ISSN : 0974 - 7419 Volume 11 Issue 7,8

Trade Science Inc.

Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ, 11(7,8), 2012 [274-281]

Simultaneous determination of two antispasmodic drugs in bulk, pharmaceutical products and body fluid by a validated, acetonitrile free, cost effective and stability indicating reverse phase high performance liquid chromatographic method

Najmul Hasan[1], Nawab Sher Afridi[2], Sauleha Khan*[2], Muhammad Zain Siddiqui[3], Mathurot Chaiharn[4]

[1]Department of Microbiology, Faculty of Science, University of Karachi, Karachi - 75270, (PAKISTAN)

[2]Department of Chemistry, Faculty of Science, University of Karachi, Karachi - 75270, (PAKISTAN)

[3]Department of Chemistry, Faculty of Science, Federal Urdu University of Science & Technology, Karachi - 75270, (PAKISTAN)

[4]Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai - 50290, (THAILAND)

E-mail: sauleha.chem@hotmail.com

Received: 18th March, 2012 ; Accepted: 18th April, 2012

ABSTRACT

A stability indicating, accurate, specific, precise, and simple reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated for the simultaneous determination of Phloroglucinol (PGD) and Trimethylphloroglucinol (TMP) in bulk, pharmaceutical dosage forms of tablet and in body fluid. Methanol: Buffer: Sulfuric Acid 0.1mol/l (60:40:0.3) was the mobile phase at flow rate 1.0 ml/min using a Symmetry C18 column monitored at 234nm. The API's were subjected to stress conditions of hydrolysis (acid, base, oxidation, and thermal degradation). Maximum degradation was observed in acid and 35% H_2O_2 while found stable in the other stress conditions. The calibration curve was linear with a correlation coefficient of more than 0.9995 for both drugs. The averages of the absolute and relative recoveries were found to be 100.154% and 99.993% for Phloroglucinol and Trimethylphloroglucinol respectively, with 30pg/ml LOD and with 300pg/ml LOQ. The developed method was validated with respect to linearity, accuracy, precision, system suitability, specificity and robustness. The forced degradation studies prove the stability indicating power of the method. The proposed HPLC method was successfully applied to quantify the amount of Trimethylphloroglucinol and Phloroglucinol in bulk, dosage form and body fluid in quality control.

KEYWORDS

Trimethylphloroglucinol;
Phloroglucinol;
Acetonitrile-free;
RP-HPLC;
Body fluid.

INTRODUCTION

Phloroglucinol is white or yellowish white crystals, with melting point 215-219°C, slightly soluble in water, soluble in alcohol and in ether, chemically benzene-1,3,5-triol having molecular formula $C_6H_6O_3$(Figure 1a) while trimethylphloroglucinol is 1,3,5-trimethoxybenzene with molecular formula $C_9H_{12}O_3$(Figure 1b). Phloroglucinol is used as an antispasmodic, often in combination with trimethylphloroglucinol and is regarded to be effective in decreasing smooth muscle contraction and in alleviating the pain consequent to smooth muscle spasms. Under spastic conditions the antispasmodic effect is greater against muscle than against smooth muscle under physiological condition. Phloroglucinol is indicated for symptomatic treatment of colics due to renal and biliary calculi, acute pain, urinary passages or in gastrointestinal tract, pain of uncertain genesis in the abdominal region, spastic conditions of the female genital system; dysmenorrheal[1-3]. Phloroglucinol is a non atropinic antispasmodic agent which is used by oral route, rectal route, intramuscular or intravenous injection for its spasmolytic properties against digestive or urinary tract. Phloroglucinol is primarily used to treat the pains in the digestive functional disorders, in the renal colics (pains related to urinary conditions) and in certain pains in gynaecology. The drug was extensively used in the past for augmentation of labour[4].

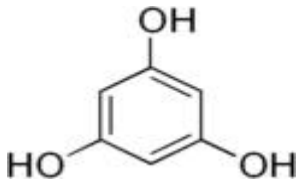

Figure 1a : Phloroglucinol.

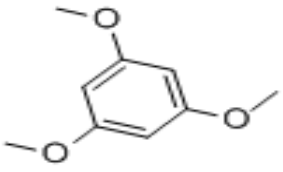

Figure 1b : Trimethylphloroglucinol.

Some methods of analysis of phloroglucinol are available including supercritical fluid extraction and high-performance liquid chromatographic determination of phloroglucinol in St.John's Wort[5]. Determination of phloroglucinol in human plasma by HPLC-mass spectrometry[6] and by gas chromatography-mass spectrometry[7-8], fast HPLC analysis of naphthodianthrones and phloroglucinols from hypercium perforatum extracts are also reported[9-10] determined by Titrimetric method. Other methods reported includes[11-14].

However there is no simple and sensitive method to be followed in general quality control laboratories especially on industrial basis. Therefore still there was a need for an analytical method which helps to determine the APIs in bulk drug, commercial products and body fluid. Keeping this point into consideration, an attempt was made to develop a simple, sensitive and validated stability indicating RP-HPLC method using UV detection. The applicability of the method was confirmed for analysis in pharmaceutical products. The results of analysis were validated in accordance with ICH guidelines[15].

MATERIAL AND METHODS

The present method was designed to be easy to use, sensitive, rapid and cost effective with simple sample preparation for PGD & TMP. Separation and quantification of PGD & TMP in pharmaceutical drug formulations and urine were achieved with an isocratic elution.

Material and reagents

Trimethyl Phloroglucinol and Phloroglucinol were a kind gifts from National Pharmaceuticals, sulphuric acid Merck, (Germany), Methanol (HPLC grade) were purchased from Fisher Scientific. The tablets containing PGD and TMP were obtained from commercial source (SPASFON®, SPADIX®, FUROSINOL®, and ANAFORTAN PLUS®) labeled to contain 80mg of both drugs/ tab. Distilled and deionized water was obtained by passage through RO plant (Waterman, Pakistan) and was further filtered through a 0.45 µm membrane filter (Millipore, Bedford, MA, USA).

Apparatus

For chromatography a SIL 10A auto injector HPLC system comprising of SCL 10A system controller, SPD 20A prominence UV/VIS detector, with a Shimadzu LC 20 AT pump with LC

Solutions software was used. Separation was performed on a Hibar® µBondapak® ODS C18 HPLC column, (4.6 x 250 mm; 5 µm bead size) maintained at 25°C. A UV-visible Shimadzu 1650 PC spectrophotometer with UV Probe software, ultrasonic cleaner (Elmasoni E 60 H), Jenway 3240 pH meter and Sartorious TE2145 analytical balance were used in the research work. Through out the work only amber glass flasks were used to avoid light effect on the solution of PGD & TMP standards and samples.

Chromatographic conditions

The HPLC analysis was carried out at ambient temperature. The compound was chromatographed isocratically with a mobile phase consisting of Methanol (HPLC grade): Heptane Sulfonate Buffer (0.005mol/l): Sulfuric acid 0.1mol/l (60:40:0.3, v/v/v) with the pH adjusted if required to 3.0 ± 0.1 using Sulfuric acid 0.1mol/l. While for sample and standard preparation diluent was prepared from Methanol (HPLC grade): D.I. Water (50:50, v/v %). The mobile phase was filtered by passing through a 0.45µm membrane filter (Millipore, Bedford, MA, USA). The flow rate was 1.0 ml/min, and the injected volume was 20µl. The effluent was monitored spectrophotometrically at lmax: 234 nm.

Analytical procedure

Standard preparation

In a 100ml volumetric flask, weighed accurately about 20.0 mg of PGD & TMP reference standards. Dissolved up to 50ml in diluent sonicated for 5 minutes let it cool to room temperature and make up volume with the same solvent, stirred well for 20 minutes to get 200ug/ml working standard solution of both the API's. Filter through 0.45micron filter paper.

Sample preparation

Assay of tablets containing the drug

For making sample of 200ug/ml PGD & TMP, 20 tablets were weighed and ground to get an evenly homogenized powder. The sample was weighed accurately equivalent to 80mg of PGD & TMP and taken in 100ml volumetric flask and 50 ml of diluent was added. The sample was sonicated for 10 minutes and then added diluent up to the mark, placed for stirring for 20 minutes to cool down the temperature. The solution was diluted in a 50 ml volumetric flask to get 200ug/ml working standard solution by adding 12.5 of stock solution. The sample was then filtered through 0.45µm filter paper and injected into the HPLC system.

Assay of tablets for degradation studies of drugs

For this purpose the 12.5ml of stock sample solution was diluted in four individual 50 ml volumetric flasks and 15 ml of degrading agent were added to each flask separately, with exception of one to which only diluent was added; these included 0.1 N HCL, 0.1 N NaOH, 35% H_2O_2 and then to each flask diluent was added up to the mark. All the four samples were placed in water bath at 60ºC for one hour. The samples were then filtered through 0.45µm filter paper and injected into the HPLC system.

Sample preparation for body fluid

Urine samples were collected from three healthy volunteers and were stored at 4°C for 24 hrs. For making sample 12.5ml of stock solution was taken in 50ml flask and 10 ml of diluent was added followed by 27.5ml of urine sample. The sample was sonicated for 05 minutes and the placed for stirring for 10 minutes to cool down the temperature and then added diluent up to the mark. The sample was then filtered through 0.45µm filter paper and injected into the HPLC system.

Stability and forced degradation studies

Stability testing is an important part of the process of drug product development. The purpose of stability testing is to provide evidence of how the quality of a drug substance or drug product varies with time under a variety of environmental conditions, for example temperature, humidity, and light and enables recommendation of storage conditions, retest periods, and shelf life to be established. The two main aspects of drug product that play an important role in shelf-life determination are assay of the active drug and the degradation products generated during stability studies.

The commercially available tablet samples were placed at accelerated conditions of temperature that is at 40°C with 75% relative humidity and at 30°C with 65% relative humidity in environmental chamber for six months. The stability protocol mentioned elsewhere was followed and assays were made as mentioned in method development.

Intentional degradation was attempted to stress conditions exposing the drugs with acid (0.1mol/l HCl), alkali (0.1mol/l NaOH), hydrogen peroxide (35%), heat (60ºC) to evaluate the ability of the proposed method to separate drugs from its degradation products. For all conditions the study period was one hour.

Method validation

The method validation was performed in accordance with ICH guideline. For the assay validation various procedures were performed including specificity, linearity, range, accuracy, intra-day and inter-day precision[15].

To study the linearity of standard solutions, twenty dilutions were prepared to give standards solution in range of 10% to 200 %. The standard calibration curve was generated using regression analysis with Microsoft excel. For specificity commonly used excipient in tablet preparation were spiked in a pre-weighed quantity of drugs and then peak areas were measured and calculations done to determine the quantity of the drugs.

The precision was studied to find out intra and inter day variations in the test method of PGD & TMP in the concentration range of 160-240 µg/ml for three times on the same day and three different days. Precision was determined by analyzing corresponding standard daily for a period of three days. Analysis was performed at three different days with three separate samples from same homogeneous bulk. For determining accuracy the PGD & TMP reference standard were accurately weighed and added to a mixture of the tablet excipients, at three different concentration levels (160, 200 and 240ug/ml of PGD & TMP). At each level, samples were prepared in triplicate and the recovery percentage was determined.

Standard solutions were prepared by sequential dilutions at decreasing concentrations, in the range of 10–500 pg/ml of PGD & TMP and injected onto the chromatographic system. The limit of detection was defined as the concentration for which a signal-to-noise ratio of 3 was obtained and, for quantitation limit; a signal-to-noise ratio of 10 was considered.

The robustness was studied by analyzing the same samples of PGD & TMP by deliberate variation in the method parameters, such as in the chromatographic conditions, like mobile phase, flow rate, temperature etc.suitability of the method was evaluated by analyzing the symmetry of the standard peaks, resolution and theoretical plates of the column.

RESULTS AND DISCUSSION

The HPLC method development and its validation are the most prioritized requirement for any drug available in the market to have high quality products. A few methods are available for determination of PG and TMP as described earlier, but many of them are used only for certain definite objectives and no one can be generalized for their simultaneous determination in form of pharmaceutical products and body fluid. Similarly none of them is as much sensitive as ours is; in terms of its Precision, accuracy, %recovery and LOD & LOQ especially as compared to (9, 12, and 14) whose LOD or LOQ were in microgram range only.

Method development

For developing an efficient method for the simultaneous analysis of PGD & TMP, parameters such as detection wavelength, mobile phase composition, optimum pH and concentration of the standard solutions were comprehensively studied. Both PGD & TMP were diluted in dilution solvent and then run through UV spectrophotometer in UV range of 190nm – 400nm to get maxima and a wavelength i.e. 234nm was determined where maximum absorbance was gained (Figure 2). Mobile phase was selected in terms of its components and their proportions. The chromatographic parameters were evaluated using a Symmetry C18 column, the mobile phase composed of Methanol: Buffer of given proportion promoted a short run time (10 min) without any interference, so this condition was adopted in sub-sequent analyses.

Full Paper

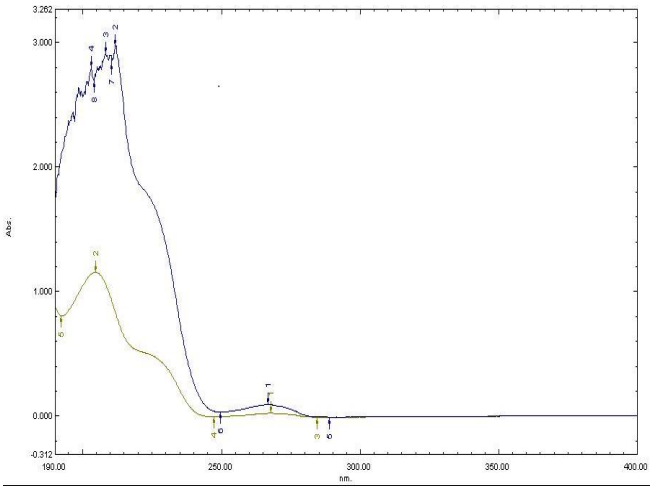

Figure 2 : UV Scan of PGD and TMP.

The literature survey also revealed that almost all the methods developed so far have utilized acetonitrile as a major constituent in mobile phases. ACN is always preferred due to the supreme solubility properties and UV absorbance characteristics of acetonitrile, and there is no counterpart substitute for acetonitrile in the reverse-phase HPLC, UV application., keeping in view the increasing shortage of acetonitrile "Great Acetonitrile Shortage", and high cost, laboratories are in search of cost-effective solutions to manage the impact on their research and business timelines. Also considering the chromatography type and the detection wavelengths in use, it may be possible to replace Acetonitrile with methanol or with a longer chain alcohol. Also as Methanol is less expensive than acetonitrile, therefore the use of methanol as an alternative solvent to acetonitrile was evaluated in analysis on large industrial basis, and hence a cost effective and easy to use method has been developed.

Method validation

The linearity ranges were found in the range of 20-400 µg/ml. The assay was judged to be linear as the correlation coefficient was greater than 0.995 by the least-square method. A linear correlation was found between the peak areas and the concentrations of API's, in the assayed range. The regression analysis data are presented in TABLE 1 and Figure 3a, 3b.

Chromatogram shown in Figure 4a proves specificity or selectivity of the assayed method, as the chromatogram in samples were found identical with standard chromatogram and no interfer-

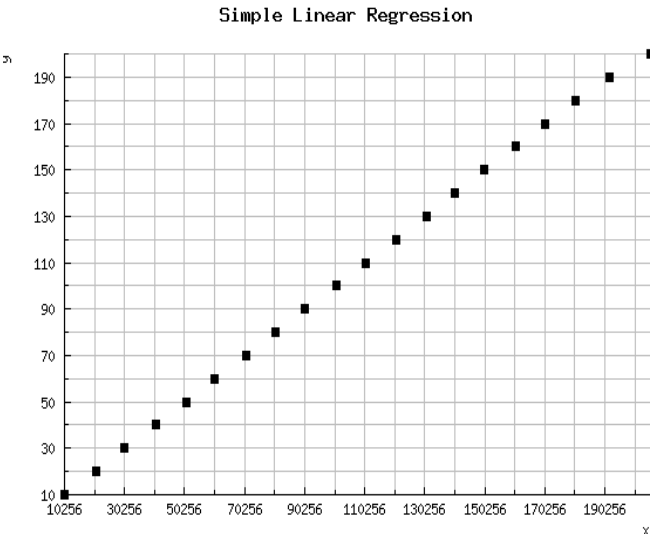

Figure 3a : Linearity of the method for PGD.

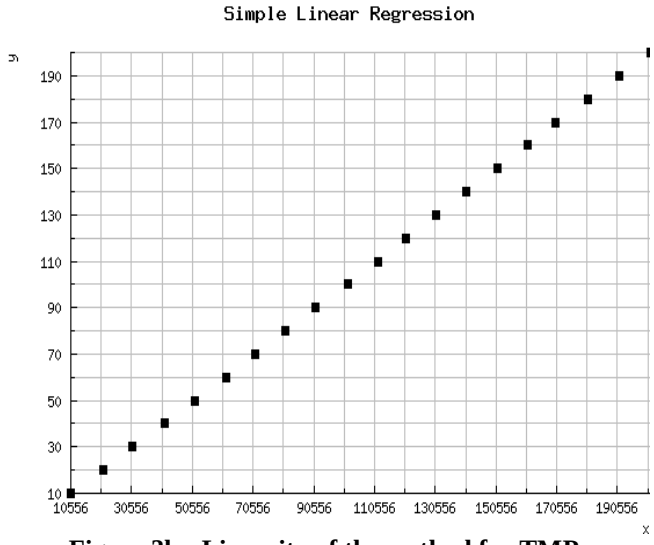

Figure 3b : Linearity of the method for TMP.

ence peak was observed in sample chromatogram. Peak purities higher than 98.0% were obtained in the chromatograms of sample solutions, demonstrating that other compounds did not co-elute with the main peaks. The chromatogram obtained with the mixture of the tablet excipient proves that there is no interference from excipient and peak of interest fulfill all the requirements of symmetrical peak, and hence the specificity is confirmed. The LOQ in picogram-per-milliliter (Figure 4b) shows the sensitivity as well as selectivity of the method which is a major clinical advantage in body fluids analysis.

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogeneous sample. Intra-day precision of the method was evaluated at three different

independent concentrations i.e. 160, 200, and 240 µg/ml (n=3) by determining their assay. Inter-day precision of the method was tested for 3 days at the same concentration levels. Solutions for calibration curves were prepared every day on freshly basis. Since the inter-day and intra-day precision obtained % RSD was less than 2% it assures that the proposed method is quite precise and reproducible

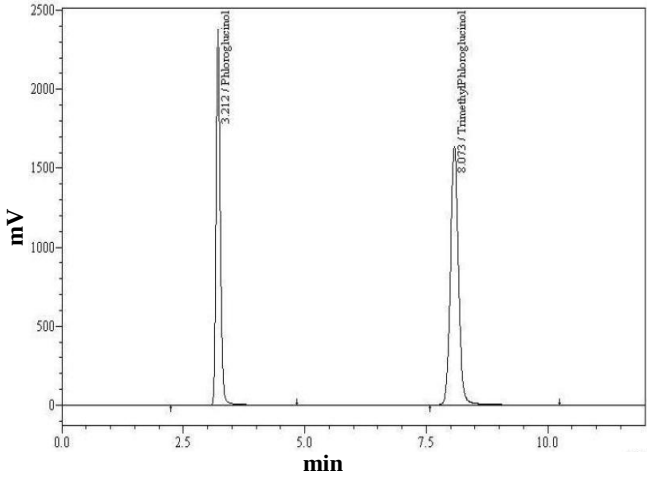

Figure 4a : Typical Chromatogram of sample.

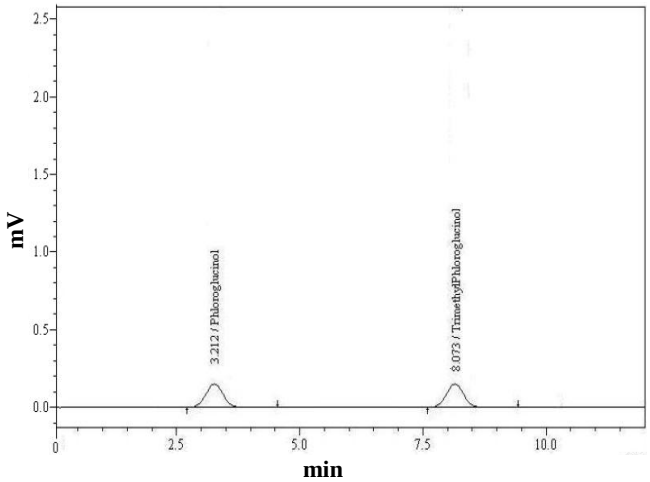

Figure 4b : Chromatogram of LOQ.

TABLE 1 : Calibration curve data and validation-parameters

Parameter	Inference	
	Phloroglucinol	Trimethyl phloroglucinol
Linearity range (µg /ml)	20-400	20-400
Correlation coefficient(r)	0.999968712	0.999962694
Regression equation (y=mx+c) Slope (m)	1027.331579	1004.7
Intercept (c)	-4321.842105	711.8421053
Limit of detection(LOD) (pgml)	30	30
Limit of quantification (LOQ) (pg /ml)	300	300

TABLE 2 : Inter-day and intra-day precision.

Nominal concentration (µg /ml)	Day 1		Day 2		Day 3	
	% Recovered		% Recovered		% Recovered	
	PGD	TMP	PGD	TMP	PGD	TMP
160	99.375	99.482	100.115	99.785	99.915	99.715
200	100.373	100.819	100.491	99.997	100.195	100.378
240	100.917	99.799	100.008	100.177	99.991	100.157
Mean	100.222	100.033	100.205	99.863	100.034	100.083

as shown in TABLE 2.

The accuracy was investigated by means of addition of reference standards to a mixture of the tablet excipient. The mean recovery (n = 9) was 98.25% - 100.75% (RSD%=0.259) demonstrating the accuracy of the method. Recovery studies of the drug were carried out for the accuracy parameter at three different concentration levels i.e. multiple level recovery studies. A known amount of API's standards were added into pre-analyzed sample and subjected to the proposed HPLC method. Percentage recoveries for marketed

TABLE 3 : Contents of PGD and TMP in the fixed dose combination tablets

Sample tablet	Content (%) ± S.D.	
	PGD	TMP
SPASFON®,	99.12%± 0.13	99.92%± 0.13
SPADIX®,	99.92 %± 0.62	98.97% ± 0.74
FUROSINOL®	98.89%± 0.13	100.19%± 0.14
ANAFORTAN PLUS®	98.91 %± 0.93	99.48% ± 0.46

S.D. = standard deviation.

products were found to be within the limits (TABLE 3).

The statistical analysis showed no significant difference between results obtained employing the analytical conditions established for the method and those obtained in the experiments in which variations of some parameters were introduced. The parameters used in system suitability test were asymmetry of the chromatographic peak, theoretical plates and capacity factor, as RSD of peak area for replicate injections. Thus, the method showed to be robust for changes in mobile phase methanol proportion, mobile phase, flow rate, and column temperature (TABLE 4).

During the study it was observed that upon treatment of PGD and TMP with base (0.1M NaOH), acid (0.1M HCl) and hydrogen peroxide (35%) the degradation was observed in acid and H_2O_2 whereas no peculiar degradation was observed with other stress conditions. TABLE 5 indicates the extent of degradation

Full Paper

TABLE 4 : Robustness of the method.

Chromatographic Conditions	Variation	Retentiontime	
		PGD	TMP
Temperature (ºC)	23	3.316	7.931
	27	3.212	8.073
Flow rate (ml/min.)	0.9	3.537	8.514
	1.1	2.89	7.89
Amount of Methanol (%)	58	3.47	8.49
	62	2.87	7.89

of both the drugs under various stress conditions.

Figure 5a to 5d shows the chromatograms of forced degraded samples. Further it is important to note that from the chromatograms, it is evident that although the degraded peaks are observed, under the applied stress conditions like acid and oxidative degradation states. The drug is stable under alkali and other stress conditions.

The proposed assay method was applied to stability study of commercially available tablets, for which the samples were placed at 30°C with

TABLE 5 : Summary of forced degradation results.

Stress conditions	Time / h	Assay of active Substance		Degradation (%)	
		PGD	TMP	PGD	TMP
Acid hydrolysis(0.1 mol L-1 HCl)	1	105.557%	106.340%	5.557%	6.3 40%
Base hydrolysis(0.5 mol L-1 NaOH)	1	125.834%	104.001%	25.834%	4.001%
Oxidation(35% H2O2)	1	644.138%	105.869%	544.138%	5.869%
Thermal (60ºC)	1	108.507%	107.417%	8.507%	7.417%

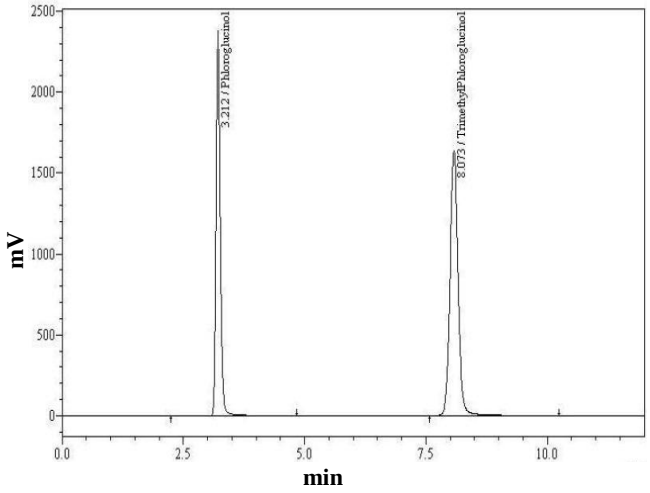

Figure 5a : Chromatogram of Heat treated Sample.

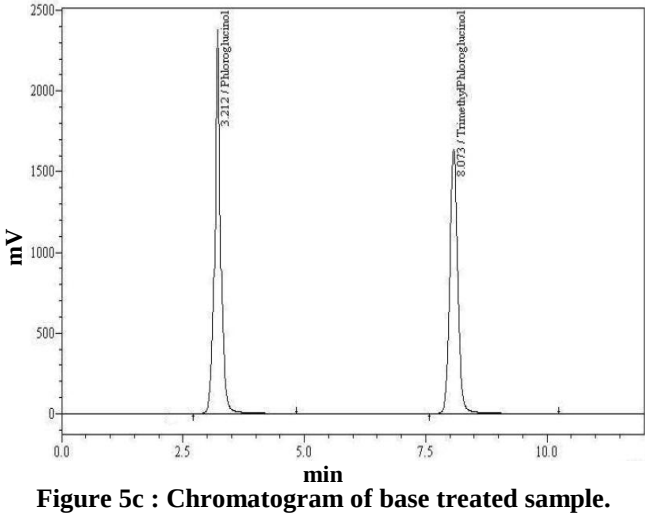

Figure 5c : Chromatogram of base treated sample.

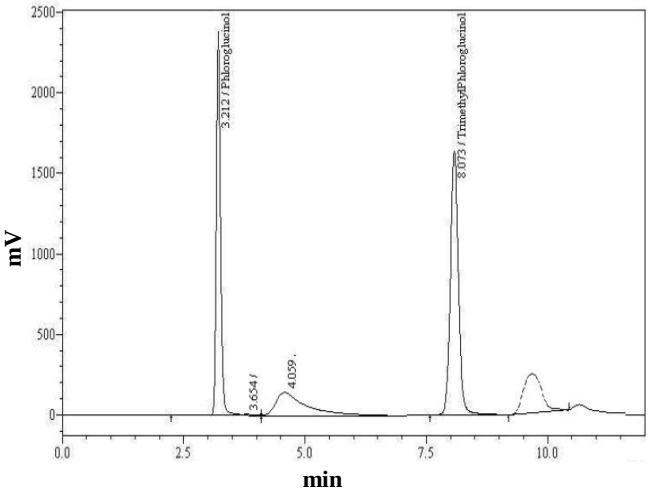

Figure 5b : Chromatogram of acid treated sample.

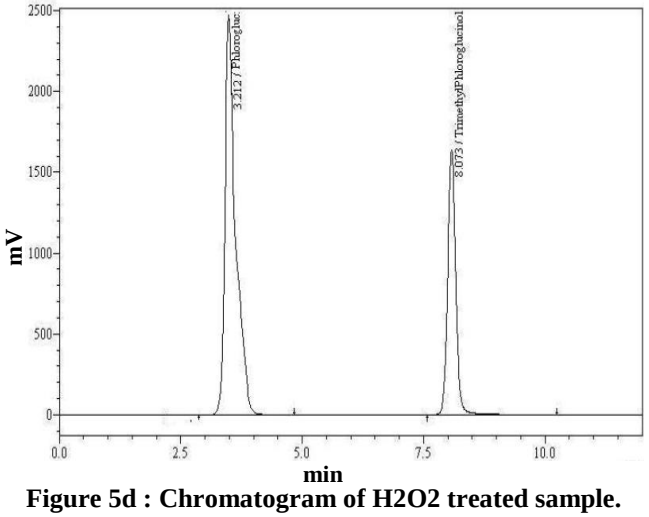

Figure 5d : Chromatogram of H2O2 treated sample.

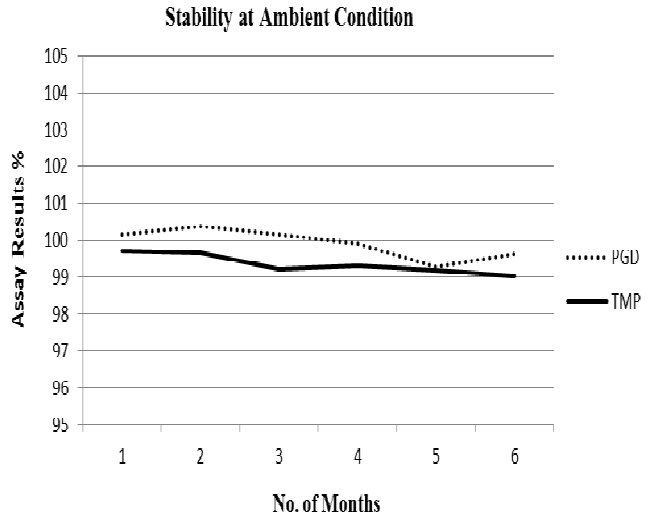

Figure 6a : Chromatogram of Sample stability at ambient conditions.

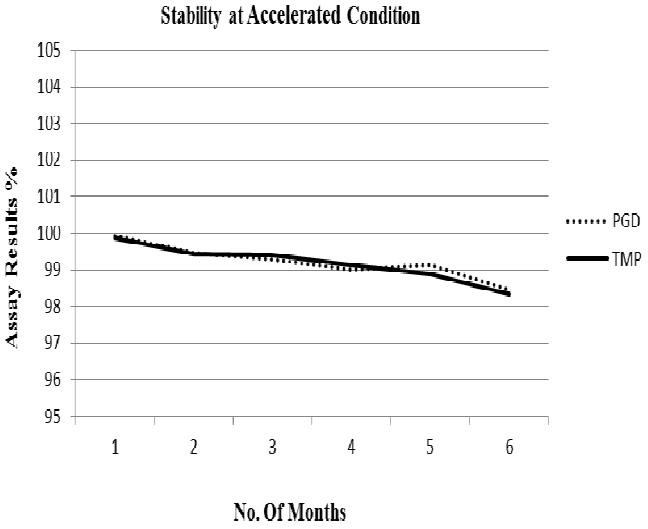

Figure 6b : Chromatogram of Sample stability at accelerated conditions.

TABLE 6 : Summary of stability studies.

Stress conditions	Accelerated (40ºC+75%H)		Ambient (30ºC+65%H)	
No. of Months	PGD	TMP	PGD	TMP
1	99.926	99.871	100.159	99.713
2	99.467	99.418	100.391	99.671
3	99.279	99.387	100.173	99.219
4	98.995	99.115	99.893	99.323
5	99.125	98.893	99.279	99.182
6	98.459	98.335	99.619	99.013

relative humidity of 65% (Figure 6a) and at 40°C with relative humidity of 75% relative humidity (Figure 6b). Stability study was performed according to stability protocol as described in previous section. Samples were analyzed and percentage of contents was measured (TABLE 6). According to the results obtained both API's were found to be stable at applied conditions of temperature and relative humidity, and were accurately analyzed with the proposed method.

REFERENCES

[1] J.E.F.Reynolds, (Ed); London:Royal Society of Pharmacology, 1741 **(1996)**.

[2] S.Budavari; The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals, M.J.O'Neill, (Ed); (Annals of Internal Medicine, Merck and Co., Inc) 113 **(2006)**.

[3] United States Pharmacopoeia; The United State Pharmacopeial Convention, 2702 **(2004)**.

[4] S.Tabassum, B.Afridi, Z.Aman; J.Pak.Med.Assoc., **55**, 270-273 **(2005)**.

[5] Y.Cui, C.Y.Ang; J.Agric Food Chem., **50**, 2755-2759 **(2002)**.

[6] H.Kim, H.Roh, H.J.Lee, S.Y.Chung, S.O.Choi, K.R.Lee, S.B.Han; J.Chromatogr.B.Analyt.Technol. Biomed.Life Sci., **792**, 307-312 **(2003)**.

[7] C.Lartigue-Mattei, C.Lauro-Marty, M.Bastide, J.A.Berger, J.L.Chabard, E.Goutay, J.M.Aiache; J.Chromatogr., **617**, 140-146 **(1993)**.

[8] S.T.Cargnin, J.de Matos Nunes, J.S.Haas, L.F.Baladao, E.Cassel, R.F.Vargas, S.B.Rech, G.L.von Poser; J.Chromatogr.B.Analyt.Technol. Biomed.Life Sci., **878**, 83-87 **(2010)**.

[9] A.Tolonen, A.Hohtola, J.Jalonen; Phytochem. Anal., **14**, 306-309 **(2003)**.

[10] D.Amin, W.A.Bashir; Talanta., **31**, 283-284 **(1984)**.

[11] A.K.Chatterjee, L.N.Gibbins; Anal.Biochem., **30**, 436-442 **(1969)**.

[12] H.Cui, C.G.Xie, C.Z.Lai; Luminescence, **18**, 318-323 **(2003)**.

[13] A.Penttila, J.Sundman; J.Pharm.Pharmacol., **13**, 531-535 **(1961)**.

[14] C.G.Xie, H.F.Li; Guang Pu Xue Yu Guang Pu Fen Xi. **24**, 1521-1523 **(2004)**.

[15] Validation of Analytical Procedure: ICH-Q2B, Methodology, ICH, Geneva, **(2005)**.