

RP-HPLC Method Development and Validation of Gallic acid in Polyherbal Tablet Formulation

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ABSTRACT

A new simple, accurate, precise, sensitive and validated RP-HPLC method was developed for the estimation of Gallic acid in bulk and pharmaceutical tablet dosage form. The chromatographic conditions used for the separation was Phenomenex Luna C18 (2) (4.6 x 250mm, 5 μ), rheodyne manual injector with capacity of 20 μ L and mobile phase comprised of Water: Acetonitrile (80: 20%v/v) and pH is maintained at 3.00 using O-phosphoric acid (OPA). The flow rate was 1.0mL/min with detection at 272nm. The retention time was found to be 3.60min. The linearity was found to be in the range of 0.5-50 μ g/mL for Gallic acid with correlation coefficient of 0.9994. The proposed method is accurate with 99.97% - 100.58 % recovery and precise (%RSD of repeatability, intra-day and inter-day variations were 1.26%, 0.48-0.95%, 0.80-1.83%). The Limit of Detection (LOD) and Limit of Quantification (LOQ) were found to be 0.0178 μ g/mL and 0.0539 μ g/mL respectively. The amount of Gallic acid in Polyherbal tablet was found to be 1.63%.

INTRODUCTION

Gallic acid is a Poly phenolic compound having an antioxidant property. Chemically it is a 3, 4, 5-trihydroxybenzoic acid [Figure-1]. Gallic acid and its derivatives are a group of naturally occurring polyphenol antioxidants which have recently been shown to have potential health effects. Gallic acid and its derivatives have antioxidant activities, and neuroprotective effects with free radical scavenging effects (Zhongbing et al, 2006 & Kubo et al 2010).

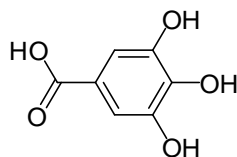


Fig. 1: Structure of Gallic acid.

Herbs that contains Gallic acid as a major constituent are citrus fruits like *Phyllanthus emblica* (Amalaki), *Terminalia bellirica* (Bibhitaki), *Terminalia chebula* (Haritaki), etc. which is useful in common cold and fever, diuretic, laxative, liver tonic,

refrigerant, stomachic, restorative, alterative, antipyretic, anti-inflammatory (Kroes et al, 1992), hair tonic, to prevent peptic ulcer and dyspepsia, and as a digestive. Preclinical studies have shown that amla possesses antipyretic, analgesic (Perianayagam et al, 2004), antitussive, antiatherogenic, adaptogenic (Sai Ram et al, 2003), cardioprotective, gastroprotective, antianemia, antihypercholesterolemia, cytoprotective (Gulati et al, 1995), wound healing, antidiarrheal, antiatherosclerotic, hepatoprotective (Jose et al, 2001), Antimutagenic, anticarcinogenic (Calixto et al, 1998), nephroprotective, memoryenhancing activity and neuroprotective properties. In addition, experimental studies have shown that amla and some of its phytochemicals such as gallic acid, ellagic acid, pyrogallol, some norsesquiterpenoids, corilagin, geraniin, elaeocarpusin, and prodelphinidins B1 and B2 (Yadav, 1987) also possess antineoplastic effects. Amalaki is also reported to possess radiomodulatory (Ghoshal et al, 1996), chemomodulatory, chemopreventive effects, free radical scavenging (Jose & Kuttan, 1995), antioxidant (Miller, 1996), anti-inflammatory, antimutagenic and immunomodulatory activities and properties that are efficacious in the treatment and prevention of cancer. Polyherbal formulation contains more than one herb or a mixture of different herbal powders or extracts, so being difficult to identify a particular component.

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This Polyherbal Trichup tablet is used as essential nutrition to hair roots, stimulates repair of hair follicles thus enhancing natural hair growth, helps control hair fall, helps prevent premature greying and falling of hair, which contains Amla and Triphala as a major constituent of gallic acid, so an attempt is being made to develop a specific, accurate, precise and simple method for estimation of particular compound from Polyherbal tablet formulation. Validation of developed method was carried out as per ICH Q2R1 guideline to maintain the reproducibility and consistency of the method.

MATERIALS & METHODS

Apparatus

The chromatography was performed on a Shimadzu LC-20AT, SPD-20A HPLC instrument equipped with UV detector UV-20A with wavelength Range 190-700nm and Spinchrom LC Solution software.

Phenomenex Luna C18(2) (4.6 x 250mm, 5 μ particle size) was used as stationary phase. Rheodyne manual injector with 20 μ L capacity loop was used. Shimadzu Unibloc AUX220 with Capacity of 10mg -220g as an analytical balance. Toshcon pH meter and Sonicator Toshcon SW-2 were used in the study.

Reagents and Materials

The reference standard of Gallic acid was purchased from Hi-media Laboratories Pvt. Ltd. Mumbai. HPLC grade Water, Methanol, Acetonitrile and OPA made of Merck specialities Pvt. Ltd. Mumbai were used in study.

Trichup tablet (An Ayurvedic Proprietary Formulation) is manufactured and provided by Vasu Healthcare Pvt. Ltd., Vadodara- Gujarat., which contains extracts of *Eclipta alba* (Bhringraj), *Glycyrrhiza glabra* (Yashtimadhu), *Emblica officinalis* (Amalaki), *Centella asiatica* (Mandukparni), *Hibiscus rosa-sinensis* (Japa), *Tinospora cordifolia* (Guduchi), *Tribulus terrestris* (Gokshur), and Powder of Triphala Churna and Shukti Bhasma.

Selection of Mobile Phase

Based on sample solubility, stability and suitability various mobile phase compositions were tried to get a good resolution and sharp peaks.

The standard and sample solution was run in different mobile phases.

From the study, Acetonitrile was preferred over Methanol because it gives sharper peak than methanol. From the various mobile phases, Water: Acetonitrile (80: 20 %v/v) and pH-3.00 by O-phosphoric acid was chosen with detection wavelength 272nm.

Chromatographic conditions

The optimized parameters which were used as a final method for the estimation of Gallic acid represented in the Table-1

Table. 1: Chromatographic Condition for Gallic acid.

Mobile Phase	Water : Acetonitrile (80 :20 %v/v)
Stationary Phase	Phenomenex Luna C18 (4.6 x 250mm, 5 μ particle size)
Wavelength	272 nm
Run time	6 min
pH of Mobile Phase	3.00 with OPA
Flow Rate	1 mL/min
Injection Volume	20 μ L
Temperature	Ambient
Mode of Operation	Isocratic elution

Preparation of Standard Stock Solution

An accurately weighed quantity of Gallic acid (10mg) was transferred to a 10mL volumetric flask, dissolved and diluted to the mark with Water: Methanol (9:1 %v/v) to obtain standard stock solution of 1000 μ g/mL.

Preparation of Calibration Curve

Aliquots of 0.1, 0.3, 0.5mL standard stock solution (1000 μ g/mL) was transferred to 10mL of volumetric flasks and made up to the mark with Water : Methanol (9: 1 %v/v) to get concentration of 10, 30, 50 μ g/mL. aliquots of 0.1, 0.2, 0.5, 1mL from 50 μ g/mL was transferred to 10mL of volumetric flasks and made up to the mark with Water: Methanol (9: 1 %v/v) to get concentration of 0.5, 1, 2.5, 5 μ g/mL.

Sample preparation

20 tablets were taken and crushed in mortar pastel. From that, accurately weighed 100mg tablet powder transferred to 10mL standard flask.

Volume is made up to the mark with Water: Methanol (9: 1 %v/v), sonicated for 10 min. It is filtered with 0.22 μ filter to obtain sample stock solution. Aliquot of 1ml from this sample stock solution is transferred to 10mL standard volumetric flask. Volume is made up to the mark with Water: Methanol (9: 1 %v/v). Then it is filtered with 0.22 μ filter. Prepared sample solution was analysed.

METHOD VALIDATION

The optimized Chromatographic method was completely validated according to the procedures described in ICH guidelines Q2(R1) for the validation of analytical methods (ICH, 2005).

System Suitability Test

20 μ L of Gallic acid standard solution of 50 μ g/mL was injected under optimized chromatographic conditions to evaluate the suitability of system [Table-2].

Table. 2: System Suitability of Gallic acid.

Parameter	Result
Retention time	3.6 min
Peak Area	3190.82
Efficiency	14669.2
Asymmetry	1.167

Specificity

Specificity of the HPLC method was demonstrated by the separation of the analytes from other potential components such as impurities, degradants or excipients. A volume of 20 μ L of individual ingredients and excipients solution were injected and the chromatogram was recorded. Peaks of excipients were not found at retention time of 3.60min. Hence, the proposed method was specific for Gallic acid.

Linearity

The linearity of calibration curve in Gallic acid standard solution, over the concentration range of 0.5-50 μ g/mL through proposed HPLC method was carried out. Regression was found to be 0.9994 [Table 3].

Table 3: Linearity of Gallic acid.

Concentration (μ g/mL)	Peak Area	Slope	Intercept	R ²
0.5	38.15	72.10	2.45	0.9994
1	75.03			
2.5	175.94			
5	322.46			
10	747.86			
30	2223.76			
50	3572.22			

Accuracy

For the accuracy of proposed method, recovery studies were performed by standard addition method at three different levels (80%, 100% and 120% of final concentration). A known amount of standard pure drug was added to pre-analyzed tablet powder and the sample was then analyzed by proposed method. Results of recovery studies were found to be satisfactory [Table-4].

Table 4: Accuracy of Gallic acid.

sno	Gallic acid Amount	Amount added μ g/mL	Peak Area	Amount Recovered	% Recovery	Mean
1	16.33 μ g/mL		1177.956	16.34		
			1165.003	16.16		
			1189.979	16.50		
2	16.33 μ g/mL	14.8	2249.274	31.19	100.20	100.13
			2225.245	30.86	99.13	
			2268.908	31.46	101.07	
3	16.33 μ g/mL	18.5	2509.076	34.79	99.90	99.97
			2513.479	34.86	100.07	
			2510.456	34.81	99.95	
4	16.33 μ g/mL	22.2	2797.424	38.79	100.68	100.58
			2780.816	38.56	100.08	
			2805.470	38.90	100.97	

Precision

The precision of the method was determined by repeatability, interday and intraday precision.

Repeatability

The repeatability of the proposed method was ascertained by injecting five replicates of 50 μ g/mL concentration, within the Beer's range and finding out the peak area by the proposed method. From this peak area %RSD was calculated [Table- 5].

Table 5: Repeatability of Gallic acid.

Sr No.	Concentration (μ g/mL)	Peak Area	Mean	SD	%RSD
1	50	3155.19	3190.82	40.18	1.26
2		3210.96			
3		3186.37			
4		3248.57			
5		3153.01			

Intra-day precision

Intra-day precision was determined by injecting three different concentrations for three times in the same day. Peak area was measured and %RSD was calculated [Table- 6].

Inter-day precision

Inter-day precision was determined by injecting three different concentrations for three days in a week. Peak area was measured and %RSD was calculated [Table- 6].

Robustness

The robustness of the HPLC method was evaluated by analysing the system suitability parameters after varying the pH of the mobile phase (\pm 2%), organic solvent content (\pm 2%), Flow Rate (\pm 2%) and wavelength (\pm 2%). None of these alterations caused change in % RSD of peak area or retention time. Although the change in the retention time was significant, yet quantification was possible [Table-7].

Limit of Detection and Limit of Quantification

Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined by using the formula based on the standard deviation of the response and the slope. LOD and LOQ were calculated by using equations,

$$\text{LOD} = 3.3 \times \sigma / \text{slope} \quad \& \quad \text{LOQ} = 10 \times \sigma / \text{slope},$$

Where, σ = Standard Deviation,

Slope = Slope of the calibration curve

By these equations LOD was found to be 0.0178 μ g/mL and LOQ was found to be 0.05399 μ g/mL.

Statistical analysis

Statistical calculations were carried out with the Microsoft Excel 2007 for Windows software package. Average, Sum, Standard Deviation (STDEV), Regression (RSQ) for Statistical Calculation, and Scattered Chart were used for Linearity; P values > 0.05 were considered to be significant.

RESULTS

Developed and validated RP-HPLC method for the estimation of Gallic acid in Polyherbal formulation is found to be simple and economical. Several mobile phase compositions were tried. A satisfactory separation and good peak symmetry was obtained with Phenomenex Luna C18(2) column (4.6 x 250mm, 5 μ) and mobile phase comprising of Water: Acetonitrile (80: 20 %v/v) and pH is maintained at 3.00 using O-phosphoric acid at a flow rate of 1.0mL/min to get better reproducibility and

repeatability. Quantification was achieved with UV detection at 272nm based on peak area. The retention time was found to be 3.60min. The optimised method was validated as per ICH guidelines. In System suitability efficiency and Asymmetry were found to be 144669.2 and 1.167 respectively [Table- 2]. Linearity for range of 0.5-50 μ g/mL with correlation coefficient 0.9994 was established [Table -3, Figure- 2, 3]. The result of recovery study by standard addition method ranging from 99.97% -100.58 % suggested the good accuracy [Table- 4]. The precision of the proposed method was carried in terms of the repeatability, inter-day and intra-day time periods.

The low %RSD values of Repeatability (1.26%), Inter-day (0.80-1.83%) and Intra-day (0.48-0.95%) variations reveal that the proposed method is precise [Table- 5, 6].

The LOD, LOQ values were found to be 0.0178 μ g/mL and 0.05399 μ g/mL respectively. The Method was found to be Robust with change of \pm 2% in Wavelength, Flow Rate, pH, Mobile Phase Composition [Table- 7]. The amount of Gallic acid in Polyherbal tablet was found to be 1.63% [Figure-5]. The absence of interference peak indicates that method can be used for routine analysis of Gallic acid in bulk pharmaceutical as well as tablet dosage form.

Table. 6: Intraday & Interday Precision of Gallic acid.

Sr No	Conc. (μ g/mL)	0 Hour		3 Hour		6 Hour		Mean	SD	%RSD
		Rt (min)	Peak Area	Rt (min)	Peak Area	Rt (min)	Peak Area			
1	0.5	3.58	38	3.58	38	3.58	38	37.87	0.36	0.95
2	5	3.59	319	3.58	316	3.58	319	318.08	1.54	0.48
3	50	3.59	3543	3.59	3582	3.60	3563	3563.01	19.63	0.55
Sr No	Conc. (μ g/mL)	Day 1		Day 2		Day 3		Mean	SD	%RSD
		Rt (min)	Peak Area	Rt (min)	Peak Area	Rt (min)	Peak Area			
1	0.5	3.58	38	3.62	39	3.62	38	38.29	0.70	1.83
2	5	3.59	319	3.62	330	3.62	328	325.68	5.90	1.81
3	50	3.59	3543	3.65	3601	3.63	3571	3571.76	28.75	0.80

Table. 7: Robustness of method.

Sr No	Conc	Rt	PA	Rt	PA	Rt	PA	Rt	PA	Rt	PA	Rt	PA
		Wavelength						Flow Rate					
		267 nm		272 nm		277 nm		0.98 mL/min		1.00 mL/min		1.02mL/min	
1	10	3.58	689	3.58	740	3.57	706	3.65	746	3.58	740	3.51	718
2		3.58	695	3.58	729	3.58	707	3.65	748	3.58	729	3.51	717
3		3.58	690	3.58	729	3.58	709	3.65	746	3.58	729	3.51	719
Mean		3.58	691	3.58	733	3.58	707	3.65	747	3.58	733	3.51	718
SD		0.002	2.943	0.002	6.494	0.002	1.226	0.002	1.431	0.002	6.494	0.002	0.979
%RSD		0.048	0.426	0.048	0.887	0.065	0.173	0.047	0.192	0.048	0.887	0.049	0.136
Sr No.	Conc.	pH						Mobile Phase					
		pH-2.94		pH-3.00		pH-3.06		78.5:21.5		80:20		81.5:18.5	
		Rt	PA	Rt	PA	Rt	PA	Rt	PA	Rt	PA	Rt	PA
1	10	3.74	739	3.68	728	3.65	720	3.67	723	3.68	728	3.72	735
2		3.72	737	3.67	726	3.64	717	3.66	721	3.67	726	3.70	731
3		3.71	734	3.66	725	3.63	717	3.65	719	3.66	725	3.69	728
Mean		3.72	737	3.67	726	3.64	718	3.66	721	3.67	726	3.70	731
SD		0.014	2.636	0.010	1.649	0.010	1.637	0.010	1.794	0.010	1.649	0.017	3.398
%RSD		0.363	0.358	0.272	0.227	0.279	0.228	0.277	0.249	0.272	0.227	0.446	0.465

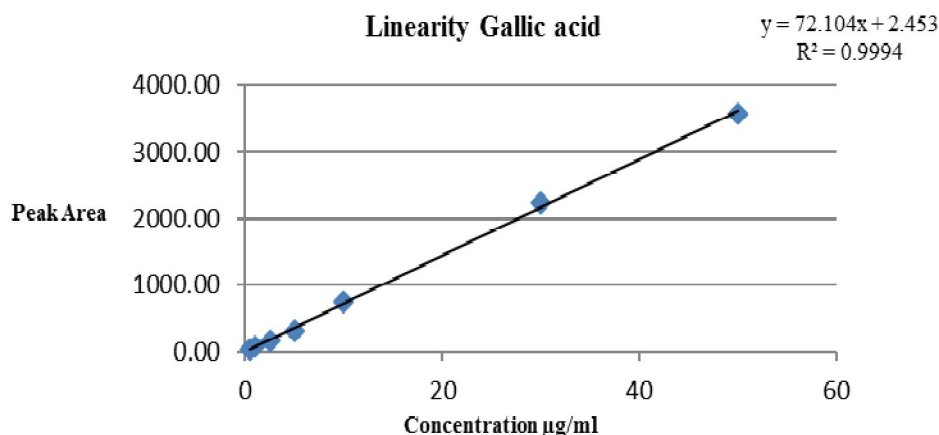


Fig. 2: Linearity Graph of Gallic acid.

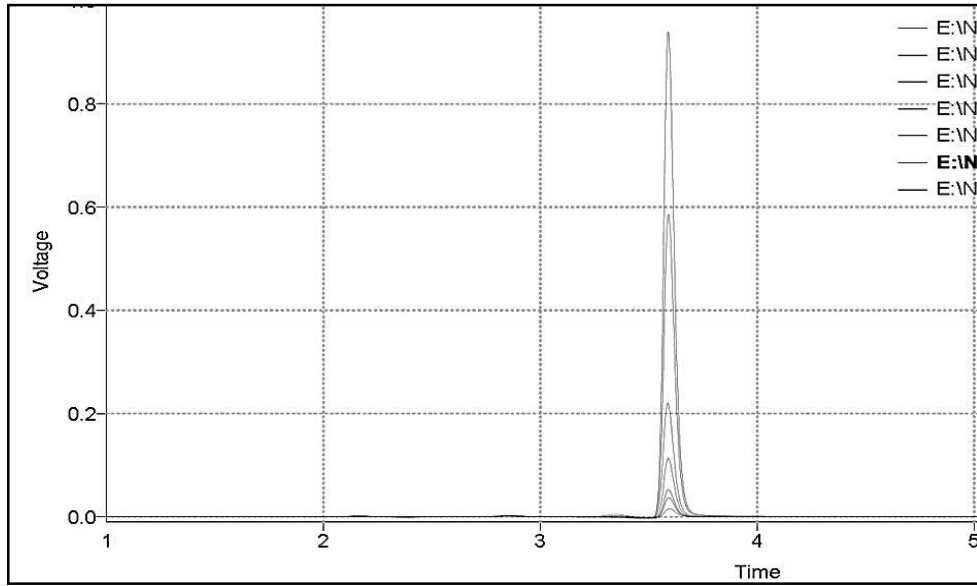


Fig. 3: Linearity chromatogram of Gallic acid.

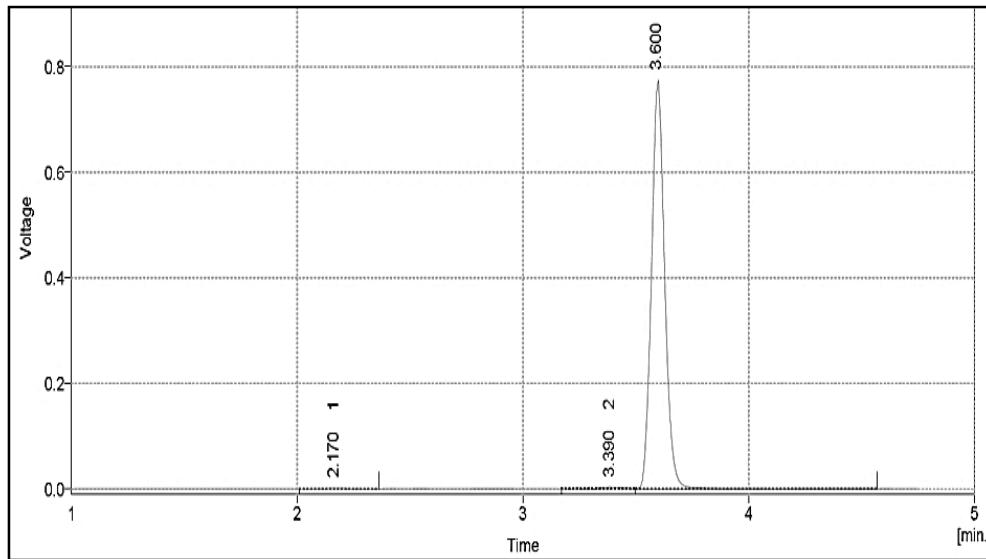


Fig. 4: Chromatogram of Gallic acid Standard 50µg/mL.

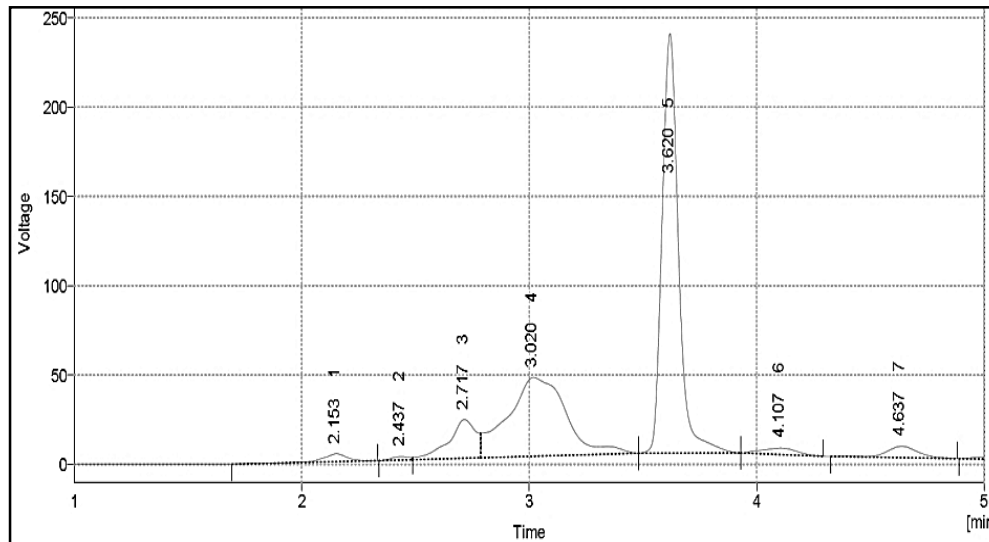


Fig. 5: Chromatogram of Polyherbal Tablet.

CONCLUSION

A specific, precise, accurate, sensitive, rapid and reliable RP-HPLC method has been developed and validated. It has short runtime (6min) and retention time (3.60min) of Gallic acid. This allows analysis of large number of samples in a short period of time. In this method, there is no interference from the excipients of tablet. So this RP-HPLC method can be used in the Quality control Department for estimation of Gallic acid in Polyherbal formulation.

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