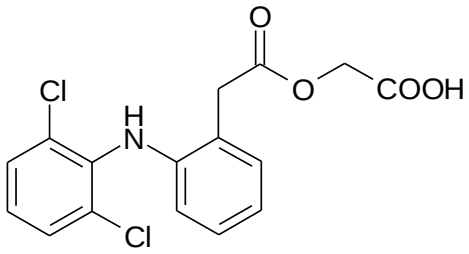

Figure 2 Structure of Aceclofenac

Thiocolchicoside is *N*-[(7*S*)-3-(beta-D-glucopyranosyloxy)-1, 2-dimethoxy-10-(methylsulf anyl)-9-oxo-5, 6, 7, 9-tetrahydrobenzo[a]heptalen-7-yl] acetamide (Figure 3). It is a muscle relaxant with anti-inflammatory and analgesic actions. Thiocolchicoside displaces both [3H] gamma-aminobutyric acid (3H) (GABA) and [3H] strychnine binding, suggesting an interaction with both GABA and strychnine-sensitive glycine receptors. It is used topically for the treatment of muscular spasms and for rheumatologic, orthopedic, and traumatologic disorders [6-8].

A tablet formulation containing Paracetamol 500 mg, Aceclofenac 100 mg and Thiocolchicoside 4 mg has been introduced in to clinical practice. A survey of literature revealed that few HPTLC, HPLC, LC-MS-MS and spectrophotometric methods are reported for determination of Paracetamol, Aceclofenac, and Thiocolchicoside individually [9-17]. However there is no HPLC method reported for simultaneous determination of Paracetamol, Aceclofenac and Thiocolchicoside from combined dosage form. The present work describes the simple,

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precise and accurate RP-HPLC method for simultaneous estimation of Paracetamol, Aceclofenac and Thiocolchicoside in tablets. It is validated by ICH guidelines [18].

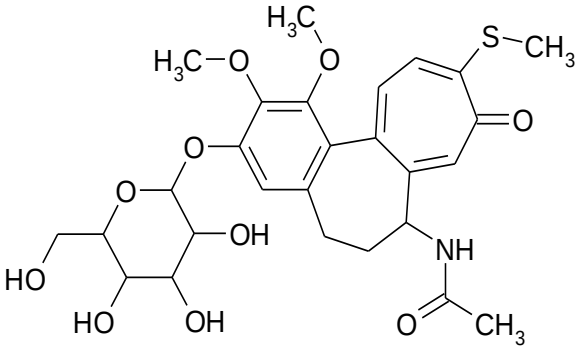

Figure 3 Structure of Thiocolchicoside

EXPERIMENTAL

Materials

Working standards of pharmaceutical grade Paracetamol (Batch no. 260738) was obtained as generous gift samples from Bal Pharmaceuticals Ltd., Pune, India, Aceclofenac (Batch no. 16043/01) was obtained from Cipla Pharmaceuticals Ltd. Mumbai, India and Thiocolchicoside (Batch no. 2148/009) was obtained from Zydus Cadila, Ahmedabad, Gujarat, India. It was used without further purification and certified to contain 99.96 % (w/w) on dry weight basis for Paracetamol, 99.98 % (w/w) on dry weight basis for Aceclofenac and 99.98 % (w/w) on dry weight basis for Thiocolchicoside. All drugs were used without further purification.

Instrumentation

The HPLC system consisted of Intelligent HPLC pump model (Jasco PU 2080 Plus) with sampler programmed at 20 µL capacity per injection was used. The detector consisted of a UV/ VIS (Jasco UV 2075 Plus). Another system consisted of Intelligent HPLC pump (Jasco PU 1580) with detector (Jasco UV-1575) and auto sampler. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system. The column used was, HiQ Sil C_{18} HS (4.6 mm I.D. × 250mm L), Japan. Mobile phase consisted of a mixture of Acetonitrile: Water (30: 70, v/v), at a flow rate of 1.0 ml/min with detection at 263 nm. The mobile phase was filtered through a 0.2 micron membrane filter and degassed. The injection volume was 20 µL and analysis was performed at ambient temperature.

Pharmaceutical formulation

Fixed dose combination tablets (Bakflex Plus) containing Paracetamol 500 mg, Aceclofenac 100 mg and Thiocolchicoside 4 mg (Batch no BEOT-137) manufactured by

Biomilicron Pharmaceuticals Ltd., Muthirayapalayam, Puducherry, India were obtained from local market.

Preparation of Standard Solutions

Standard stock solutions of concentration 1000 µg/mL of Paracetamol, 1000 µg/mL of Aceclofenac and 1000 µg/mL of Thiocolchicoside were prepared separately using methanol. The stock solution was stored at 2-8 °C protected from light. From the standard stock solution, the working standard solutions were prepared using methanol to get 500 µg/mL of Paracetamol, 100 µg/mL of Aceclofenac and 4 µg/mL of Thiocolchicoside, respectively. The stock solution was stored at 2-8 °C, protected from light.

Optimization of HPLC Method

All drugs were subjected to chromatographic analysis using mobile phases of differing pH, flow rate using the under mentioned chromatographic conditions. The changes in the retention time of all drugs were noted as a function of changing mobile phase, pH, flow rate, strength and selectivity. Initially methanol: water in the ratio of (70: 30) was tried but the peak eluted in the dead volume and all the three peaks merged. Later methanol: water in the ratio of (30: 70) was tried. It was found that the retention time and resolution was increased but sharp peaks were not obtained. Hence methanol was replaced with acetonitrile and acetonitrile: water in the ratio of (30: 70) at flow rate of 1 mL/min was tried. It was found that Paracetamol, Aceclofenac and Thiocolchicoside gave acceptable retention time, plates and good resolution.

System Suitability Studies

The resolution, number of theoretical plates and peak asymmetry were calculated for the standard solutions as shown in Table 1. The values obtained demonstrated the suitability of the system for the analysis of these drugs in combinations. The typical chromatogram of standard solution is as shown in Figure 4.

Table 1: System suitability studies

Chromatogram	Retention time (R_t) (min)	Asymmetry	Area	Plate Count	Resolution
Paracetamol	2.517	1.085	225898.20	4501.20	0.00
Thiocolchicoside	3.558	1.202	864397.66	6700.19	3.209
Aceclofenac	5.200	1.231	173504.3	9834.72	8.578

Validation of the method

Validation of optimized LC method was done with respect to following parameters.

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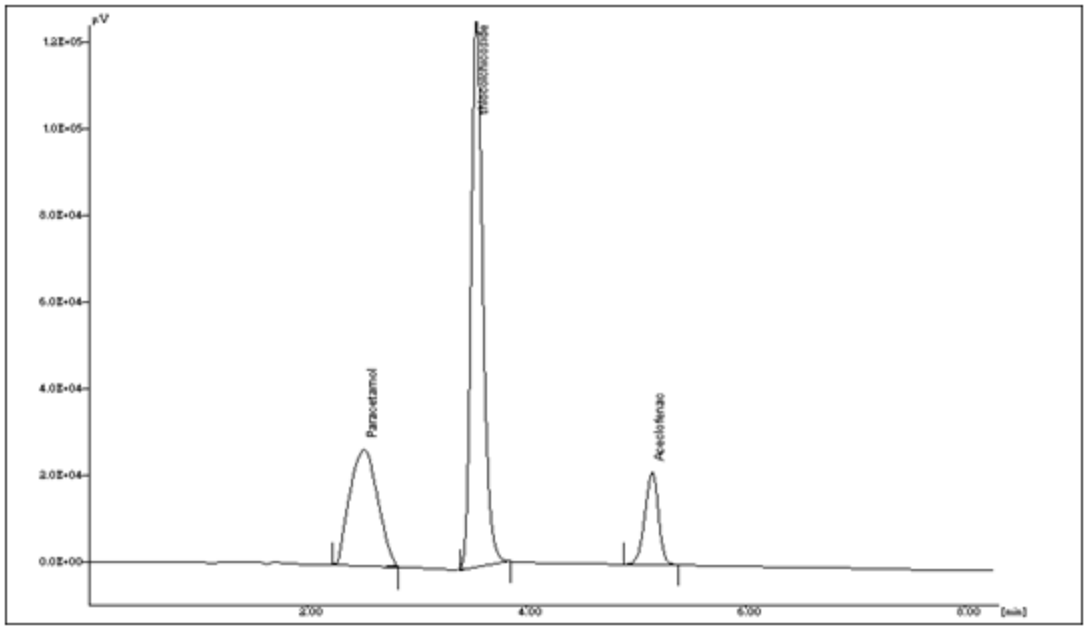

Figure 4 HPLC chromatogram of standard Paracetamol R_t (2.517), Thiocolchicoside R_t (3.558) and Aceclofenac R_t (5.200).

Mobile phase: acetonitrile: water (30: 70, v/v)

Concentration of drugs: 50 µg/mL for Paracetamol, 10 µg/mL for Thiocolchicoside and 0.4 µg/mL for Aceclofenac)
Application volume: 20 µL

Linearity and Range

Linearity of the method was studied by injecting six concentrations of the drug prepared in the mobile phase in the range of 50-175 µg/mL, 10-35 µg/mL and 0.4-1.4 µg/mL for Paracetamol, Aceclofenac and Thiocolchicoside, respectively in triplicate into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

Precision

Precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations 50, 100, 150 µg/mL for Paracetamol, 10, 20, 30 µg/mL for Aceclofenac and 0.4, 0.8, 0.12 µg/mL for Thiocolchicoside of the drug in hexaplicate on the same day. Intermediate precision of the method was checked by repeating studies on three different days. Additionally, the developed HPLC method was checked through separation studies on the mixture of reaction solutions on a different chromatographic system on a different day.

Limit of detection and limit of quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was injected six times following the same method as explained above. The signal to

noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. The LOD and LOQ were experimentally verified by diluting known concentrations of standard solution of Paracetamol, Aceclofenac and Thiocolchicoside until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

Robustness

To evaluate robustness of a HPLC method, few parameters were deliberately varied. The parameters included variation of flow rate, percentage of acetonitrile in the mobile phase and solvents from different lot were taken. Robustness of the method was done at three different concentration levels 50, 100, 150 µg/mL for Paracetamol, 10, 20, 30 µg/mL for Aceclofenac and 0.4, 0.8, 0.12 µg/mL for Thiocolchicoside.

Specificity

The specificity of the method towards the drug was established through study of resolution factor of the drug peak from the nearest resolving peak.The peak purity of Paracetamol, Aceclofenac and Thiocolchicoside 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). Effect of excipients of formulation was studied for whether it interfered with the assay.

Accuracy

Accuracy of the method was carried out by applying the method to drug sample (Paracetamol, Aceclofenac and Thiocolchicoside combination tablet) to which know amount of Paracetamol, Aceclofenac and Thiocolchicoside standard powder corresponding to 80, 100 and 120 % of label claim had been added (Standard addition method), mixed and the powder was extracted and analyzed by running chromatogram in optimized mobile phase.

Analysis of a marketed formulation

To determine the content of Paracetamol, Aceclofenac and Thiocolchicoside in conventional tablets (Brand name: Bakflex Plus, Label claim: Paracetamol 500 mg, Aceclofenac 100 mg, Thiocolchicoside 4 mg per tablet), twenty tablets were weighed, their mean weight determined and finely powdered. The average weight of the tablet triturate equivalent to 500 mg of Paracetamol, 100 mg of Aceclofenac and 4 mg of Thiocolchicoside was transferred into a 100 mL volumetric flask containing 60-65 mL methanol, sonicated for 30 min and diluted to 100 mL with methanol. The resulting solution was centrifuged at 3000 rpm for 5 min and the drug content of the supernatant was determined (5 mg/mL, 1 mg/mL, and 0.04 mg/mL for Paracetamol, Aceclofenac and Thiocolchicoside respectively). Then 1.0 mL of the above filtered solution was diluted to produce a concentration of 50 µg/mL, 10 µg/mL and 0.4 µg/mL for

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Paracetamol, Aceclofenac, and Thiocolchicoside respectively and 20 µL volume of sample solution was injected into HPLC, six times, under the conditions described above. The peak areas were measured at 263 nm and concentrations in the samples were determined using multilevel calibration developed on the same HPLC system under the same conditions using linear regression equation.

RESULTS AND DISCUSSION

The results of validation studies on simultaneous estimation method developed for Paracetamol, Aceclofenac and Thiocolchicoside in the current study involving acetonitrile: water (30: 70, v/v) are given below.

Linearity and Range

Paracetamol, Aceclofenac and Thiocolchicoside showed good correlation coefficient (r^2=0.9959 for Paracetamol, 0.9975 for Aceclofenac and 0.9984 for Thiocolchicoside) in given concentration range (50-175 µg/mL for Paracetamol, 10-35 µg/mL for Aceclofenac and 0.40-1.4 µg/mL for Thiocolchicoside). The equation for regression line was, y=11389x+7020 for Paracetamol, y =15521x+16800 for Aceclofenac and y=13144x+357.9 for Thiocolchicoside. The results show that an excellent correlation exists between response factor and concentration of drugs within the concentration range indicated above.

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.

Concentration (µg/mL)	Measured concentration ± SD, RSD (%)			
	Repeatability (n= 6)		Intermediate precision (n= 6)	
Paracetamol				
50	48.99	0.285	49.64	0.604
100	99.43	1.478	98.01	0.142
125	124.22	1.102	123.5	0.364
Aceclofenac				
10	09.03	0.996	09.44	1.27
20	18.93	1.32	19.49	0.615
30	29.77	0.26	29.56	1.72
Thiocolchicoside				
0.4	0.32	1.574	0.38	1.286
0.8	0.74	1.275	0.78	0.927
1.2	1.16	0.407	1.19	0.701

Table 2 Precision study for Paracetamol, Aceclofenac and Thiocolchicoside

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LOD and LOQ

Signal-to-noise ratios of 3:1 and 10:1 were obtained for the LOD and LOQ respectively. The LOD and LOQ was found to be 0.25 µg/mL and 0.5 µg/mL for Paracetamol, 1.0 µg/mL and 2.0 µg/mL for Aceclofenac and 0.24 µg/mL and 0.4 µg/mL Thiocolchicoside.

Robustness of the method

Each factor selected (except columns from different manufacturers) was changed at three levels (−1, 0 and 1). One factor at the time was changed to estimate the effect. Thus, replicate injections (*n* = 6) of mixed standard solution at three concentration levels were performed under small changes of three chromatographic parameters (factors). Insignificant differences in peak areas and less variability in retention time were observed (Table 3).

Table 3: Robustness Testing of Paracetamol, Aceclofenac and Thiocolchicoside (n=3)

Factor[a]	Level	Retention time	Retention factor	Asymmetry
Paracetamol				
A: Flow rate (mL/min)				
0.9	-0.1	2.521	0.008	1.091
1.0	0	2.517	0.006	1.085
1.1	+0.1	2.512	0.004	1.082
Mean ± SD (n = 3)		2.567 ± 0.05	0.006 ± 0.02	1.086 ± 0.04
B: % of acetonitrile in the mobile phase (v/v)				
29	-1	2.520	0.008	1.088
30	0	2.517	0.006	1.085
31	+1	2.515	0.006	1.082
Mean ± SD (n = 3)		2.517 ± 0.04	0.006 ± 0.01	1.085 ± 0.02
C: Solvents of different lot				
First lot		2.518	0.007	1.085
Second lot		2.520	0.008	1.087
Mean ± SD (n = 3)		2.519 ± 0.01	0.007 ± 0.01	1.086 ± 0.01

Factor[a]	Level	Retention time	Retention factor	Asymmetry
Aceclofenac				
A: Flow rate (mL/min)				
0.9	-1	5.203	1.081	1.233
1.0	0	5.200	1.080	1.231
1.1	+1	5.198	1.079	1.229
Mean ± SD (n = 3)		5.200 ± 0.2	1.080 ± 0.02	1.231 ± 0.03
B: % of acetonitrile in the mobile phase (v/v)				
29	-1	5.202	1.082	1.234
30	0	5.200	1.080	1.231
31	+1	5.198	1.078	1.227
Mean ± SD (n = 3)		5.200 ± 0.02	1.080 ± 0.02	1.230 ± 0.03
C: Solvents of different lot				

First lot		5.200	1.080	1.231
Second lot		5.202	1.082	1.233
Mean ± SD (n = 3)		5.201 ± 0.01	1.081 ± 0.01	1.232 ± 0.01
Factora	Level	Retention time	Retention factor	Asymmetry
Thiocolchicoside				
A: Flow rate (mL/min)				
0.9	-1	3.561	0.425	1.205
1.0	0	3.558	0.423	1.202
1.1	+1	3.552	0.420	1.198
Mean ± SD (n = 3)		3.557 ± 0.04	0.422 ± 0.03	1.201 ± 0.04
B: % of acetonitrile in the mobile phase (v/v)				
29	-1	3.562	0.424	1.204
30	0	3.558	0.423	1.202
31	+1	3.551	0.420	1.200
Mean ± SD (n = 3)		3.557 ± 0.04	0.422 ± 0.02	1.202 ± 0.02
C: Solvents of different lot				
First lot		3.558	0.423	1.202
Second lot		3.557	0.422	1.200
Mean ± SD (n = 3)		3.557 ± 0.01	0.422 ± 0.01	1.201 ± 0.01

Specificity

The peak purity of Paracetamol, Aceclofenac and Thiocolchicoside was assessed by comparing their respective spectra at the peak start, apex and peak end positions i.e., r (S, M) = 0.9992 and r (M, E) = 0.9989. A good correlation (r = 0.9993) was also obtained between the standard and sample spectra of Paracetamol, Aceclofenac and Thiocolchicoside respectively. Also, excipients from formulation were not interfering with the assay.

Recovery Studies

As shown from the data in Table 4 good recoveries of the Paracetamol, Aceclofenac and Thiocolchicoside in the range from 98 to 101 % were obtained at various added concentrations.

Table 4: Recovery studies of Paracetamol, Aceclofenac and Thiocolchicoside

Lable Claim (mg/ tablet)	Amount added %	Total amount	Amount recovered (mg ± % RSD)	Recovery %
Paracetamol 500 mg	80	900	899.12 ± 1.42	99.88
	100	1000	989.43 ± 0.45	98.94
	120	1100	1092.83 ± 0.78	99.31
Aceclofenac 100 mg	80	180	178.10 ± 1.61	98.77
	100	200	199.43 ± 1.27	99.93
	120	220	217.4 ± 0.46	98.63
Thiocolchicoside 4 mg	80	7.2	7.213 ± 1.03	100.15
	100	8.0	7.98 ± 0.62	99.50
	120	8.8	8.68 ± 1..25	98.27

Analysis of a formulation

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Experimental results of the amount of Paracetamol, Aceclofenac and Thiocolchicoside in tablets, expressed as a percentage of label claims were in good agreement with the label claims thereby suggesting that there is no interference from any of the excipients that are normally present. The drug content was found to be 99.90 % for Paracetamol, 98.87 % for Aceclofenac and 98.76 % for Thiocolchicoside. Two different lots of Paracetamol, Aceclofenac and Thiocolchicoside combination tablets were analyzed using the proposed procedures as shown in Table 5.

Table 5: Analysis of commercial formulation

Paracetamol (500 mg)	Paracetamol found (mg per tablet)	
	Mean ± SD (n= 6)	Recovery (%)
1st Lot	498.63 ± 0.95	99.72
2nd Lot	500.45 ± 1.03	100.09
Aceclofenac (10 mg)	Aceclofenac found (mg per tablet)	
	Mean ± SD (n= 6)	Recovery (%)
1st Lot	9.85 ± 0.76	98.54
2nd Lot	9.92 ± 0.96	99.21
Thiocolchicoside (4 mg)	Thiocolchicoside found (mg per tablet)	
	Mean ± SD (n= 6)	Recovery (%)
1st Lot	3.93 ± 1.03	98.27
2nd Lot	3.97 ± 0.91	99.25

CONCLUSION

HPLC method was developed and validated as per ICH guidelines. UV detection allowed an accurate quantitation of chromophoric compounds.

The drug was analyzed by HPLC method using HiQ Sil C_{18} (250 mm × 4.6 mm, 5.0 µ) from Japan, with a mobile phase consisting of acetonitrile: water (30: 70, v/v) at flow rate of 1.0 ml/min. Detection was carried out at 263 nm. The procedure has been evaluated for the linearity, accuracy, precision and robustness in order to ascertain the suitability of the analytical method. The method was also applied to marketed samples. It has been proved that the method is selective and linear between concentration range of 50-175 µg/mL, 10-35 µg/mL and 0.4-1.4 µg/mL for Paracetamol, Aceclofenac and Thiocolchicoside, respectively. The method was found to be accurate and precise as indicated by recovery studies and % RSD not more than 2. Moreover LOD and LOQ for Paracetamol was found to be 0.25 µg/mL and 0.5 µg/mL, for Aceclofenac it was found to be 1.0 µg/mL and 2.0 µg/mL and for Thiocolchicoside it was found to be 0.24 µg/ml and 0.4 µg/ml respectively. Thus the method is specific and sensitive.

Statistical analysis proves that the method is suitable for the analysis of Paracetamol, Aceclofenac and Thiocolchicoside as bulk drug and in pharmaceutical formulation without any interference from the excipients. It may be extended to study the degradation kinetics of Paracetamol, Aceclofenac and Thiocolchicoside and also for its estimation in plasma and other biological fluids.

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