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and Validation of Hptlc Method for the Simultaneous Estimation of Ascorbic Acid and Gallic Acid in Amla Juice Preparation

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ABSTRACT

The aim of this study was to asses a simple, selective, precise, and reproducible high performance thin-layer chromatography (HPTLC) method for the simultaneous estimation analysis of ascorbic acid (AA) and gallic acid (GA) in amla juice preparation. The aluminium-based pre-coated TLC plates (Silica gel G 60F 254) were used for the HPTLC fingerprinting analysis. The chromatograms of samples were developed in twin trough glass chamber pre-saturated with mobile phase (toluene: ethyl acetate: methanol: formic acid; 3:3:2:1, v/v/v/v) at room temperature (25 ± 2°C). The densitometric analysis was carried out in absorbance mode at 254 nm. The optimized mobile phase showed compact spots of AA and GA at 0.59 and 0.86 *Rf* respectively. The linear regression analysis data for the calibration plots of AA and GA showed good linearity (r^2= 0.992 and 0.996 respectively) with respect to peak area in the range of 200-1400 ng/spot. The method was validated as per International Conference on Harmonization (ICH) guidelines. The limits of detection and quantification (40 and 140 ng/spot, respectively) were also established. The proposed method has shown the excellent recovery (98.97–99.89%), which supports the suitability ofthe method for the analysis of AA and GA inthe amlajuice and other preparations containing these ingredients.

Keywords: Amlajuice, Ascorbic acid, Gallic acid, HPTLC, ICH guidelines, Validation.

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1. INTRODUCTION

Herbal drugs are complex in nature and inherent variability of the chemical constituents in plant based drugs so it is difficult to establish quality control parameters by using analytical techniques. Antioxidants are the substances which play a vital role and help to defend the body against cell damage caused by free radicals which are produced by various degenerative changes in our body leading to ailments such as heart disease, hardening of the arteries, inflammatory conditions, cataracts and other visual problems, arthritis and rheumatism, diabetes and cancer[1,2]. *Emblica officinalis* (Euphorbiaceae) is commonly known as Indian gooseberry or amla is found throughout the different regions of India. It is most important medicinal plant in the Indian traditional system of medicine as in the Ayurveda as various parts of the plant are used to treat wide range of diseases[3,4]. The fruit of *E. officinalis* are acidic in nature, bitter in taste and rich in vitamin C[5,6]. The amla fruit is used either alone or in combination with other plants to possess pharmacological activity like laxative, livertonic, stomachic, restorative, alterative, cardioprotective, gastroprotective, antianemia, antihypercholesterolemia, cytoprotective, hepatoprotective, antimutagenic, anticarcinogenic, nephroprotective, neuroprotective and radiomodulatory, chemomodulatory, chemopreventive effects, antioxidant, anti-inflammatory, and immunomodulatory activities[7-9]. Ascorbic acid (AA) and gallic acid (GA) are the major antioxidants found in the amla fruits. Therefore, they can be used as bioactive markers for standardization of herbal preparation[10,11]. According to the literature surveyed there are no reports on (HPTLC) method for simultaneous estimation of AA and GA in herbal amla juice. The

quality of herbal medicines and the constituents present in the final formulation has direct implication on efficacy and safety of the formulation. The aim of this work is to develop an accurate, specific, repeatable and validated method for simultaneous determination of AA and GA in amla juice formulations.

2. MATERIALS AND METHODS

2.1 Plant material

The amla fruit was procured from Dehradun in the month of January, 2014 and authenticated by botanical survey of India Dehradun. The voucher specimens were deposited in the Department of Quality Assurance at ISF College of Pharmacy, Moga (Punjab, India).

2.2 Standard markers

Standard markers of ascorbic acid and gallic acid were procured from CDH (Central Drug House) Ltd New Delhi, India.

2.3 Apparatus and chromatographic conditions

A HPTLC system (Camag, Switzerland) was equipped with a sampler applicator Linomet V (Hamilton, Switzerland), twin trough TLC plate developing chamber, TLC Scanner III, Reprostar and Wincats, integration software (Camag, Switzerland). Chromatography was performed on pre-coated silica gel 60 F254 TLC aluminium plates (20×10 cm, 0.2 mm thick) (E. Merck, Germany).

2.4 Extraction and lyophilization of amla juice

The amla fruits were cleaned and cut into pieces and immediately pressed to obtain juice using a small laboratory manual grinder, a fine paste was obtained and the paste was filtered through muslin cloth and juice was collected in a container. For freeze drying 100 ml of juice was subjected to freezing at -35° C (primary drying) for 3 h followed by freeze dehydration in a freeze dryer (Daihan Labtech Co. Ltd, Model no. LTFD- 5505) at 60° C (secondary drying) for 16 h. The dried powder was obtained which was stored in a well closed container away from moisture and light[5].

2.5 Preparation of test samples for HPTLC study

Ten mg of lyophilized amla juice powder was transferred into a 10-ml volumetric flask containing 5 ml methanol and was sonicated for 30 min. The volume was made up to 10 ml using methanol and the resulting solution was centrifuged to obtain the supernatant (10 µl) previously filtered for the evaluation. The experiment was repeated in triplicate by maintaining similar chromatographic conditions as mentioned in instrumentation.

2.6 Preparation of standards (AA and GA)

A stock solution of 1 mg/ml were prepared for the markers by dissolving accurately weighed 1 mg of standard (ascorbic acid and gallic acid) in 10 ml of methanol separately followed by sonication for 30 min at ultra sonicator.

2.7 Sample application

Methanolic solutions of samples and standard marker (AA and GA) of known concentrations were applied to the plate as 8 mm bands positioned 15 mm from the bottom and 15 mm from side of the plate, using Camag Linomat 5 automated TLC applicator. The rate of application was kept 90 nl/s from the application syringe. The TLC scanner III was used for the analysis purpose. These conditions were kept constant throughout the analysis of the samples.

3. BIOMARKERS ESTIMATION

3.1 Estimation of AA and GA

The mobile phase toluene: ethyl acetate: methanol: formic acid (3: 3: 2: 1,v/v/v/v) was selected after many trials.

3.2Method Validation

3.2.1 Linearity

The linearity range of AA and GA was obtained by plotting the peak area of AA and GA against its varied concentrations over a range (400-1400 ng/spot).

3.2.2 Precision

The precision of the instrument was evaluated by performing repeatability of the sample application and peak area measurement in six replicates of the same spot (800 ng/spot) for both the markers. The intra- and inter-day precision were determined using three different concentration levels 400, 600, and 800 ng/spot of AA and GA and expressed as % RSD.

3.3.3 Specificity

This was ascertained by analyzing the chromatograms of markers and similar R f chromatogram in the samples and spectra pattern of reference marker and drug samples. The peak purity of samples was determined by spectral comparison of sample peak at three different levels, viz. peak start (S), peak apex (M), and peak end (E) positions[12].

3.4.4 Robustness

In this study, small deliberated changes were made in the composition, volume presaturation time for mobile phase, and their effects on the results were examined. The effect of different analysts was also evaluated. The study was done in triplicate at a single concentration, (800 ng/spot) of both markers.

3.5.5 Accuracy

The standard addition method was followed as mention in ICH guidelines[1]. The pre-analyzed sample was spiked with extra 80,100 and 120% of the standards and the resultant mixture was re-analyzed by the proposed method. The experiment was repeated for six times. The results were expressed as percent recovery.

3.6.6 Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ were experimentally determined by visual detection method recommended in ICH guidelines[1].

3.7.7 Recovery studies

The pre-analyzed sample of AA and GA were spiked with extra 80%, 100%, and 120% of the standard AA and GA, and resultant mixtures were reanalyzed by the proposed method. The experiment was repeated for six times.

4. RESULTS

4.1. Estimation of AA and GA

The mobile phase toluene: ethyl acetate: methanol: formic acid (3: 3: 2: 1, v/v/v/v), pre saturated for 20 min had shown the sharp and well defined peaks for standards, ascorbic acid and gallic acid (Rf = 0.59 and 0.86). The standard chromatogram for ascorbic acid and gallic acid at 254 nm has been shown in the Fig. 1

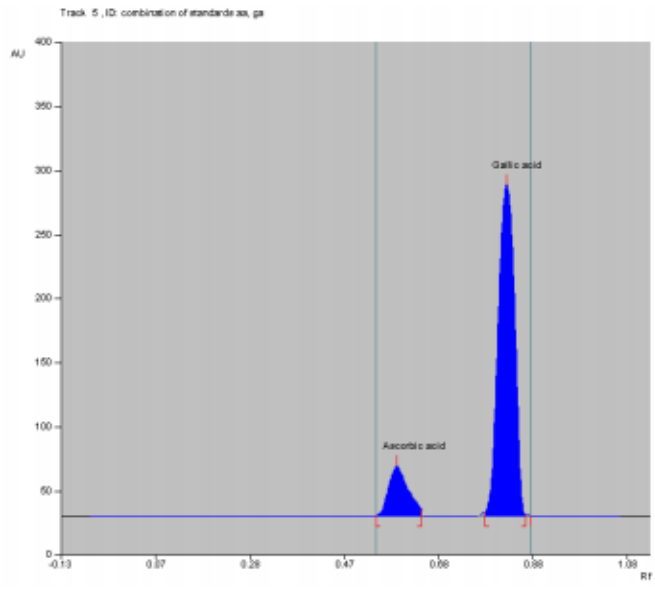

Fig. 1: Chromatogram of standard markers ascorbic acid and gallic acid by HPTLC

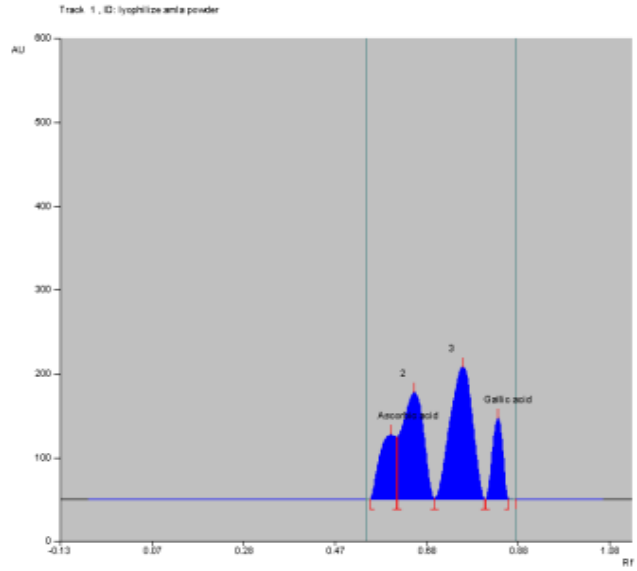

Fig. 2: Chromatogram of lyophilized Amla juice powder by HPTLC

4.2. Method validation

4.2.1. Linearity Curve

The developed TLC method for estimation of AA and GA showed a correlation co-efficient (0.992 and 0.996) respectively at the concentration range of 400–1400 ng/spot with respect to the peak area (Fig.3 and Fig.4).

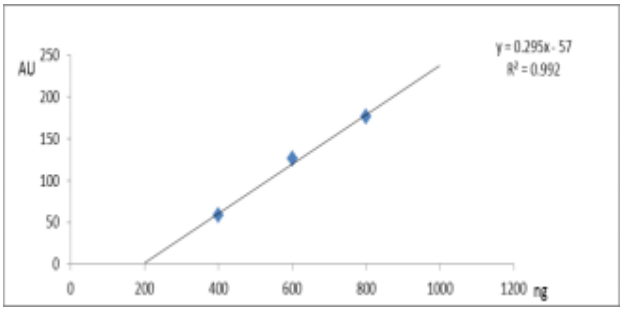

Fig. 3: Calibration curve of standard ascorbic acid over a range of concentrations from 400-1400 ng/band

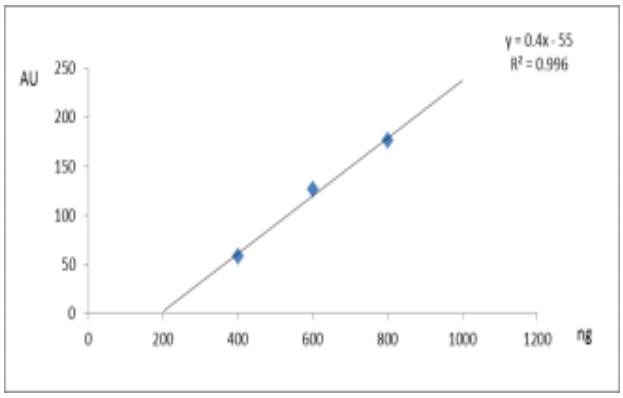

Fig. 4: Calibration curve of standard gallic acid over a range of concentrations from 400-1400 ng/band

4.2.2. Specificity

The wavelength maxima for the ascorbic acid and gallic acid was found to be 245 nm and 280 nm respectively, but during the study 254 nm was selected for both the markers as they showed better peaks simultaneously at this wavelength. The specificity of method was ascertained by analyzing the standard markers and the sample. The peak for ascorbic acid and gallic acid (*Rf* 0.59 and 0.86) in sample was confirmed by comparing the *Rf* value and overlain spectra of the peak obtained from the sample with that of the standard markers (Fig. 5). The spectra overlain of the standard and some content present in the sample drug supports the specificity of the developed method.

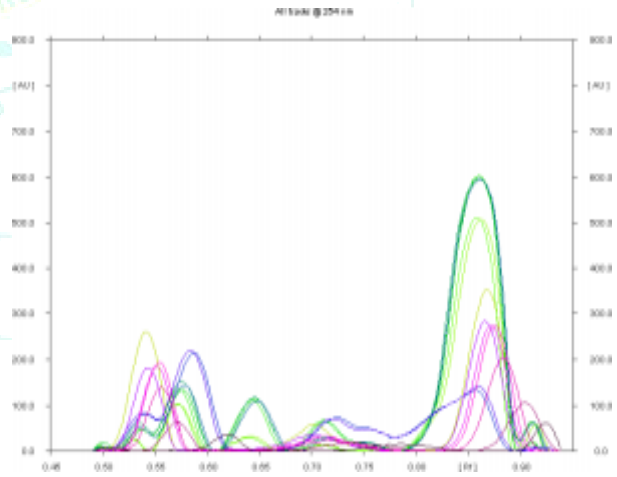

Fig. 5: Overlay UV absorption spectra of the lyophilized Amla juice powder and marketed formulations formulations with respect to the standard

4.2.3. Repeatability studies

The proposed method had shown a % RSD of repeatability (800 ng/spot of AA and GA) studies to be 1.53% and 1.50% respectively. Repeatability of the sample application and measurement of peak area can be carried out using six replicates of the same spot (e.g. 800 ng/spot of marker) and expressed in terms of percent relative standard deviation (% RSD) as shown in Table 1.

Table 1: Repeatability studies of standard ascorbic acid(AA) and gallic acid(GA)

Track	Amount (ng)	Area for AA	%RSD	Area for GA	%RSD
1	800	4571.6	1.53	8381.3	1.50
2	800	4536.4		8325.5	
3	800	4482.9		8296.8	
4	800	4421.5		8215.2	
5	800	4372.3		8188.6	
6	800	4315.8		8113.1	
7	800	4247.2		8098.4	
8	800	4226.1		8026.2	
9	800	4194.5		7992.7	

4.2.4. Precision

In precision studies on intra and inter-days, the resultant peak area for AA and GA, determined at three different concentration levels, showed low values of SD and % RSD (< 2 %) for inter- and intra-day variations. This suggested an excellent precision of the method, which shows that method as well as proper working of an instrument (Table 2).

Table 2: Intra-day and inter-day precision study for ascorbic acid and gallic acid at 254 nm (n=3)

Intra-day precision				Inter-day precision		
Concentration (ng)	Mean area (AA)	Mean area (GA)	% RSD	Mean area (AA)	Mean area (GA)	%RSD
400	1471.13	3033.01	0.18	1396.38	2992.21	0.19
600	3343.89	5881.07	0.22	3342.92	5562.14	0.21
800	4896.93	7974.64	0.23	4997.46	8030.56	0.18

4.2.5. Robustness

Robustness study of the method was done in six replicates at a concentration of 800ng/band. The % RSD of peak areas and *R*fwere calculated.

4.2.6 Accuracy

The results expressed as percent recovery is shown in Table 3 and Table 4.

Table 3: Accuracy studies for estimation of ascorbic acid (n=3)

Excess drug added to analyte (%)	Theoretical content (ng)	Found Amount (ng)	Recovery (%)	%RSD
0	400	398.6	99.25	0.12
80	720	715.4	99.86	0.19
100	800	797.2	99.97	0.11
120	880	872.5	99.82	0.16

Table 4: Accuracy studies for estimation of gallic acid

Excess drug Added to analyte (%)	Theoretical content (ng)	Found Amount (ng)	Recovery (%)	%RSD
0	400	394.2	99.89	0.72
80	720	712.8	99.76	0.53
100	800	798.6	99.82	0.38
120	880	873.4	99.74	0.58

4.2.7. LOD and LOQ

Detection limit and limit of quantification were found to be 40 and 140 ng/spot respectively, which indicate adequate sensitivity of the method.

4.2.8 Recovery studies

The proposed method afforded recovery in the range of 99.97-99.89 % as shown in Table 5. This confirms that the proposed method can be used for the determination of AA and GA in herbal juices at different concentration levels.

Table 5: Recovery study of ascorbic acid, gallic acid (n=3)

Excess drug Added to analyte (%)	Theoretical content (ng)	Found Amount (ng)		Recovery (%)		% RSD	
		AA	GA	AA	GA	AA	GA
0	400	398.6	394.2	99.25	99.89	0.12	0.72
80	720	715.4	712.8	99.86	99.76	0.19	0.53
100	800	797.2	798.6	99.97	99.82	0.11	0.38
120	880	872.5	873.4	99.82	99.74	0.16	0.58

5. DISCUSSION

The HPTLC methods were developed for the analysis of AA and GA in amla juice preparations. The HPTLC method was successfully validated for precision, recovery and robustness with respect to International Conference on Harmonization (ICH) guidelines. The statistical analysis of the data showed that the proposed method is precise, reproducible and accurate which can be employed for the standardization of amla juices (qualitative and quantitative analysis). Results of the study showed high extraction efficiency of (AA) and (GA) from the samples. Low % RSD of peak area values has proven the ruggedness of the method, indicating that (AA) and (GA) were stable throughout the extraction procedure and also during analysis.

6. CONCLUSION

The developed method is specific, accurate, and robust for the determination of AA and GA content in the samples. Statistical data analysis proved the reproducibility and selectivity of the developed method for the analysis of AA and GA. The method can be used for qualitative as well as quantitative analysis of AA and GA in herbal amla juice marketed formulations containing amla. Further, the proposed method can be extended to study the degradation

of AA and GA under different stress conditions, as per the recommendations of ICH guidelines.

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Conflicts of Interests: None

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