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Original Article

Stability indicating Simultaneous estimation of Thiocolchicoside, Paracetamol and Diclofenac sodium in bulk drug and formulation by RP-HPLC

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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, Thiocolchicoside and Diclofenac Sodium. The method was carried out on a Kromasil, C18 ODS (100 mm x 4.8 m, 5µ), with a mobile phase consisting of acetonitrile: Phosphat buffer adjusted pH 3 with Ortho Phoosphoric acid at flow rate of 1.0 ml/min. Detection was carried out at 228 nm. This method has been applied to formulation also and it gives expected results 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 100 - 500 µg/mL for Paracetamol, for Thiocolchicoside and for Diclofenac Sodium. The mean values of the correlation coefficient, slope and intercept were found to be 0.99, 80865 and 34739 for Paracetamol and 0.99, 10284 and 41683 for Thiocochicoside and 0.99, 59407, 29275 for Diclofenac Sodium respectively. The method was validated for precision, robustness and Linearity. Stability indicating method also performed for the formulation and for the mixture of Active Pharmaceutical Ingredients by using 0.1 N HCl, 0.1 N NaOH, 1% H_2O_2, % H_2O_2. Thermal degradation was performed at 105 C for 48 hr. and Photolytic degradation were also performed for 48 hrs. Validation data showed that the method is repeatable and selective for the estimation of Paracetamol, Thiocolchicoside and Diclofenac Sodium.

Keywords: Paracetamol, Diclofenac Sodium, Thiocolchicoside, RP-HPLC, Validation, Stability indicating assay Method, PTD (Paracetamol, Thiocolchicoside, Diclofenac Sodium).

INTRODUCTION

Paracetamol: Paracetamol is chemically 4-hydroxy acetanilide. It is a weak inhibitor of peripheral cyclooxygenase and its analgesic effects may arise from inhibition of prostaglandin synthesis in the central nervous system. The antipyretic effects of paracetamol are due to its action at the level of the hypothalamus to reduce pyrogen-initiated alterations in body temperature by inhibiting prostaglandin synthesis. While generally safe for use at a recommended dose, toxicity of paracetamol is the foremost cause of acute gastro intestinal problems. [1]

Thiocolchicoside: Thiocolchicoside (THC) chemically, N-[(7S)-3-(beta-D glucopyranosyloxy)- 1, 2-dimethoxy-10-(methyl sulfanyl)-9- oxo-5, 6, 7, 9-tetrahydrobenzo[a]heptalen-7-yl]acetamide. It is a semi-synthetic derivative of the naturally occurring compound colchicoside with a relaxant effect on skeletal muscle, has been found to displace both [3H] gamma-amino butyric acid ([3H] GABA) and [3H] strychnine binding, suggesting an interaction with both GABA and strychnine sensitive glycine receptors. THC is potent competitive antagonist of GABA function, thereby acting as potent muscle relaxant and displays anti-inflammatory and analgesic properties. [2]

Diclofenac Sodium: Diclofenac is an anti-inflammatory agent. The exact mechanism of action is unknown but is thought to be inhibiting prostaglandin synthesis by inhibiting cyclooxygenase. It appears to exhibit bacteriostatic activity by inhibiting bacterial DNA synthesis. [3]

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A capsule formulation containing Paracetamol 325 mg, Thiocolchicoside 8 mg and Diclofenac Sodium 50 mg has been introduced in to clinical practice. A survey of literature revealed that there is not a single HPTLC, HPLC, LC-MS and spectrophotometric methods are reported for determination of Paracetamol, Thiocolchicoside and Diclofenac Sodium simultaneously from combined dosage form. [4-8] The present work describes the simple, precise and accurate RP-HPLC method for simultaneous estimation of Paracetamol, Thiocolchicoside and Diclofenac Sodium in tablets. It is validated by ICH guidelines. [9]

EXPERIMENTAL WORK

Materials: Working standards of pharmaceutical grade Paracetamol, Thiocolchicoside and Paracetamol were obtained as generous gift samples from Micro Labs, Mumbai, 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 Diclofenac Sodium and 99.98 % (w/w) on dry weight basis for Thiocolchicoside.

Instrumentation: The HPLC system consisted of Intelligent HPLC pump model (Perkin Elmer 200 B/250). 20 µL capacity per injection was used for sampling purpose. The detector consisted of a UV/ VIS. Data was integrated using Total Chrome Navigator system. The column used was, Kromasil, C_{18} ODS, (100 mm X 4.8 mm, 5 µm). Mobile phase consisted of a mixture of Acetonitrile: Phosphat buffer adjusted pH 3 with Ortho Phoosphoric Acid. A flow rate of 1.0 ml/min with detection at 228 nm. The mobile phase was filtered through a 0.45 micron membrane filter and degassed. The injection volume was 20 µL and analysis was performed at ambient temperature.

Pharmaceutical formulation: Fixed dose combination Capsules (ThioQuest DP 8) containing Paracetamol 325 mg, Thiocolchicoside 8 mg, and Diclofenac Sodium 50 mg (Batch no A120429) manufactured by Accent Pharma., Mettupalayam, 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 Thiocolchicoside and 1000 µg/mL of Diclofenac Sodium were prepared separately using Water. 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 325 µg/mL of Paracetamol, 8 µg/mL of Thiocolchicoside and 50 µg/mL of Diclofenac Sodium 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 specificity. Initially methanol: water in the ratio of (70: 30) was tried but baseline and peak were not proper separated. Later methanol: water in the ratio of (50: 50) was tried but peaks were not separated properly. Then ACN:Water in the ratio of (80:20) was tried, but In that case third Peak was not found. Later ACN:Water in the ratio of (60:40) was tried, but shape of the peaks were not proper. Later Methanol:ACN:Buffer (50:25:25) was used, but peaks were not separated properly. So from all these trials concluded that isocratic method can not be used for the separation of this drugs. Later Acetonitrile and phosphate buffer adjusted the pH 3 were used with gradient method to separate this drugs. By this method this formulation can be separated. The retention time are 5.33 mins, 9.61 mins, 21.47 mins. found for Paracetamol, Thiocolchicoside and for Diclofenac Sodium Respectively. It was found that Paracetamol, Thiocolchicoside and for Diclofenac Sodium gave acceptable retention time, theoritical plates, tailing factor and good resolution.

System Suitability Studies: The resolution, number of theoretical plates, tailing factor and peak asymmetry were calculated for the standard solutions as shown in Table. 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.

Validation of the method

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

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 100-500 µg/mL of each Paracetamol, diclofenac sodium and Thiocolchicoside in triplicate into the HPLC system keeping 20 µL the injection volume20 µL constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

Precision: Precision of the method was verified by intraday and interday precision studies. Repeatability studies were performed by analysis

of three different concentrations 100, 200, 300 µg/mL for Paracetamol, Diclofenac sodium and for Thiocolchicoside of the drug on the intraday. 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.

Robustness: To evaluate robustness of a HPLC method, few parameters were deliberately varied. The parameters included variation of flow rate, Change in wavelength were taken. Robustness of the method was done at three different concentration levels 100, 200, 300µg/mL for Paracetamol, Diclofenac sodium and 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.

Analysis of a marketed formulation: To determine the content of Paracetamol, Diclofenac sodium and Thiocolchicoside in capsule (Brand name: ThioQuest DP 8 , Label claim: Paracetamol 325 mg, Diclofenac 50 mg, Thiocolchicoside 8 mg per tablet). Twenty Capsule were weighed, their mean weight determined. The average weight of the capsule equivalent to 325 mg of Paracetamol, 50 mg of Diclofenac Sodium and 8 mg of Thiocolchicoside was transferred into a 100 mL volumetric flask containing 60-65 mL water, sonicated for 15 min and diluted to 100 mL with water. Then 1.0 mL of the above filtered solution was diluted to produce a concentration of 325 µg/mL, 50 µg/mL and 8 µg/mL for Paracetamol, Diclofenac sodium, 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 228 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.

Forced Degradation Study

The ICH guidelines indicate that stress testing is designed to help determine the intrinsic stability of the molecule by establishing degradation pathway in order to identify the likely degradation products and to validate the stability indicating power of the analytical procedure used.

Preparation of Standard solution: The stock solution (1000µg/ml) of Paracetamol was prepared by dissolving accurately weighed 10 mg of the drug, transferred into 10 mL volumetric flask, dissolved and made up to the volume by using water. The stock solution (1000µg/ml) of Thiocolchicoside was prepared by dissolving accurately weighed 10 mg of the drug, transferred into 10 mL volumetric flask, dissolved and made up to the volume by using water. The stock solution (1000µg/ml) of Diclofenac sodium was prepared by dissolving accurately weighed 10 mg of the drug, transferred into 10 mL volumetric flask, dissolved and made up to the volume by using water. Take 1ml of Paracetamol stock solution, 1ml of Thiocolchicoside stock solution, & 1ml of Diclofenac sodium stock solution using volumetric pipett transferred into 10 mL volumetric flask and diluted and made up to 10 ml volume by using water.

Preparation of Sample solution:

Take 1 Capsules accurately weighed, transferred into 100 mL volumetric flask, dissolved in water, sonicated using ultrasonicator for 30 mins, made up to the volume by using water. Take 10 ml of solution and transferred into 100 mL volumetric flask & made up to the volume by using water. (Concentration 325 µg/ml, 50 µg/ml, 8 µg/ml of PTD Respectively).

Then In all degradation studies the average peak area of PTD after application of six replicates was obtained. Forced degradation was performed for PTD in acidic, alkali oxidative, thermal, and photolytic conditions.

Acid and Alkali induced-degradation: To 5 ml of stock solution, 5 ml of each 0.1M HCl and 0.1M of NaOH respectively were added to obtain the working standards of 500 µg/ml. The degradation was performed at 50^0 C for 4 hrs. Samples were withdrawn at 30 mins., 1 hr., 2 hr. and at 4 hr. From the resultant solutions 200 µl was taken and made volume 1 ml using diluent to obtain the concentration of 100 µg/ml and 20 µl were injected into the system.

Thermal degradation: Pure PTD drugs when subjected to dry heat at 105°C for 24 hours. To 10 ml of stock solution of 100 µg/ml pure PTD, to study the degradation under thermal conditions.

Hydrogen peroxide induced-degradation: To 5 ml of stock solution of 100 µg/ml pure PTD, 5 ml of 1 % Hydrogen peroxide solution was added. Samples were withdrawn at 30 mins., 1 hr., 2 hr. and at 4 hr. From the resultant solutions 200 µl was taken and made volume 1 ml using diluent to obtain the concentration of 100 µg/ml and 20 µl were injected into the system.

Photochemical degradation: The photochemical stability of the drug was studied by exposing the stock solution of 1000 µg/ml. PTD were kept in photo stability chamber for 5 days. The resultant solution was diluted to obtain concentration of 100 µg/ml and 20 µl were injected into the system under optimized conditions.

RESULTS AND DISCUSSION

The results of validation studies on simultaneous estimation method developed for Paracetamol, Diclofenac sodium and Thiocolchicoside in the current study involving acetonitrile and Phosphate buffer are given below.

Linearity and Range: Paracetamol, Diclofenac Sodium and Thiocolchicoside showed good correlation coefficient (r2=0.99 for Paracetamol, 0.997 for Diclofenac Sodium and 0.99 for Thiocolchicoside) in given concentration range (100-500 µg/mL for Paracetamol, Diclofenac Sodium for Thiocolchicoside). The equation for regression line was, y = 80865x + 34739 for Paracetamol, y = 59407x + 29275 for Diclofenac Sodium and y = 10284x + 41683 for Thiocolchicoside. The results show that an excellent correlation exists between response factor and concentration of drugs within the concentration range indicated as above.

Precision: The results of the repeatability and intermediate precision experiments are shown in table 3 to 5. 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.

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. Result have been show in table 6 and 7.

Specificity: The specificity of the given HPLC method was determined in presence of its degradation products along with other parameters like retention time (tR), capacity factor (k), tailing or asymmetrical factor (T) etc. Result Shown in Table 8. From the above results it was found that there is no interference of the drug peaks, therefore the method is specific.

Forced Degradation Studies: Forced degradation was performed for drug product in acidic, alkaline, oxidation, thermal and photolytic conditions. All degradants peaks were well resolved from Paracetamol, Thiocolchicoside, Diclofenac sodium peaks in the chromatograms of all stressed samples.

Acid induced degradation studies

Paracetamol, Thiocolchicoside, Diclofenac sodium when treated with 0.1M HCl at 50°C for 4 hrs. The chromatograph is shown below.

Alkali induced degradation studies

Paracetamol, Thiocolchicoside, Diclofenac sodium when treated with 0.1M NaOH at 50°C for 4hrs. The chromatograph is shown below.

Hydrogen peroxide induced degradation studies

Drug product was treated with hydrogen peroxide (1% H_2O_2 & 3% H_2O_2) solution for 4hr. Paracetamol, Thiocolchicoside, Diclofenac sodium. The chromatograph is shown below.

Thermal degradation studies

PTD when subjected to placed at 105°C for 48 hrs. This indicated that PTD was stable in dry heat. Chromatogram is shown below.

Photolytic degradation studies

PTD showed no significant degradation when subjected to photolytic degradation. PTD was found to be stable. The chromatogram is shown in figure below.

All the results obtained from forced degradation studies are summarized in Table 9

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 Kromasil, C_{18} ODS, (100 mm X 4.8 mm, 5 µm), with a mobile phase consisting of acetonitrile and buffer adjusted pH 3 with ortho phosphoric acid at flow rate of 1.0 ml/min. Detection was carried out at 228 nm. The procedure has been evaluated for the Specificity, linearity, 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 100 -500 µg/mL, for Paracetamol, Diclofenac Sodium and Thiocolchicoside. The method was found to be accurate and precise as indicated by results and % RSD not more than 2. Thus the method is specific and sensitive. Statistical analysis proves that the method is suitable for the analysis of Paracetamol, Diclofenac sodium and Thiocolchicoside as bulk drug and in pharmaceutical formulation without any interference from the excipients. Force degradation study was also found to be satisfactory.

Table1: Gradient programme for method Develpoment

Time (min.)	A %	B %
0.5	5	95
3	17	83
10	25	75
8	90	10
3	5	95
3	5	95

Table 2: System Suitability Report

Chromatogram	Retention time	Tailing Factor	Area	Plate Count
Paracetamol	5.33	1.18	4073703	30145
Thiocolchicoside	9.61	1.15	5533601	41577
Diclofenac Sodium	21.47	1.16	5891643	628860

Table 3 Precision for Paracetamol

Concentration (µg/mL)	Intraday Precision		Interday Precision	
	Area	%RSD	Area	%RSD
200	1644142.14	1.84	1626127.69	1.78
300	2470591.23	1.71	2518108.27	1.63
400	3117121.23	1.99	3194712.76	1.88

Table 4 Precision for Thiocolchicoside

Concentration (µg/mL)	Intraday Precision		Interday Precision	
	Area	%RSD	Area	%RSD
200	2135485.69	1.75	2136845.52	1.71
300	3091919.98	1.93	3102596.10	1.58
400	4138882.10	1.89	4201395.63	1.88

Table 5 Precision for Diclofenac Sodium

Concentration (µg/mL)	Intraday Precision		Interday Precision	
	Area	RSD	Area	RSD
200	2417771.57	1.99	2310174.91	1.95
300	3720121.50	1.96	3723560.48	1.69
400	4764262.14	1.89	4851926.14	1.84

Table No. 6 Change in flow rate

Level	Retention time (minute)		
	Paracetamol	Thiocolchicoside	Diclofenac Sodium
-1	**5.43**	9.69	21.56
0	**5.33**	9.60	21.47
+1	**5.28**	9.54	21.23
S.D	0.076	0.075	0.17
%RSD*	1.42	0.78	0.79

Table No. 7 Change in wavelength

Level	Retention time (minute)		
	Paracetamol	**Thiocolchicoside**	**Diclofenac Sodium**
-1	5.30	9.61	21.49
0	5.34	9.62	21.45
+1	5.35	9.66	21.51
S.D	0.026	0.026	0.030
%RSD*	0.49	0.27	0.14

Table No. 8 Peak results of specificity

Name	RT	Area	Theoretical Plate count	Tailing
Paracetamol	5.33	4073703	30145	1.189
Thiocolchicoside	9.6	5533601	41577	1.159
Diclofenac Sodium	21.47	5891643	628860	1.167

Table No. 9 - Summary of forced degradation study

Stress Condition		% Degradation		
		Paracetamol	**Thiocolchicoside**	**Diclofenac Sodium**
Acid	0.1M HCl (4hr at 50°C)	5.76	29.44	21.42
Alkali	0.1M NaOH (4hr at 50°C)	7.69	8.33	17.33
Oxidation	3% H_2O_2 (4hr at 50°C)	7.67	12.5	17.40
Thermal	48 hr at 105°C	23.07	12.5	4.66

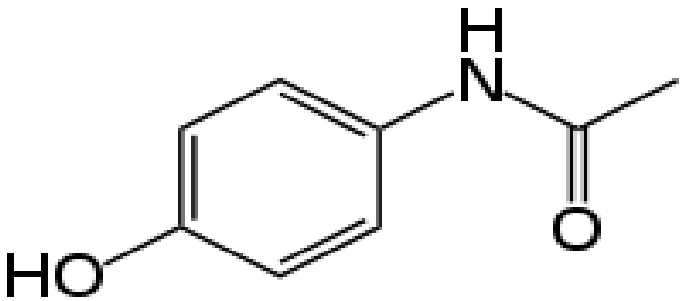

Figure 1 Structure of Paracetamol

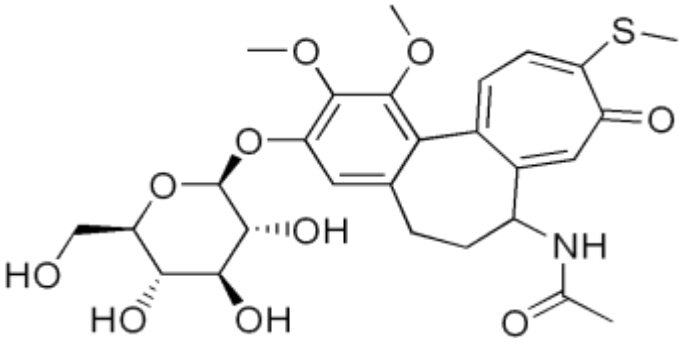

Figure 2 Structure of Thiocolchicoside

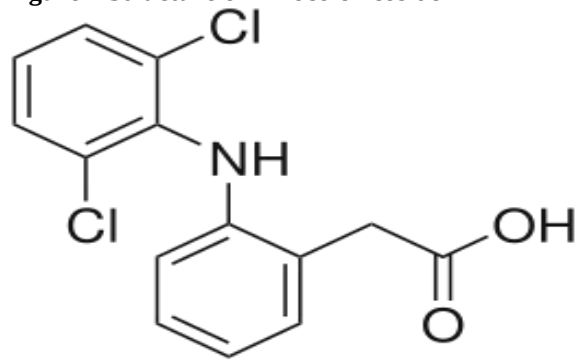

Figure 3 Structure of Diclofenac Sodium

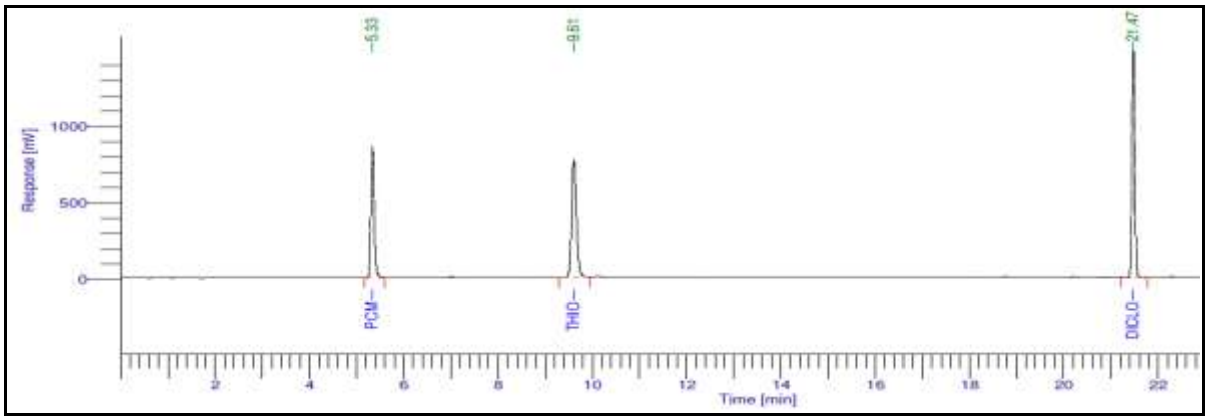

Figure 4 Chromatogram of the separated peak

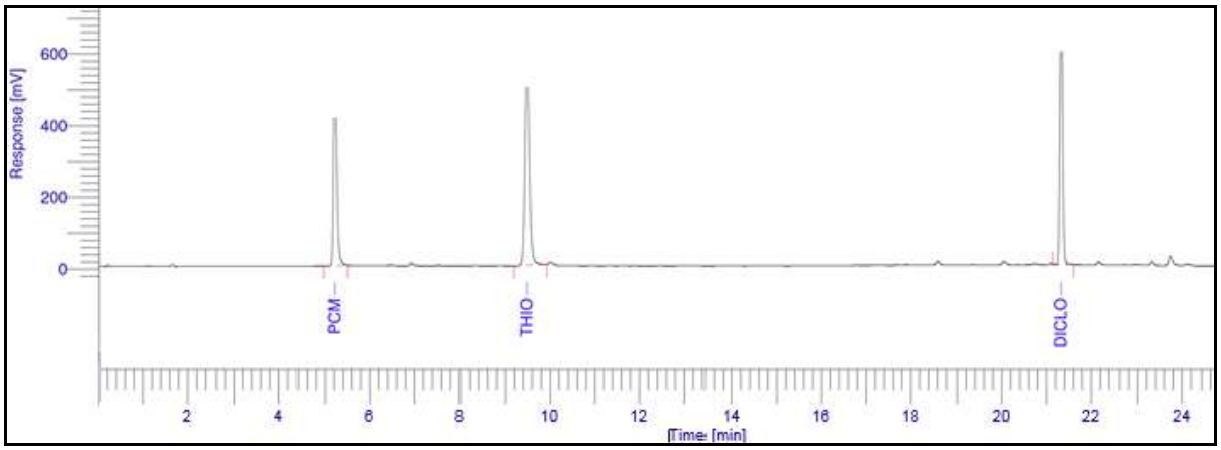

Figure 5 Chromatograph of Sample preparation

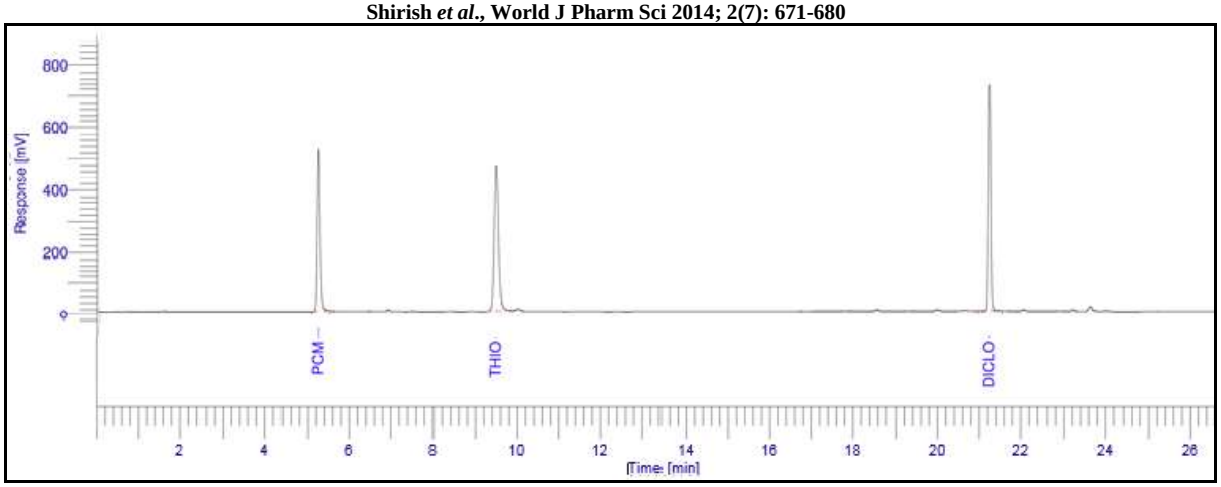

Figure 6 Chromatograph of Standard preparation

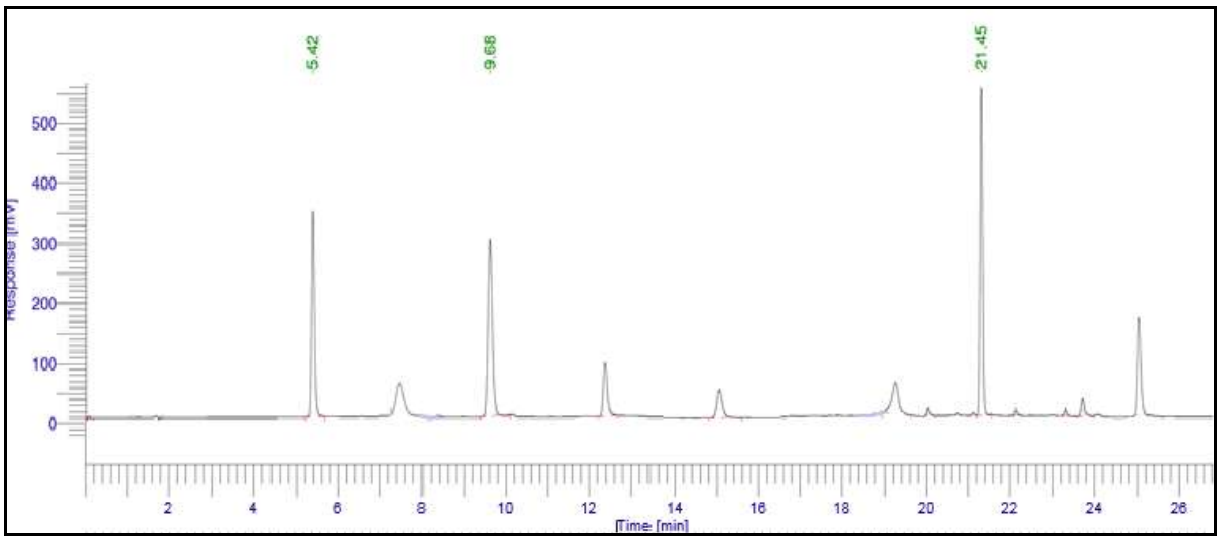

Figure 7 Chromatograph of acid (0.1M HCl, 4hrs at 50°C) treated PTD

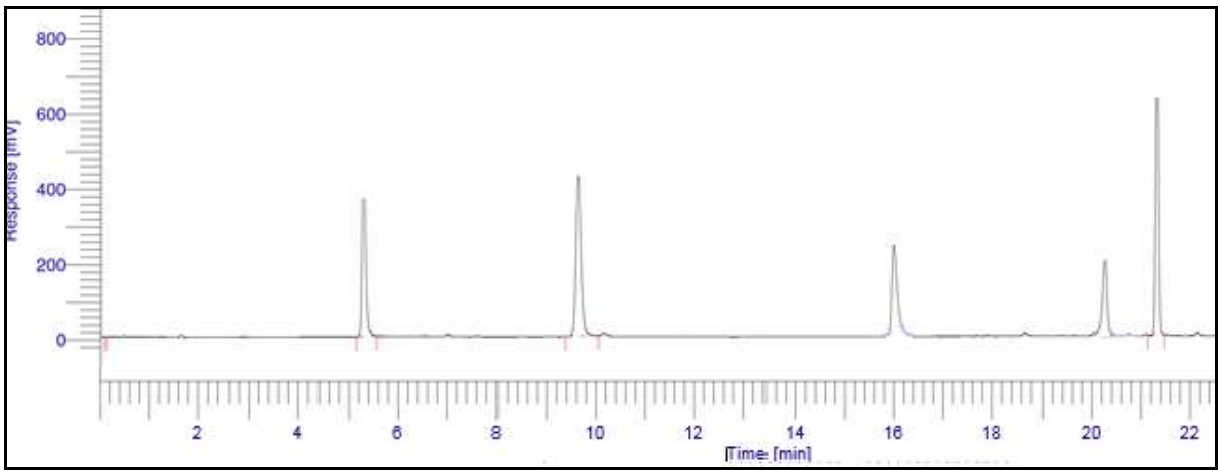

Figure 8 Chromatograph of alkali (0.1M NaOH, 4hrs at 50°C) treated PTD

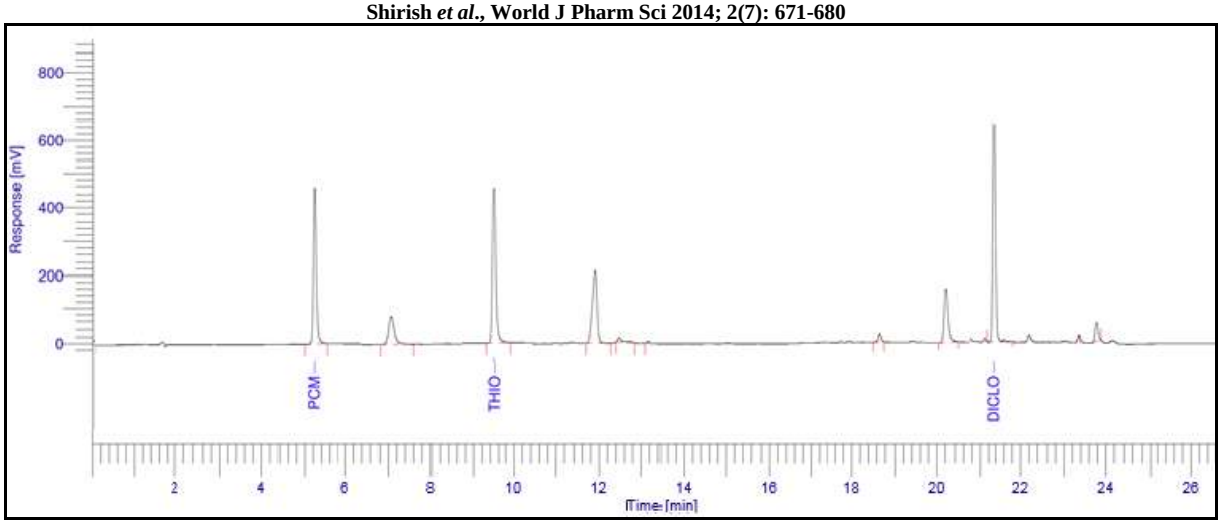

Figure 9 Chromatograph of H_2O_2 (3% H_2O_2, 4hrs) treated PTD.

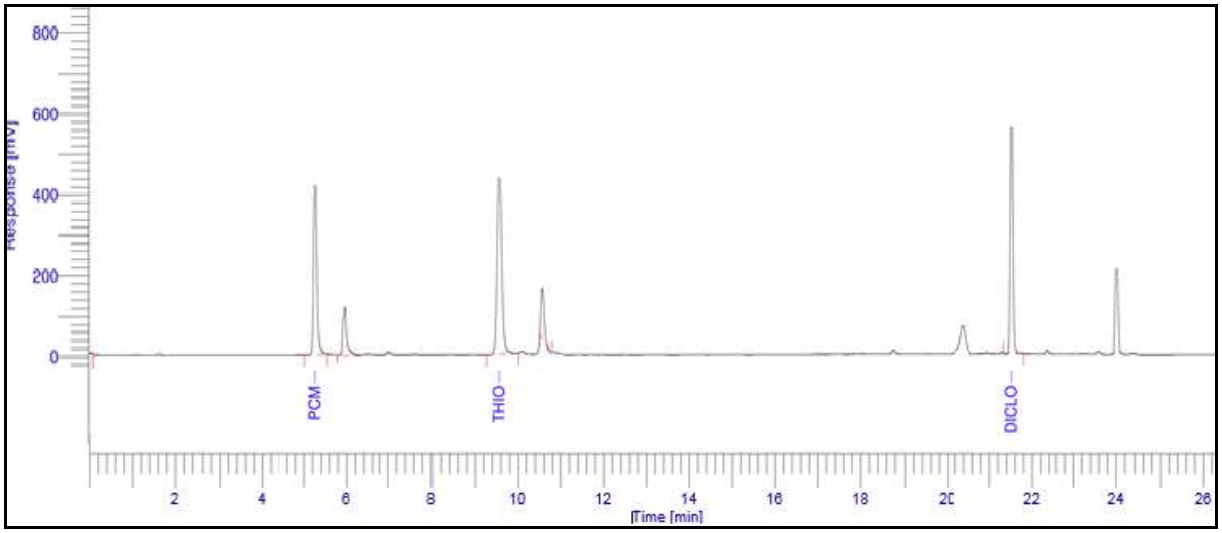

Figure 10 Chromatograph of thermal (48 hrs at 105°C) treated PTD.

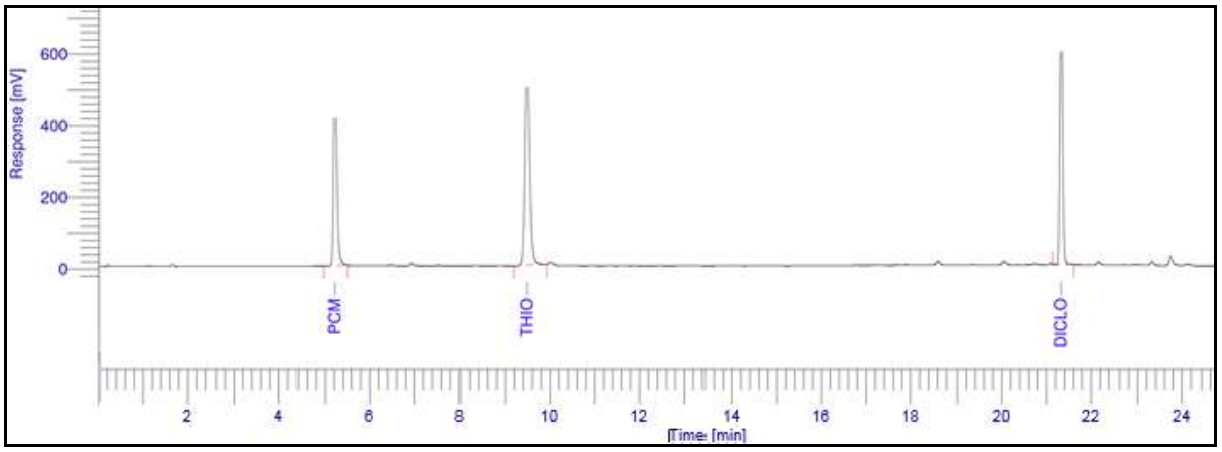

Figure 11 Chromatograph of photolysis (photolytic chamber, 2days) treated PTD

REFERENCES

1. Adewuyi G. O., Ogunneye A. L. UV-Spectrophotometry and RP-HPLC methods for the simultaneous estimation of acetaminophen: Validation, comparison and application for marketed tablet analysis in South West, Nigeria. Journal of Chemical and Pharmaceutical Research, 2013; 5(5):1-11.
2. M. T. Harde et al. Development and Validation of UV-Visible Spectrophotometric Methods for Simultaneous Estimation of Thiocolchicoside and Dexketoprofen in Bulk and Tablet Dosage Form. International Journal of Pharmaceutical Sciences and Drug Research 2012; 4(2): 160-163.
3. M. D. azeez1. et al. Spectrophotometric Determination of Paracetamol and Diclofenac in combined dosage form by Simutaneous Equation Method using UV-VIS Spectrophotomeetry. World Journal of Pharmaceutical Research. 2013; 2 (4): 917-923.
4. B. Gowramma, S. et al. A Vaiated RP-HPLC Method for simultaneous Estimation of paracetamol and Diclofenac potassium in Pharmaceutical formulation. International Journal of ChemTech Research. 2010; 2(1): 676-680.
5. Sagar B.Wankhede, et al. RP- HPLC Method for Simultaneous Estimation of Thiocolchicoside and Ketoprofen in Combined Dosage Form. Scholars Research Library. 2010; 2(3): 315-320.
6. Rachana R Joshi., Krishna R Gupta. UV-Spectrophotometric Determination of Thiocolchicoside in capsule. Scholars Research Library. 2010; 2(2): 384-391.
7. Venkatachalam T., Lalitha K.G., RP –HPLC Method for Simultaneous analysis of Thiocolchocoside and Ketoprofen in Tablet Formulations. International journal of Pharmacy and Pharmaceutical sciences. 2013; 5(4): 679-682.
8. Madhukar A. Badgujar et al. Mangaonkar. Simultaneous Determination of Paracetamol, Chlorzoxazone and Diclofenac Sodium in Tablet Dosage Form by High Performance Liquid Chromatography. E-Journal of Chemistry. 2011; 8(3): 1206-1211.
9. ICH, Guidance for industry; Q2B Validation of analytical procedure: Methodology.
10. G.D. Christian, Analytical Chemistry, 6th ed., John Wiley and Sons Pvt., 2007; 1-5.
11. A.H. Becket, J.B. Stenlake, Practical Pharmaceutical Chemistry, CBS Publications and Distributors, New Delhi, 2005; 2(4):157-168.
12. M.W. Dong, Modern HPLC for Practicing Scientists, A John Wiley & Sons,inc.,New Jersey, 2006; 135-155.
13. Skoog, West, Holler, Crouch, Fundamentals of analytical chemistry, 8th ed., cengage learning, 920-921.
14 Willard, Instrumental method of analysis, 7th ed., CBS publisher, 580-582.
15 R.D. Braun, Introduction to instrumental analysis, Mc-Graw-Hill, 839-889.
16. Skoog, West, Holler, Crouch, Fundamentals of analytical chemistry, 8th ed., cengage learning, 973-995.
17. Y Kazakevich, R Lobrutto, HPLC for Pharmaceutical Scientist, 2nd ed., A John Wiley & Sons, inc. 2007; 687-688.
18. Y Kazakevich, R Lobrutto, HPLC for Pharmaceutical Scientist, 2nd ed., A John Wiley & Sons, inc. 2007; 691-695.
19. L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, 2nd ed., Wiley-Interscience Publication, New York. 1997; 7-56.
20. G.D. Christian, Analytical Chemistry, 6th ed., John Wiley and Sons Pvt., 2007; 613-618.
21. ICH, Q2 (R1): Validation of analytical procedures: Text and methodology, International Conference on Harmonization, Geneva, 2005.