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Quantitative estimation of Gallic acid and Ascorbic acid in a marketed herbal medicine: Triphala Churna by High Performance Thin Layer Chromatography

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Abstract: Triphala is an age old commonly used Ayurvedic powdered preparation in Indian systems of medicine. This well known formulation is made by combining *Terminalia chebula, Terminalia belerica* and *Embellica officinalis*, in equal proportions. The formulation is prescribed in the first line treatment of many aliments and is used as laxative, detoxifying agent and rejuvenator. *Terminalia chebula* and *Terminalia belerica* consist of gallic acid and *Embellica officinalis* consist of ascorbic acid as marker constituent.

A HPTLC- densitometric method of analysis for these markers i.e. gallic acid and ascorbic acid was developed. Water was selected as a solvent for preparing standard solutions. Quantitative estimation of gallic acid and ascorbic acid was performed separately on aluminum backed silica gel 60 F_{254} TLC plates (10 cm x 10 cm plate size, layer thickness 0.2 mm, E-Merck, Darmstadt, Germany).

Ascorbic acid shows R*f* value of 0.74 ± 0.1 using ethanol: glacial acetic acid: toluene (5.5:1:1.5) and gallic acid showed R*f* value of 0.54 ±0.1, using ethyl acetate: toluene: acetone (4.5:4:1) as mobile phase. The polynomial regression data of ascorbic acid and gallic acid were interpreted separately for its linearity at 500-3500 µg/ml with R^2 = 0.9986 and 0.9931 respectively.

The literature survey reveals that, there is no such chromatographic method available for quantitative estimation of gallic acid and ascorbic acid.

Keywords: Gallic acid, Ascorbic acid, High Performance Thin Layer Chromatography.

INTRODUCTION:

The use of plants, parts of plants and isolated phytochemicals for the prevention and treatment of various health ailments has been in practice from time immemorial. [1] About 75–80% of the world population, mainly in the developing countries, uses herbal medicines for primary health care because of better cultural acceptability, better compatibility with the human body and lesser side effects. [2]

Triphala is a traditional ayurvedic herbal formulation, consisting equal parts of three medicinal plants namely Terminalia chebula, Terminalia belerica and Embellica officinalis. Triphala has been reported to possess antioxidant activity, improves mental and physical power and also assist in weight loss. [3]

Triphala is neither a harsh purgative nor a lubricating laxative. A nutritive and cleansing property of triphala makes it special. Triphala gently stimulates the cleansing of accumulated toxins from all tissues of the body, reduces cholesterol and high blood pressure, and improves circulation.

Terminalia chebula contains 30% tannins and possess anthelmintic, aphrodisiac and astringent activity; Terminalia belerica contains 17% tannins, gallic acid, and ellagic acid and possess hepatoprotective activity. Embellica officinalis is the highest known source of vitamin C in nature, cures bleeding diseases, used in treatment of scurvy. [4]

Triphala also claimed to have various biological activities like heart protective, cardio tonic, improves digestion, liver function and hepatoprotective. In this study gallic acid is used as marker constituent for Terminalia chebula and Terminalia belerica, and ascorbic acid is used as marker for Embellica officinalis.

The aim of present research study is to carry out quantitative estimation of ascorbic acid and gallic acid in triphala churna and comparison between three renowned manufacturers (A, B, C) by using HPTLC method.

EXPERIMENTAL:

INSTRUMENTATION:

The method was developed on CAMAG HPTLC system consisting of Linomat V applicator (Camag, Muttenz, Switzerland) CAMAG twin trough chamber, CAMAG TLC scanner, equipped with Wincats software (version 1.4.2) , CAMAG syringe of 100 µL capacity.

Separation and identification of gallic acid and ascorbic acid were performed separately on aluminum backed silica gel 60 F_{254} (20cm x10cm of plate size, layer thickness 0.2 mm, E-Merck, Darmstadt, Germany).

REAGENTS AND CHEMICALS:

The mobile phase, composed of ethyl acetate: toluene: acetone (4.5:4:1) separated the gallic acid with good resolution. And the mobile phase, composed of ethanol: glacial acetic acid: toluene (5.5:1:1.5), separated the ascorbic acid with good resolution. *Triphala churna* of three different manufacturers (A, B, C) were procured from local market.

PREPARATION OF WORKING STANDARD SOLUTIONOF ASCORBIC ACID:

Standard stock solution of ascorbic acid was prepared by dissolving 5 mg of ascorbic acid up to 10 ml of water, to get stock solution containing 500µg/ml of ascorbic acid. Further dilutions were made as 500-3500 ng/µl to perform linearity study.

PREPARATION OF WORKING STANDARD SOLUTIONOF GALLIC ACID:

Standard stock solution containing 5 mg of gallic acid was prepared in 10 ml of water, to get stock solution containing 500µg/ml of gallic acid. Further dilutions were made as 500-3500 ng/µl to perform linearity study.

PREPARATION OF SAMPLE SOLUTION OF ASCORBIC ACID:

4 g of triphala churna (each from three different manufacturers) was macerated separately with water for 24 hours, resulting extract was filtered and filtrate was treated with lead acetate to remove unwanted impurities and filtered, filtrate was treated with ether to isolate ascorbic acid. The ascorbic acid thus obtained was dissolved up to 10 ml of distilled water separately.

PREPARATION OF SAMPLE SOLUTION OF GALLIC ACID:

1 g of triphala churna (from three different manufacturers) were extracted separately with 20 ml 2M HCL and filtered, and the filtrate was treated with diethyl ether in a separating funnel. The ethereal part was collected and the solvent was allowed to evaporate to get gallic acid. The gallic acid thus obtained was dissolved up to 50 ml of distilled water separately.

CHROMATOGRAPHIC CONDITIONS

The experiment was performed on a silica gel 60 F_{254} (0.2 mm thickness) HPTLC plates (20×10cm) without prewashing. Samples were applied to the plates as 8 mm bands, 8 mm apart and 10 mm from the edges of the plate, with a Camag Linomat V automatic sample applicator. The plates were developed by the ascending technique, to a distance of 80 mm, at 25 ± 5°C, relative humidity 50–60%, in a Camag twin-trough glass chamber with a stainless steel lid, using a mobile phase, composed of ethanol: glacial acetic acid: toluene (5.5:1:1.5), for ascorbic acid and ethyl acetate: toluene: acetone (4.5:4:1) for gallic acid respectively. The chamber saturation time was kept as 20 min. After development, plates were dried with a hot-hair dryer, viewed in a Camag UV cabinet, and then scanned with a Camag TLC Scanner, using winCATS software (version 1.4.2) , in absorbance mode, with slit dimensions 6.00 x 0.45 mm, Micro. The detection wavelength 254 nm was selected. The *R*f values were found to be 0.74 ±0.01 and 0.54±0.01 for ascorbic acid and gallic acid, respectively.

METHOD VALIDATION: [5]

LINEARITY:

The linearity of the method was evaluated by triplicate analysis of standard solutions containing 500 ng to 3500 ng of ascorbic acid. Peak area was recorded for each concentration and a calibration plot was obtained by plotting peak area against concentration. Correlation coefficients (r^2) was 0.9986 for ascorbic acid, using ethanol: glacial acetic acid: toluene (5.5:1:1.5) as solvent system. The average linear equations were Y = 6.078X – 5796 (Table 1).

Similarly the linearity of the method was evaluated by triplicate analysis of standard solutions containing 500 ng to 3500 ng of gallic acid. Peak area was recorded for each concentration and a calibration plot was obtained by plotting peak area against concentration. Correlation coefficients (r^2) was 0.9931 for gallic acid, using ethyl acetate: toluene: acetone (4.5:4:1) as solvent system. The average linear equations were Y = 5.842X – 2282 (Table 2).

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION:

The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the slope (s) of the calibration plot and the standard deviation of the response (SD). The values were found to be 25.248*µg/ml and* 76.510 *µg/ml,* 13.129 *µg/ml and* 39.388 *µg/ml* for ascorbic acid and gallic acid respectively.

PRECISION:

To establish intra-day precision, the stock solution of ascorbic acid and gallic acid was applied on the plates separately, analysis was carried out in triplicates (3µl i.e. 1500ng of ascorbic acid and 3µl i.e. 1500ng of gallic acid) at an interval of 2 hours (Table-3 & 4) (Figure 1 & 2).

Table 1: Results of linearity of ascorbic acid

Track	Sample volume in µl	Amount fraction in ng	Mean area (AU)	±SD	%RSD	Correlation coefficient (r^2)
1	1	500	-	-	-	0.9986
2	2	1000	572.1767	10.6386	1.8593	
3	3	1500	3114.167	55.55811	1.7840	
4	4	2000	6270.46	35.5992	0.5677	
5	5	2500	9242.13	33.4247	0.3616	
6	6	3000	12405.53	59.2220	0.4773	
7	7	3500	15678.77	84.6112	0.5396	

Table 2: Results of linearity of gallic acid:

Track	Sample volume in µl	Amount fraction in ng	Mean area (AU)	±SD	%RSD	Correlation coefficient (r^2)
1	1	500	471.353	4.09196	0.8681	0.9931
2	2	1000	2979.39	5.6276	0.1888	
3	3	1500	6753.89	26.3163	0.3896	
4	4	2000	9970.743	11.6462	0.1168	
5	5	2500	12827.47	46.52899	0.3627	
6	6	3000	15350.32	47.75708	0.3111	
7	7	3500	17463.52	20.78701	0.1190	

Table 3: Results of intraday precision for ascorbic acid.

Sample volume in µl	Concentration of Ascorbic acid in ng	R*f* value	Area (AU)	Mean area (AU)	± SD	%RSD
3	1500	0.74	3078.0 3075.1 3082.0	3078.4	3.4645	0.1125
3	1500	0.74	3369.8 3373.6 3369.7	3371.3	2.2233	0.6594
3	1500	0.74	3073.2 3064.6 3068.0	3068.6	4.3312	0.1411

Table 4: Results of intraday precision for gallic acid

Sample volume in µl	Concentration of Ascorbic acid in ng	R*f* value	Area (AU)	Mean area (AU)	± SD	%RSD
3	1500	0.53	8442 8460 8451.3	8451.1	9.0001	0.1064
3	1500	0.53	8427.1 8426.1 8419.2	8424.2	4.3015	0.0510
3	1500	0.53	8659.1 8658.3 8651.0	8656.5	4.1139	0.0475

Fig 1: Densitogram of ascorbic acid was measured at 254 nm using mobile phase ethanol: glacial acetic acid: toluene (5.5:1:1.5)

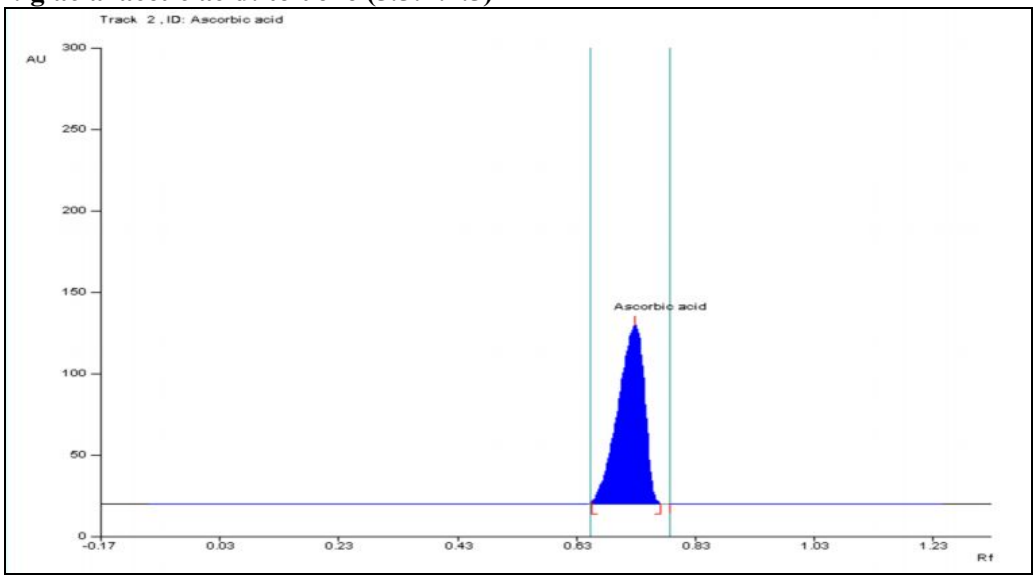
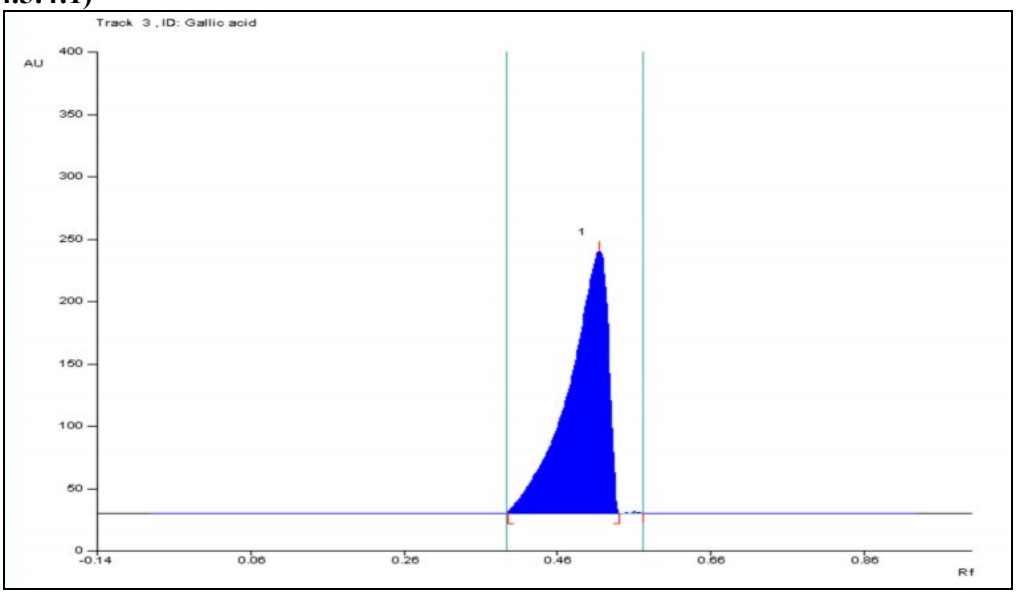


Fig 2: Densitogram of gallic acid spot was measured at 254 nm using mobile phase, ethyl acetate: toluene: acetone (4.5:4:1)



ANALYSIS OF MARKETED FORMULATION:

The sample solution prepared from extraction of triphala churna of different manufactures were applied on the plates separately, for quantitative determination of ascorbic acid and gallic acid, analysis was carried out in triplicates (Table 5 & 6)

RECOVERY STUDIES:

To confirm the accuracy of the proposed method, recovery experiments were carried out by standard addition technique by adding a known amount of standard to the sample. Each level was repeated three times (n = 3). Results and statistical parameters are reported in Table 7 & 8. From the amount of drug found, the percentage recovery was calculated.

Table 5: Results of isolated ascorbic acid of three different manufacturers of triphala churna

Sample	Sample volume in µl	Area (AU)	Mean area (AU)	± SD	%RSD	Unknown concentration in ng
A	3	3550.9	3550.8	2.5161	0.0709	1741
		3548.9				
		3553.6				
B	3	3364.6	3357.7	6.0827	0.1812	1647
		3354.8				
		3353.7				
C	3	3067.7	3068.3	4.5824	0.1493	1505
		3064.1				
		3073.0				

Table 6: Results of isolated gallic acid of three different manufacturers of triphala churna

Sample	Sample volume in µl	Area (AU)	Mean area (AU)	± SD	%RSD	Unknown concentration in ng
A	4	9795.1 9796.2 9790	9793.98	3.2145	0.0328	2067
B	4	9569.0 9562.1 9567.0	9566.1	3.6055	0.0377	2028
C	4	9276.5 9276.3 9275.2	9276.0	0.5773	0.0062	1978

Table 7: Recovery studies of ascorbic acid:

Sample	Recovery Level (%)	Initial amount in ng	Amount added in ng	Amount found in ng	Mean Area (AU)	±SD	%RSD	% recovery
A	80	2500	2000	4496.79	21535.6	33.005	0.1532	99.9281
	100	2500	2500	4989.90	24527.6	15.394	0.0627	99.7815
	120	2500	3000	5488.69	27564.3	36.143	0.1311	99.7944
B	80	2500	2000	4466.60	21352	30.413	0.1424	99.2577
	100	2500	2500	4960.34	24353	21.283	0.0873	99.2069
	120	2500	3000	5432.05	27220.6	20.984	0.7708	98.7645
C	80	2500	2000	4435.34	21162	32.186	0.1520	98.5631
	100	2500	2500	4973.01	24430.2	32.654	0.1336	99.4603
	120	2500	3000	5439.45	27265.2	20.792	0.0762	98.8991

Table 8: Recovery studies of gallic acid

Sample	Recovery level (%)	Initial amount in ng	Amount added in ng	Amount found in ng	Mean Area (AU)	±SD	%RSD	% recovery
A	80	2500	2000	4586.23	24538.3	29.022	0.1182	101.916
	100	2500	2500	5066.45	27346.6	48.045	0.1756	101.329
	120	2500	3000	5490.77	29828.3	25.146	0.0843	99.830
B	80	2500	2000	4502.73	24050.0	27.024	0.1123	100.060
	100	2500	2500	4995.21	26930.3	18.556	0.0689	99.904
	120	2500	3000	5491.10	29830.6	34.990	0.1172	99.838
C	80	2500	2000	4502.05	24046	40.632	0.1689	100.045
	100	2500	2500	4982.48	26855.6	25.324	0.0942	99.649
	120	2500	3000	5457.07	29631	22.113	0.0746	99.216

RESULT AND DISCUSSION:

A chromatographic method was developed for separation of ascorbic acid, using ethanol: glacial acetic acid: toluene (5.5:1:1.5) as a mobile phase. Similarly another chromatographic method was developed for separation of gallic acid, using ethyl acetate: toluene: acetone (4.5:4:1) as a mobile phase.

The polynomial regression data of ascorbic acid and gallic acid were interpreted separately for its linearity at 500-3500 µg/ml with regression coefficient of (R^2 = 0.9986 and 0.9931) respectively.

The result of recovery study clearly indicates that the percentage recovery for ascorbic acid and gallic acid were found to be within range of 98.5631-101.916% for all the three products.

Amount of ascorbic acid present in 3µl sample of product A, B and C was found to be 1741ng, 1647ng and 1505ng respectively. Amount of gallic acid present in 4µl sample of product A, B and C was found to be 2067 ng, 2028 ng and 1978 ng respectively.
Thus amount of ascorbic acid and gallic acid present in 100 g of Triphala churna of different manufacturers (i.e. A, B and C) were found to be 145.08, 137.25, 125.43 mg and 2.583,2.535,2.472 g respectively using HPTLC.

CONCLUSION:

In the present study, a simple, precise and accurate method for quantitative estimation of ascorbic acid and gallic acid in herbal medicine *(triphala churna)* by HPTLC was developed. The gallic acid and ascorbic acid content in *triphala churna* was quantified. The new developed HPTLC methods can be used for quantitative estimation of ascorbic acid and gallic acid in herbal medicines. The result of quantitative estimation of ascorbic acid and gallic acid by HPTLC reveals that the product A is more efficacious as compared to product B and C.

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