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# *IN VITRO* AVAILABILITY OF TRIMETHYLPHLOROGLUCINOL AND ITS DEGRADATION PRODUCT FROM DOSAGE FORMULATIONS BY RP-HPLC

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A simple, sensitive, reliable, and rapid high-performance liquid chromatographic method for the simultaneous determination of phloroglucinol and trimethyl phloroglucinol has been developed. Acetonitrile-water (1:1 v/v) was used as mobile phase, with flow rate 2 ml/minutes. pH was adjusted to 3 with phosphoric acid. U.V detection was performed at 242 nm. The results obtained showed a good agreement with the declared content. Recovery values for phloroglucinol were from 99.91 % to 100.62 % and recovery values for trimethylphloroglucinol were from 98.44 % to 100.04 %. The proposed method is rapid, accurate, and selective, it may be used for the quantitative analysis of phloroglucinol and trimethylphloroglucinol. The method was found to be specific, accurate, precise and reliable for the determination of phloroglucinol and trimethylphloroglucinol in the form of raw materials, in bulk drugs and formulation. It was possible to determine both phloroglucinol and trimethylphloroglucinol in the concentration range of 5 nano gram to 30 nano grams. The detection limit of both phloroglucinol and trimethylphloroglucinol were 0.4 nano gram.

**Keywords:** Reversed-phase HPLC, phloroglucinol, trimethylphloroglucinol, 1,3,5-trimethoxybenzene, 1,3,5-trihydroxybenzene, UV detection.

## INTRODUCTION

Phloroglucinol or 1,3,5-benzene, tri,ol (*structure 1*) is white or yellowish white crystals, slightly soluble in water, soluble in alcohol and in ether, melting point 215-219°C, while trimethylphloroglucinol is 1,3,5-trimethoxybenzene (*structure 2*). Phloroglucinol is used as an antispasmodic, often in combination with trimethylphloroglucinol. It is regarded to be effective in reducing smooth muscle contraction and in relieving the pain consequent to smooth muscle spasms. The antispasmodic effect is greater against muscle under spastic conditions than against smooth muscle under physiological condition. Phloroglucinol is indicated for symptomatic treatment of colics due to renal and biliary calculi, acute pain due to spasm of bile ducts, urinary passages or in gastrointestinal tract, pain of uncertain genesis in the abdominal region, spastic conditions of the female genital system; dysmenorrheal [The Merck index. (2001) Martindale, The extra pharmacopoeia (1996) United State Pharmacopoeia 2004].

There are a few references of phloroglucinol which were analyzed by HPLC method (Tolonen *et al.,* 2002). A single method was developed for the separation and quantification of hexahydro-1,3,5-trinitro-1,3,5-triazine, 2,4,6-trinitrotoluene (TNT), and most of the known and suspected biodegradation intermediates of TNT by RP-HPLC and diode array detection. The known biodegradation intermediates of TNT analyzed were 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 2,6-diamino-4-nitrotoluene, 2,4-diamino-6-nitrotoluene, 2,4,6-triaminotoluene, 2,2',6,6'-tetranitro-4,4'-azoxytoluene, and 4,4',6,6'-tetranitro-2,2'-azoxytoluene. The suspected biodegradation intermediates of TNT included 1,2,3-benzenetriol (pyrogallol), 1,3,5-benzenetriol (phloroglucinol), 2-methyl-1,3,5-benzenetriol (methyl phloroglucinol) and 4-methylphenol (p-cresol). Mobile phases consisting of aqueous buffers adjusted to three different pH values in a gradient with acetonitrile were examined for their efficiency in separating the intermediate compounds and for the minimization of speciation of the ionizable intermediates (e.g. 2,4,6-triaminotoluene). A final aqueous buffer pH of 3.2 was selected to minimize the interference to the separation caused by 2,4,6-triaminotoluene speciation. Solvent consumption was minimized by the use of a narrow-bore column. All of the known reduction products as well as p-cresol and methyl phloroglucinol were identified in culture supernatants from TNT-degrading cultures while pyrogallol and phloroglucinol were not (Ahmad *et al.,* 1995). A method of analysis of proanthocyanidin cleavage products after acid-catalysis in the presence of excess phloroglucinol was investigated (Kennedy *et al.,* 2001). Supercritical fluid extraction and high-performance liquid chromatographic determination of phloroglucinol in St. John's Wort (Cui *et al.,* 2002). Determination of phloroglucinol in human plasma by HPLC-mass spectrometry (Kim *et al.,* 2003) and fast HPLC analysis of naphthodianthrones and phloroglucinols from hypercium perforatum extracts are also reported (Tolonen *et al.,* 2003).
The aim of present investigation was to develop reversed-

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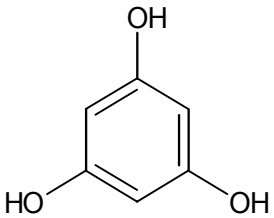
**Table 1**
Recovery of phloroglucinol (PH) and trimethylphloroglucinol (TPH) in reference drug and dosage form

| Conc. Inject | <——— Reference Drug ———> | | | | <————— Dosage Form ———> | | | |
|---|---|---|---|---|---|---|---|---|
| | <—PH—> | | <—TPH—> | | <—PH—> | | <—TPH—> | |
| (ppm) | Found | % Recov. | Found | % Recov. | Found | % Recov. | Found | % Recov. |
| 0.5 | 0.502 | 100.424 | 0.500 | 100.042 | 0.502 | 100.341 | 0.497 | 99.442 |
| 1 | 0.999 | 99.91 | 0.999 | 99.880 | 1.007 | 100.659 | 0.996 | 99.606 |
| 1.5 | 1.508 | 100.517 | 1.499 | 99.941 | 1.502 | 100.124 | 1.490 | 99.323 |
| 2 | 2.012 | 100.620 | 1.969 | 98.441 | 2.008 | 100.394 | 1.986 | 99.297 |
| 2.5 | 2.507 | 100.267 | 2.488 | 99.534 | 2.507 | 100.274 | 2.487 | 99.472 |
| 3 | 3 | 100 | 3 | 100 | 3.015 | 100.492 | 2.993 | 99.781 |

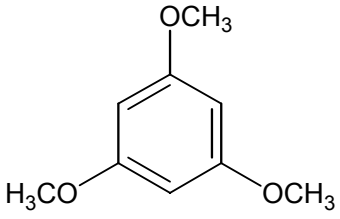
**Table 2**
Recovery and regression characteristic of phloroglucinol by proposed method

| Injected Concentration (ppm) | Recovered concentration | | | |
|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 |
| 0.5 | 0.5021 | 0.5017 | 0.4993 | 0.4994 |
| 1 | 0.9991 | 1.0066 | 0.9943 | 0.9966 |
| 1.5 | 1.5078 | 1.5019 | 1.4986 | 1.5007 |
| 2 | 2.0124 | 2.0079 | 2.0058 | 1.9887 |
| 2.5 | 2.5067 | 2.5069 | 2.4961 | 2.4982 |
| 3 | 3 | 3.0148 | 3 | 3 |
| Correlation Coefficient (R ) | 0.9998 | 0.9999 | 0.9999 | 0.9998 |
| Standard Error of estimate | 0.00568 | 0.003158 | 0.004305 | 0.004957 |
| Standard Error | | 0.005289 | 0.002939 | 0.004003 |
| P Value | | 0 | 0 | 0 |
| Intercept | | 0.002993 | 0.00062 | 0.002621 |
| Slope | | 1 | 1 | 1 |

phase high-performance liquid chromatographic (RP-HPLC) method for the simultaneous determination of phloroglucinol and its trimethyl derivative, a mixture of substances with so similar structure but different polarity and to investigate the linearity, precision, limit of quantification and limit of detection. The developed method is useful for the analysis of these drugs in the form of raw materials, bulk drug samples, its tablets formulations and also in human serum simultaneously and as well as separately.




Structure 1




Structure 2

## EXPERIMENTAL

***Material and reagents***

Samples of phloroglucinol and trimethylphloroglucinol reference standards were of analytical grade. Anafortan plus tablets (manufactured by AGP, Pakistan) containing phloroglucinol and trimethylphloroglucinol (each 80 mg) and Spasfon tablets (manufactured by Laboratoire L. LAFON, France) containing phloroglucinol (62.233 mg) and trimethylphloroglucinol (80 mg) were purchased from the market. HPLC grade acetonitrile and phosphoric acid

**Table 3**
Recovery and regression characteristics of trimethylphloroglucinol by proposed method

| Injected concentration (ppm) | Recovered concentration | | | |
|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 |
| 0.5 | 0.5002 | 0.4972 | 0.4965 | 0.4987 |
| 1 | 0.9988 | 0.9961 | 1.0019 | 1.0015 |
| 1.5 | 1.4991 | 1.4898 | 1.4901 | 1.5089 |
| 2 | 1.9688 | 1.9859 | 2.0098 | 1.9795 |
| 2.5 | 2.4884 | 2.4868 | 2.4657 | 2.4828 |
| 3 | 3 | 2.9934 | 3 | 3 |
| Correlation coefficient (R) | 0.9999 | 0.9999 | 0.9998 | 0.9999 |
| Standard error of estimate | 0.013477 | 0.004392 | 0.016631 | 0.01203 |
| Standard error | | 0.012539 | 0.004083 | 0.015489 |
| P value | | 0 | 0 | 0 |
| Intercept | 0.001495 | 0.003427 | -0.0007 | -0.00292 |
| Slope | 1 | 1 | 1 | 1 |

**Table 4**
Accuracy and precision of proposed method

| Inj. conc. (ppm) | Day 1 | | Day 2 | | Day 3 | | Day 4 | |
|---|---|---|---|---|---|---|---|---|
| | RSD | % Recov. | RSD | % Recov. | RSD | % Recov. | RSD | % Recov |
| **A - Phloroglucinol** | | | | | | | | |
| 0.5 | 0.0079 | 100.42 | 0.0067 | 100.34 | 0.0084 | 99.85 | 0.0082 | 98.83 |
| 1 | 0.0085 | 99.91 | 0.0059 | 100.66 | 0.0055 | 99.43 | 0.0037 | 99.66 |
| 1.5 | 0.0023 | 100.52 | 0.004 | 100.12 | 0.0055 | 99.91 | 0.002 | 100.04 |
| 2 | 0.0031 | 100.62 | 0.0026 | 100.39 | 0.0174 | 100.29 | 0.0034 | 99.43 |
| 2.5 | 0.0022 | 100.27 | 0.0015 | 100.27 | 0.0019 | 99.84 | 0.0018 | 99.27 |
| 3 | 0.0013 | 100 | 0.0036 | 100.49 | 0.008 | 100 | 0.0016 | 100 |
| **B - Trimethylphloroglucinol** | | | | | | | | |
| 0.5 | 0.0037 | 100.04 | 0.004 | 99.44 | 0.0048 | 99.30 | 0.008 | 99.73 |
| 1 | 0.0013 | 99.88 | 0.0019 | 99.61 | 0.0062 | 100.19 | 0.0024 | 100.15 |
| 1.5 | 0.001 | 99.94 | 0.0022 | 99.32 | 0.0016 | 99.34 | 0.0018 | 100.59 |
| 2 | 0.0112 | 98.44 | 0.006 | 99.30 | 0.0036 | 100.49 | 0.0034 | 98.973 |
| 2.5 | 0.0027 | 99.53 | 0.0066 | 99.47 | 0.0036 | 98.63 | 0.0059 | 99.31 |
| 3 | 0.0072 | 100 | 0.0044 | 99.78 | 0.0042 | 100 | 0.0041 | 100 |

were obtained from Merck. The mobile phase and solution were prepared in deionized double distilled water. Stock solutions of the compounds were prepared in acetonitrile:water (1:1) and stored at room temperature. Fresh working solutions were prepared daily. All solutions were filtered (0.45 μm) and degassed by sonicator.

***Chromatographic system***

An HPLC system was used of LC-10 AT VP shimadzu pump, SPD-10AV VP Shimadzu U.V visible detector, a μ Bondapak 125 A C18 10 μm column (particle size 10 um) was used for separation. The chromatographic and integrated data were recorded using a CBM-102 communication Bus Module Shimadzu.

***Chromatographic conditions***

The mobile phase was acetonitrile-water (1:1), the pH of this mobile phase was adjusted to 3 with phosphoric acid (85 %). 1:1 acetonitrile and water was mixed in a conical flask and put it at the magnetic stirrer, and electrode of pH meter was dipped into this solution and then pH was adjusted to 3 with phosphoric acid by the help of pH meter. The analysis was carried out under isocratic conditions using a flow rate 2 ml/minutes at room temperature.

Chromatograms were recorded at 242 nm using a detector SPD-10AV VP Shimadzu UV visible. The samples were introduced by injector with a 10 µ liter sample loop.

***Analytical procedure***

10 mg phloroglucinol and 10 mg of trimethylphloroglucinol were dissolved in acetonitrile:water (1:1) in 100 ml volumetric flask and made up volume up to the mark (stock solution 100ug/ml) and aliquots were diluted as required. Twenty tablets of anafortan plus were weighed to obtain the average tablets weight (665 mg) and were then powered. The powdered tablets equivalent to 10 mg of active substance was mixed with 100 ml acetonitrile:water (1:1). This mixture were allowed to stand for 1 hour with intermittent sonication to ensure complete solubility of the drug. This stock solution was filtered to obtain clear filtrate and working solutions were prepared of desire concentrations. Same procedure was repeated for the spasfon tablet. 1 ml of anafortan plus injection was diluted to100 ml (stock solution). These solutions were diluted to desired concentrations.

## RESULT AND DISCUSSION

The goal of this study was to develop a rapid, more accurate, precise reliable, less expensive and least time consuming HPLC method for the analysis of phloroglucinol and trimethylphloroglucinol in the form of raw materials, bulk drug samples injections and its tablets formulations simultaneously and as well as separately using the most commonly employed C-18 column with U V detection.

Despite the fact that physical characteristics of closely related substances are the most important factors that can predict chromatographic behavior. In present investigation, the best separation of these two compounds was achieved using a µ-Bondapak 125 A C18 (10 µm) column. For the separation and determination of phloroglucinol and trimethylphloroglucinol the best results were obtained using mobile phase acetonitrile:water (1:1 v/v). The lower percentage of acetonitrile in mobile phase results in peak tailing of both components and long analysis duration in trimethyl phloroglucinol peak, while higher percentage of acetonitrile in mobile phase results in very little analysis duration. Optimal retention times (phloroglucinol - 1.29 minutes and trimethylphloroglucinol - 2 minutes) were achieved when the pH of mobile phase was adjusted to 3 with 85 % phosphoric acid. Small changes in pH of the mobile phase had a great influence to the chromatographic behavior of these substances.

***Accuracy***

Accuracy of the method was evaluated in the concentration range 0.5 ppm to 3 ppm. Six concentrations of samples were injected in the chromatographic system, and each concentration was injected four times in the system in a day. This work was repeated on four different days. Good recovery showed that method can be applied successfully in the concentration range 0.5 - 3 ppm. Table 1 show recovery of phloroglucinol and trimethylphloroglucinol in reference drug and in dosage forms. Regression characteristics and recovered concentration of phloroglucinol and trimethylphloroglucinol during four different days are presented in table 2 and 3 respectively.

Correlations coefficient, standard error of estimate, standard error, p value, intercept, and slope of both the components are also presented in tables 2-3. Recovery of phloroglucinol and trimethylphloroglucinol in dosage formulations is given in table 5.

The accuracy of the method was evaluated by analyzing independently prepared solutions of phloroglucinol and trimethylphloroglucinol. The recovery data expressed in tables 1-5 show that the method is accurate for determination of both of these drugs up to 0.5 ppm concentrations. All calibration curves have a correlation value of at least 0.9998. The accuracy was calculated as a percentage of the nominal concentration.

***Precision***

The precision of the method was investigated with respect to repeatability. For intra-day precision, six concentrations of each compound were analyzed on the same day. Each concentration of sample was injected 10 times. Tables 2-3 show the regression characteristics while table 4 summarizes the relative standard deviation (RSD). Generally, acceptable repeatability of the results with in one day and day-to-day was observed. Data of the relative retention times obtained in a series of four consecutive injections also showed acceptable repeatability when analyzed same day as well as on three consecutive days.

***System suitability***

System suitability of the method was evaluated by analyzing the symmetry of the phloroglucinol and trimethylphloro-glucinol peaks, theoretical plates of the column (>2000) and the resolution between the peaks of phloroglucinol and trimethylphloroglucinol, tailing factor of both the peak was < 2, capacity factor of column was found in between 2 to 8, dead time was 0.3 minutes.

***Specificity***

In order to apply the method to dosage formulations, any peak of excepients was not found in chromatogram, which proves that method can be applied successfully to dosage formulation of pharmaceuticals. Recovery of the method and very little value of RSD further proved that method could be applied in pharmaceutical dosage formulations in the presence of some excepients.

The specificity of the method was evaluated to ensure separation of phloroglucinol and trimethylphloroglucinol.

**Table 5**
Recovery of phloroglucinol and its trimethyl derivative from by proposed method

| | Label claim | Found | % Recovery | % Error |
|---|---|---|---|---|
| **A Anafortan™ Plus Tablet** | | | | |
| Phloroglucinol.2$H_2O$ | 80 mg | 80.27 | 100.34 | 0.34 |
| | | 80.10 | 100.12 | 0.12 |
| | | 80.52 | 100.66 | 0.66 |
| | | 80.31 | 100.39 | 0.39 |
| | | 80.22 | 100.27 | 0.27 |
| | | 80.39 | 100.49 | 0.49 |
| Trimethylphloroglucinol | 80 mg | 79.55 | 99.44 | 0.56 |
| | | 79.68 | 99.61 | 0.39 |
| | | 79.46 | 99.32 | 0.68 |
| | | 79.44 | 99.30 | 0.70 |
| | | 79.58 | 99.47 | 0.53 |
| | | 79.82 | 99.78 | 0.22 |
| **B Spasfon™ Tablets** | | | | |
| Phloroglucinol | 63.33 mg | 63.95 | 100.98 | 0.98 |
| | | 64.1 | 101.22 | 1.22 |
| | | 63.68 | 100.55 | 0.55 |
| Trimethyl phloroglucinol | 80 mg | 80.69 | 100.86 | 0.86 |
| | | 81.06 | 101.32 | 1.32 |
| | | 80.48 | 100.6 | 0.6 |
| **C Anafortan™ Plus Injections** | | | | |
| Phloroglucinol.2$H_2O$ | 10 mg/ml | 10.19 | 101.9 | 1.9 |
| | | 10.12 | 101.2 | 1.2 |
| | | 10.08 | 100.8 | 0.8 |
| Trimethyl phloroglucinol | 0.01mg/ml | 0.00985 | 98.5 | 1.5 |
| | | 0.0102 | 102 | 2.0 |
| | | .0103 | 103 | 3.0 |

The specificity of the method was demonstrated by assaying a sample of phloroglucinol and trimethylphloroglucinol. The method demonstrated resolution between phloroglucinol and trimethylphloroglucinol.

***Linearity, calibration and quantification limit***
The quantification limit is a characteristic of quantitative assays for low levels of compound in sample. It is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions.

Newly developed method was applied in the concentration range 0.5 ppm to 3 ppm, good recovery and very little RSD showed that method can be applied in the concentration range as mentioned above successfully.

***Detection limit***
The detection limit is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. In the case of instrumental analytical procedures that exhibit background noise, which is to compare measured signals from samples with known low concentration of analyte with those of blank samples. The detection limit of the method was calculated to compare sample solution with blank sample, signal to noise ratio was found > 10.

Injected concentrations of phloroglucinol and trimethylphloroglucinol against recovered concentration respectively in the concentration range 0.5 - 3.0 ppm were linear. The limit of quantification was found 5 ng and detection limit was 0.4 ng. All calibration curves had coefficient correlation value of at least 0.9998. Typical chromatogram is shown in figure 1.

***Ruggedness***
The ruggedness of an analytical method is the degree of reproducibility of the test results obtained by the same samples under a variety of conditions, such as different

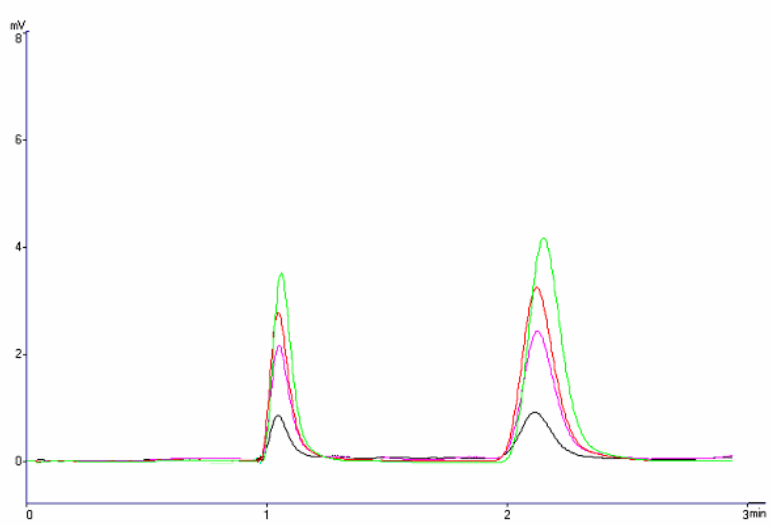



Fig. 1: Typical chromatogram showing separation of phloroglucinol (peak 1) and its trimethyl derivative (peak 2).

laboratories, different analysts, different instruments, different lots of reagents, and different days.

Ruggedness of this method was evaluated in two different labs with two different instruments, different analyst, and different days and with different reagents. Lab 1 was in the Department of Chemistry, University of Karachi while Lab 2 was Brookes Lab1 at Research institute of Pharmaceutical Sciences, Faculty of Pharmacy, University of Karachi. Results of RSD and % recovery of four different days prove the ruggedness of developed method.

## CONCLUSION

The paper is the first to describe a simultaneous determination of phloroglucinol and trimethylphloroglucinol by RP-HPLC. Our initial intent in the present study was to develop an efficient chromatographic system for the analysis of these drugs, which are used in combination by many manufacturers. The method has been applied to raw materials as well as drugs of different formulation from Pakistan and France. The proposed RP-HPLC method has an advantage of good separation and resolution of the chromatographic peaks. The obtained results are in a good agreement with the declared contents. Results are accurate, precise, and confirmed by the statistical parameters. The proposed method is rapid, precise and estimates phloroglucinol and trimethylphloroglucinol in raw material, oral dosage form, and injections formulation.

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