



**DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC  
METHOD FOR METHYL GALLATE**

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**ABSTRACT**

An isocratic stability indicating RP-HPLC method was developed and validated for the assay of methyl gallate (MG). The marker and other degradation components were separated on a Thermo Hypersil BDS-C18 (250 x 4.6 mm, 5.0 μ) column. The mobile phase composed of water acidified with o-phosphoric acid (0.01%): acetonitrile (88:12 v/v). The mobile phase flow rate was optimized at 1.0 mL/min. The detection wavelength was selected as 271 nm. The method was linear over the concentration range of 5–50.0 μg/mL. Forced degradation studies of MG were carried out under conditions of acid, base and oxidative hydrolysis, sun light irradiation and in water to detect the major degradation products of the MG. The MG was found to be stable under acidic, neutral and in sun light irradiation. The alkaline conditions resulted in higher degradation of MG than oxidative conditions.

**KEYWORDS:** Methyl gallate (MG), HPLC, stability indicating, forced degradation.

**INTRODUCTION**

During the past decade, an increasing acceptance and public interest in herbal medicines has been observed all over the world. However, one of the problems for acceptance of herbal formulations is the lack of standard quality control profiles. The quality of herbal medicine is depend upon the concentration of active component and its implications for efficacy and safety. Because of the complex nature of the chemical constituents of the plant based products, it is difficult to establish their quality control parameters. The modern analytical techniques are expected to help in circumventing this problem. Various Ayurvedic formulations have been found to be useful remedies for a number of disorders. Although herbal formulations are effective but their use is often associated with a number of undesirable side effects that may be due to the degradation products of drug formed during storage.<sup>[1]</sup>

The International Conference on Harmonization (ICH) drug stability test guideline Q1A requires that analysis of stability of samples is to be done through the use of validated stability-indicating analytical method, but this concept is often used for synthetic drugs and is not commonly used for herbal drugs. The results of stress test often help to guide the pharmaceutical dosage forms and provide information about the instability of drugs, enabling the development of stability –indicating analytical methods that may be used in the subsequent stability studies of the active ingredient. Hence these

studies are also important for herbal dosage form containing the active markers.<sup>[2]</sup>

Methyl gallate is a phenolic compound found in various medicinal plants such as *Acacia nilotica*, *Toona sinensis*, *Archidendron jiringa*.<sup>[3,4]</sup> It is also found in wine. It is the methyl ester of gallic acid. Methyl gallate is a phenolic compound found in *Terminalia myriocarpa* and *Geranium niveum*.<sup>[5,6]</sup> Methyl gallate exhibits potent antitumor activities by inhibiting tumor infiltration of T cells.<sup>[4]</sup> It has been reported to show antioxidant property in triglyceride of Kilka fish oil.<sup>[5]</sup> It is reported to inhibit biofilm formation is of high importance for developing health care products.<sup>[6]</sup> Various high-performance liquid chromatographic methods are reported for determination of methyl gallate which showed that the retention time for methyl gallate is around 2-3 min.<sup>[7,8,9,10]</sup> For stability indicating method the retention time must be higher, for elution of more polar degraded products formed during stress testing of methyl gallate.

Therefore the present work, aims to develop and validate stability indicating HPLC method of methyl gallate and qualitative analysis of its components for subsequent use in the quality control of herbal medicine.

**Experimental Reagents**

Methyl gallate was purchased from Sigma (purity of 95.0% by HPLC), methanol was of HPLC grade and

purchased from S.D. Fine chemicals. Water was purified by doubled distillation and filtered by using 0.2 µm nylon membrane filters.

**Instrumentation:** DIONEX Ultimate 3000 consisting of a quaternary pump and UV-Vis. Detector. Chromatographic Conditions Analysis was performed using a column Hypersil RP-C18 (250 x 4.6 mm, 5.0 µm column) with an isocratic run, using the mobile phase consisting of aqueous o-phosphoric acid (0.01%) : acetonitrile (88:12 v/v) at a flow rate of 1 mL/min. The detection was carried out at wavelength 271 nm.

#### Preparation of standard stock solution

Methyl gallate (5 mg) was dissolved in 50 mL of methanol, sonicated for 30 min (100µg/mL). This solution was diluted appropriately with mobile phase to produce a working solution (20 µg /mL) for assay of the samples. Both solutions were freshly prepared and filtered through a 0.45 µm modified PTFE membrane, prior to injection.

#### Forced Degradation Studies

A standard stock solution of methyl gallate (1 mg/mL) was prepared by dissolving 10 mg of the methyl gallate in 10 ml of methanol. From the above stock solution, 2 ml was diluted up to 10 ml with 5 M HCl, 0.1 M NaOH, 6% H<sub>2</sub>O<sub>2</sub> and HPLC water in separate volumetric flasks to achieve a concentration of 200µg/ml. The above solutions were exposed to stress tests under acidic condition (2 ml in 8 mL of 5 M HCl, 24 h), alkaline condition (2 mL solution in 1 ml of 0.1 M NaOH, 1 h), oxidative condition (2 ml in 8 mL of 6% H<sub>2</sub>O<sub>2</sub>, 1hr) and in water for 12h. All the solutions were refluxed at 80 °C. After the degradation time was achieved, 1 mL of the solution was transferred to another 10 mL volumetric flask, neutralized and volume was made up to 10 mL with mobile phase. For photostability studies the methyl gallate solution in water was exposed to sunlight for 30 days. After filtration through a 0.45 µm modified PTFE membrane, the degraded samples were injected into the HPLC, and compared with the fresh, non – degraded sample solution.

#### Method Validation

The developed method was validated as per ICH guidelines. The validation parameters evaluated were as follows.<sup>[9]</sup>

1. Linearity
2. Precision
3. LOD & LOQ
4. Robustness
5. Accuracy
6. System suitability

**Linearity:** The linearity of analytical procedure is its ability (within given range) to obtain the test results which are directly proportional to concentration in the sample. This was studied by analyzing six concentrations

in triplicate with the concentration in range of 5-50 µg/mL solution of methyl gallate.

#### Precision

The precision of the method was examined by performing the intra- and inter-day assays of six replicate injections at three concentration levels (5, 10 and 20 µg/mL). The intraday assay precision test was performed at the intervals of 4 h in 1 day, while the inter-day assay precision test was performed over 6 days.

#### LOD & LOQ

The Limit of Detection (LOD) and limit of Quantification (LOQ) were mathematically determined through the calibration curve. The aforementioned factor (3.0 and 10, for LOD and LOQ respectively) were multiplied by the standard deviation of the linear coefficient and divided by the slope, according to the guideline.

#### Robustness

The robustness of the method was evaluated by changing: (i) the mobile phase flow rate (0.9, 1, 1.1 mL/min), (ii) the mobile phase pH (2, 3 and 4) and (iii) the solvents of different lots. Standard solutions were injected six times for each change. The % RSD was calculated for each component during each change.

#### Accuracy

Recovery study was performed for determining the accuracy of the method. Sample solution was analyzed in triplicate for each concentration level (80%, 100% and 120%). The results of recovery study are depicted in the Table 3.

#### System Suitability

A system suitability test was performed to evaluate the chromatographic parameters (capacity factor, separation factor, number of theoretical plate, asymmetry of the peak and resolution between two consecutive peaks. Triplicate injections of the standard solution (20 µg/mL) were analysed.

#### Analysis of marketed formulations

To determine the content of methyl gallate in three different herbal formulations (Triphla churna, Dabour Chywanprash, Arjunarishta) were procured from local market.

#### Triphla churna

Triphla churna 0.5 g powder was weighted and transferred to 50 ml volumetric flask. Add 25 ml of methanol and shake it for 15 min. then make up the volume to 50 ml with methanol and sonicated it for 30 min. Filtered the above solution through 0.45 micron filtered. From the above solution suitable dilution were made to obtain the working solution for analysis.

A 20 µL volume of sample solution was injected into HPLC, three times under the condition above. The peak

area was measured at 271 nm and the concentration in the sample was determined.

#### Dabour Chywanprash

0.5 g of chywanprash was weighted and transferred to 50 ml volumetric flask to it 5 ml of methanol was added and sonicated for 30 min. After that volume was make up to 50 ml with methanol and again sonicated for 20 min. Filtered the above solution through 0.45 micron filter, 1ml of filtrate was taken and diluted to 10 ml with mobile phase.

#### Arjunarishta

10 ml of arjunarishta was transferred to 50 ml volumetric flask to it 20 ml of methanol was added and sonicated for 30 min. After that volume was make up to 50 ml with methanol and again sonicated for 20 min. Filtered the

above solution through 0.45 micron filter, 1ml of filtrate was taken and diluted to 10 ml with mobile phase.

#### RESULT AND DISCUSSION

A simple stability- indicating RP-HPLC method was developed for determination of MG and its degradation products. To optimize the proposed HPLC methods, different mobile phases, were tried for the chromatographic separation of the components and retaining MG. The best resolution was achieved using a mobile phase consisting of o-phosphoric acid (0.01%): Acetonitrile (88:12).

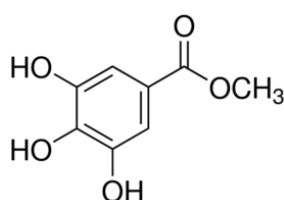


Fig. 1: Structure of Methyl gallate.

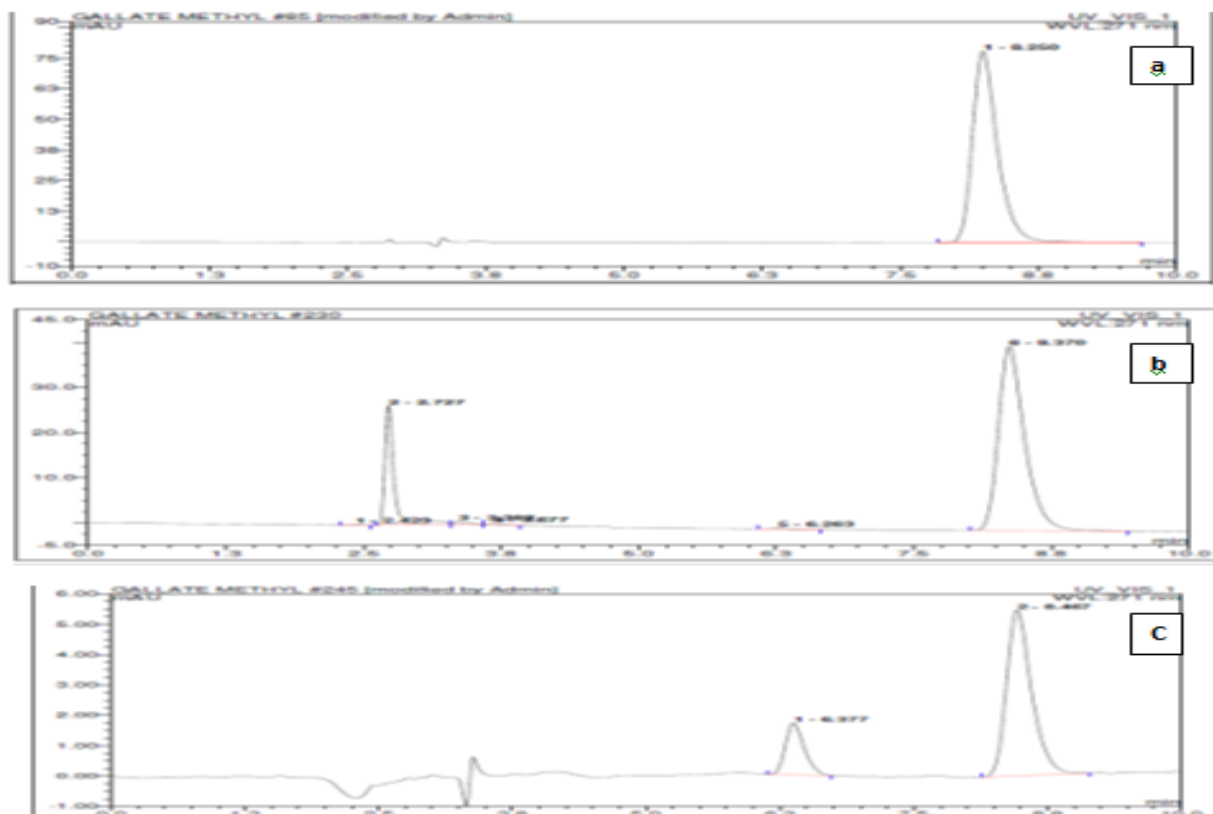


Figure 2: HPLC Chromatograms at 271 nm (a) Methyl gallate (MG) at 20 µg/mL (b) MG after stress with 0.1 M NaOH 1 hr (c) MG after stress with 6% H<sub>2</sub>O<sub>2</sub> 1 hr.

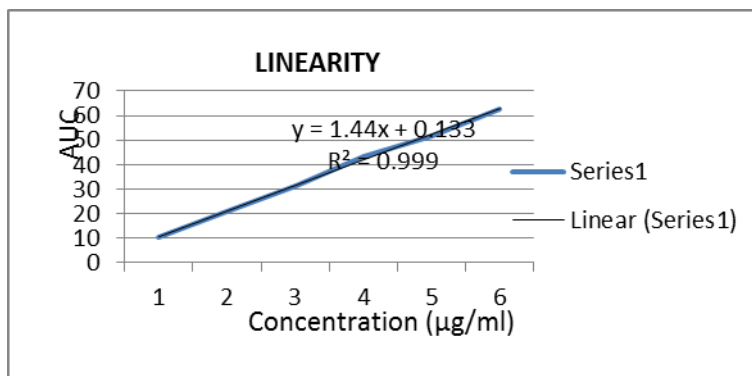


Figure 3: Calibration curve of methyl gallate.

Table 1: Linear regression data for calibration curves (n= 3).

Parameters	HPLC
Linearity range	10-60 µg/ml
R <sup>2</sup>	0.999
Slope	1.44
Intercept	0.133

Table 2: Intra- and inter-day precision of the developed method (n=6)

Concentration (µg/mL)	Repeatability(n=6)			Intermediate precision(n=6)		
	Measured concentration±SD	(%) RSD	Recovery (%)	Measured concentration	(%) RSD	Recovery (%)
5	4.8495±0.01	0.372	96.99	4.811±0.02	0.465	96.22
10	13.731±0.04	0.321	137.31	13.778±0.04	0.320	137.78
20	21.127±0.04	0.221	105.63	22.334±0.43	1.941	111.67

Table 3: Recovery study of MG added to the pre- analyzed samples using the proposed method (n=3)

Components	% Quantity added(µg/mL)	Total quantity present(µg/mL)	Amount Quantity found (µg/mL)	%Recovery	%RSD
Methyl Gallate (MG)	4 (80%)	9	9.438	104.86	±0.67
	5 (100%)	10	10.222	102.22	±0.29
	6 (120%)	11	11.074	100.67	±0.41

Table 4: Robustness evaluation of proposed HPLC method (n=3).

Factor	Level	Retention time	Asymmetry
<b>A:Flow rate(ml/min)</b>			
0.9	-1	9.27	1.38
1.0	0	8.40	1.36
1.1	+1	7.64	1.40
Mean± SD(n=3)		8.37± 0.90	1.38 ±0.02
<b>B:% of acetonitrile in mobile phase (v/v)</b>			
11	-1	9.19	1.36
12	0	8.40	1.36
13	+1	7.57	1.40
Mean± SD(n=3)		8.38± 0.81	1.37± 0.02
<b>C: Solvent of different lot</b>			
First lots		8.20	1.37
Second lots		8.18	1.36
Mean± SD(n=2)		8.17± 0.82	1.35± 0.02

**Table 5: Results of system suitability parameters of the proposed HPLC method (n=3).**

Sr.No	Parameter	MG
1	Retention time (min)	8.2 min
2	Capacity factor (k')	1.5
3	Separation factor	2.3
4	No. of theoretical plate	6975
5	Resolution (Rs)	2.8
6	Asymmetry (As)	1.37

**Table 6: Assay results of marketed herbal formulations using the developed HPLC method (n=3).**

Marketed formulation	Content of methyl gallate (%w/w)
Triphala Churna	0.071
Dabur Chyawanprash	0.076
Arjunarishta	0.05

**Forced Degradation Studies**

Conditions used for forced degradation were to achieve degradation in the range of 20-80% of the drug. The following degradation behaviour of the drug was observed during the stress degradation studies.

**Acidic condition**

The MG was refluxed in a specified concentration of 6 M HCl for 24 h. MG was found to be stable to acid degradation.

**Alkaline condition**

MG was found to undergo alkaline degradation faster as compared with acid degradation. In 0.1 M NaOH (1 h), the drug decomposed by 20%. A major degradation peak was found at 2.7 min. (Fig.2b).

**Oxidative condition**

MG was found to be highly labile in terms of oxidation in 6% H<sub>2</sub>O<sub>2</sub> at room temperature for 1 h. The major degradation peak was observed at 6.3 min. (Fig. 2c).

**Neutral (water) condition**

MG was found to be stable on refluxing the drug in water at 80 °C for 12 h.

**Photolytic condition**

MG was found to be stable to photolytic degradation.

**HPLC–UV method validation**

The method was validated for linearity, accuracy, precision, robustness, system suitability, LOD, LOQ and by the following procedure.

**Linearity**

The calibration curve was linear over the concentration range 5-50 µg/mL for MG. The correlation coefficient ( $r^2$ ) was found to be 0.999. Linearity data and calibration curve for MG are reported in the Table 1 and Fig. 3.

**Precision**

The precision of the method was examined by performing the intra- and inter-day assays of six replicate injection of the mixture of the standard solution at three

concentration levels 10, 20 and 30µg/mL). The % RSD of the assay was less than 2. (Table 2).

**LOD and LOQ**

LOD and LOQ of the method was determined by  $K \text{ sd}/s$  where K is the constant (3 for LOD and 10 for LOQ), sd is the standard deviation of the analytical signal and s is the slope of the concentration /response graph. The LOD and LOQ 0.05 µg/ml and 0.07 µg/ml respectively.

**Accuracy**

The accuracy of the method was determined by calculating the recoveries of MG by the method standard addition. Known amounts of the standard (80, 100, 120%) were added to the pre- analyzed sample solution and the amounts of these standards were estimated by measuring the peak areas. The results from recovery study for accuracy determination are given in the Table 3. The percentage recovery of MG was found to be within the limit 100.67 to 104.86 %).

**Robustness**

The robustness of the method was evaluated by changing: (i) the mobile phase flow (0.9, 1, 1.1 ml/min), (ii) % of acetonitrile in mobile phase (v/v) (11 ml, 12 and 13) and (iii) the solvents of different lots. Standard solutions were injected six times for each change. The % RSD was found to be less than 2. The results are summarized in the Table 4.

**System Suitability**

All the values of parameter i.e. capacity factor, separation factor, number of theoretical plate, asymmetry of the peak and resolution between two consecutive peak of system suitability were found to be within in the acceptable limits. It is concluded that the method and system are adequate for the analysis to be performed. The results are summarized in the Table 5.

**Analysis of marketed formulations**

The developed and validated method was applied to determine the content of methyl gallate in three marketed herbal formulations (Triphala churna, Dabour Chywanprash, Arjunarishta). (Table 6). The methyl

gallate content was highest in Dabour Chywanprash as compared to the other two formulations.

### CONCLUSION

A stability indicating HPLC method was successfully developed and validated for estimation of methyl gallate as per ICH guidelines. The method was applied for quantitative estimation of methyl gallate in three marketed herbal formulations.

### ACKNOWLEDGEMENT

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