A novel high-performance liquid chromatography-electron spray ionization-mass spectrometry method for simultaneous determination of guggulsterones, piperine and gallic acid in *Triphala guggulu*

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ABSTRACT

"*Triphalaguggulu*" is an important Ayurvedic formulation comprising of *Guggulu*, that is, *Commiphora* wightii (Arn.) Bhandari as a base wherein powdered fruits of *triphala*, that is, *Phyllanthus emblica* L., *Terminalia bellirica* (Gaertn.) Roxb and *Terminalia chebula* Retz, along with powdered fruit of *Piper longum* L. are compounded. This polyherbal preparation has been strongly recommended in chronic inflammation, piles, and fistula. However, due to the complexity of compound formulation standardization of commercial products is challenging. In the present communication marker-based standardization of "*Triphalaguggulu*" preparation using gallic acid (for *triphala*), piperine (for *P. longum* L.) and guggulsterones (for *guggulu*) is reported. These compounds of diverse chemistry were successfully separated on a Waters HR-C18 column by isocratic elution with methanol and water (80:20 v/v) as mobile phase at the flow rate of 1.0 mL/min coupled with photodiode array detector. These optimal chromatographic conditions were used for simultaneous quantification of gallic acid, guggulsterones (*E and Z*) and piperine in commercial samples by high-performance liquid chromatography-electron spray ionization-mass spectrometry and method was validated as per ICH guidelines.

Key words: Gallic acid, guggulsterones, high-performance liquid chromatography-electron spray ionization-mass spectrometry, piperine, *Triphalaguggulu*

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INTRODUCTION

Herbal medicine and home remedies are in practice since man recognizing their use as a cure for various ailments of the body, and currently the inclination to use herbal remedies is on increased mode. This increase in demand for herbal medicine has put pressure on the supply of natural resources. This ultimately results in use of substandard materials or substitution and adulteration. To control the adulteration and maintain the quality and the efficacy of the product analytical tool play a major role.[1]

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Triphalaguggulu is a traditional Ayurvedic polyherbal preparation has been a time tested medicament for chronic inflammatory conditions such as piles and fistula.[2] It uses guggulu, that is, resin of *Commiphora wightii* (Arn.) Bhandari as base to carry other fruit powders. This includes fruit of *Piper longum* L. and *triphala* (i.e. fruits of *Phyllanthus emblica* L., *Terminalia chebula* Retz, and *Terminalia bellirica* (Gaertn.) Roxb). This preparation is made in accordance with the authorized methodology provided in Ayurvedic Formulary of India.[3] Standardization of this compound ayurvedic formulation is challenging as it is an admixture of different phytochemicals with diverse chemistry. However, in order to ensure quality and efficacy of the compound formulation, it is desired to have accurate analytical tool. *Triphalaguggulu* is a commercially important formulation for which standardization method is not reported.

The basic constituent of this formulation is *C. wightii* which is an oleo-gum-resin containing biologically active steroids viz. *E* and *Z* guggulsterones.[4] The three fruits mentioned above when combined in equal ratio constitute "*Triphala*", which is well-recognized preparation of Ayurveda.[5] These three fruits are rich in tannins and have gallic acid as a common marker.[6,7] Piperine is a well-known bioavailability enhancer and is a marker for *P. longum*.[8] In the present communication, we are reporting the high-performance liquid chromatography-electron spray ionization-mass spectrometry (HPLC-ESI-MS) method for standardization of "*Triphalaguggulu*" using the above markers.

Several analytical methods, available in the literature include liquid chromatography (LC)-MS/MS, HPLC and high-performance thin layer chromatography methods for the quantification of the above mentioned markers.[9-14,18] However, in our survey we did not find any report on the simultaneous determination of gallic acid, piperine and guggulsterones (*E* and *Z*) in *Triphala guggulu*. In the present work, we have developed a new, simple, rapid and specific reversed-phase HPLC-ESI-MS method with simultaneous quantification of these four markers. This method successfully determines the compounds of diverse polarities and chemical nature, which is phenolic acid or derivative (gallic acid, ellagic acid), alkaloid (piperine) and phytosterols (guggulsterones).[15] Further, this method is used successfully to determine these compounds in a complex matrix of five different Ayurvedic herbs.[3] This method can be used for the standardization of *Triphalaguggulu* commercial preparations and also for standardization of these herbs and their extracts for respective markers.

EXPERIMENTAL

Plant material and preparation of the formulation

The materials *C. wightii* (Arn.) Bhandari (ole-gum-resin) were purchased from Gujarat Medicinal Plant Grower's Society. Other plant materials that is *T. chebula* Retz. (fruit rind), *T. bellirica* (Gaertn.) Roxb. (fruit), *P. emblica* L.(fruit) and *Piper longum* L. (fruit), (voucher specimens NPD/499/12, NPD/531/12, NPD/59/12 and NPD/119/12 resp.) were obtained locally and were authenticated by Dr. Kannan of The Himalaya Drug Company, Bangalore, India.

Chromatographic conditions

The chromatographic separation was achieved on Waters HR-C18 (300 mm × 3.9 mm, 6 μm) column using methanol and water (80:20 v/v) as mobile phase at the flow rate of 1.0 mL/min coupled with photodiode array detector. HPLC-MS studies were performed using an Agilent 1100 series online ion trap mass selective detector mass spectrometer with ESI source equipped with a degasser (G1379A), binary pump (G1312A), autosampler (G1329A), autosampler thermostat (G1329B) and diode array detector (G1315B) (Agilent Technologies, Waldbronn, Germany). The data were acquired and processed using Chemstation software 4.2 (Bruker, Waldbronn, Germany). An isocratic elution with methanol and water (80:20 v/v) as mobile phase was pumped at a flow rate of 1.0 mL/min; the sample injection volume was 20 μL with column temperature maintained at ambient conditions. The MS detection was set to electrospray (ESI) in positive ionization mode. Nitrogen was employed as the nebulizer gas. The ion source conditions were set as follows: Temperature, 335°C; nebulizer gas, 35 psi; dry gas, 10.0 L/min; skimmer 40.0V; capillary exit 128.0V; trap drive 44.5; max accu time 200 ms; Icc target 20000.

Standard solution preparation

Gallic acid (98%) and piperine (97%) were obtained from Sigma-Aldrich. Guggulsterones (*E* and *Z*, 99.6%) were obtained from Sami Labs Ltd. with *E: Z* ratio of 44:56. Accurately weighed 10 mg of each standard (piperine, gallic acid and guggulsterones (*E* and *Z*)) and dissolved in 10 mL methanol in volumetric flask, by vigorous shaking and then volume was made-up to mark with methanol to obtain a final concentration of 1 mg/mL of each compound.

Preparation of formulation

Triphalaguggulu samples marketed by different Ayurvedic medicine manufacturers (Shree Dhootapapeshwar Ltd., Baidyanath Ayurved Bhawan) were purchased locally at Bangalore.

In-house preparation of *Triphalaguggulu*

In order to ascertain the utility of the newly developed method *Triphalaguggulu* was made in-house in accordance with The Ayurvedic Formulary of India.[3] This was also intended to compare with market samples in case they differ widely from each other.

Preparation of sample

Approximately 50 mg of the formulation was weighed and transferred to 10 mL volumetric flask 5 mL of methanol was transferred and sonicated for 30 min, then transferred to a centrifuge vial and centrifuged for 20 min at a 4000 rpm, the supernatant liquid was decanted, the same cycle was repeated three times, the resultant solution was dried under vacuum and 10 mg/mL solution was prepared. The sample was injected to LC-MS system.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Optimization of chromatographic conditions is equally critical for their adequate retention and separation. In

the present work, the chromatography was performed on several reversed-phase columns such as Agilent Eclipse XDB-C18 (150 mm × 4.6 mm, 5 μm), Atlantis dC18 (150 mm × 4.6 mm, 5 μm), Waters HR-C18 (300 mm × 3.9 mm, 6 μm) and Xterra RP C18 (250 mm × 4.6 mm, 5 μm) to achieve a short run time, symmetric peak shapes, minimum matrix interference and solvent consumption. This was investigated by appropriate changes to the mobile phase composition (aqueous and organic part), and flow rate. The four compounds guggulsterones (*E* and *Z* stereoisomer) are mid polar, piperine-an alkaloid and gallic acid-a phenolic acid are polar in nature. Hence, a suitable combination of aqueous and organic solvent (methanol and water) was optimized with methanol 80% in water as the suitable mobile phase. The mobile phase was operated at a flow rate of 1.0 mL/min. The elution order and retention times were: Gallic acid (1.85 min), piperine (4.05 min) and guggulsterones (*E* and *Z*) were (6.12 and 7.06 min). This method was considered further analysis [Figure 1]. To achieve good sensitivity and accuracy for quantification, the different ultraviolet (UV) max values of four analytes were considered carefully. Thus, the PDA detection wavelengths were set at 273 nm for gallic acid, at 340 nm for piperine and 245 nm for guggulsterones according to the maximum absorption wavelength of each compound.

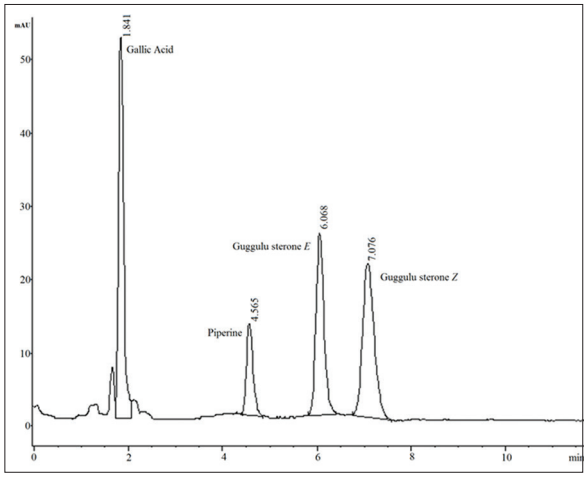

Figure 1: High-performance liquid chromatography-chromatogram of mixed standards, 1: Gallic acid (room temperature [RT] = 1.841 min), 2: Piperine (RT = 4.065 min), 3: Guggulsterone E and Z (RT = 6.068 and 7.076 min) at 254 nm

Validation

The validation process was carried out according to the ICH guidelines (ICH, 2005).[16]

Calibration curve and limit of detection and limit of quantification

Calibration standards were prepared by diluting the appropriate volume of stock solution with methanol to attain the concentration levels of 10, 20, 40, 100, 200 μg/mL for piperine and guggulsterones and 10, 20, 40, 100, 200,5 00 and 800 μg/mL for gallic acid. The data of peak area versus drug concentrations were treated by linear least-square regression. The response was linear ($r^2 = 0.997$ for gallic acid, 0.999 piperine and 0.998, 0.997 for guggulsterones) over the concentration range between 10 μg/mL and 200 μg/mL for piperine and guggulsterones, 10–800 μg/mL for gallic acid. The limit of detection was found to be 0.32 μg/mL for gallic acid, 0.40 μg/mL for piperine, 0.64 μg/mL, 0.59 μg/mL for guggulsterones. Limit of quantitation for gallic acid, piperine and guggulsterones (*E* and *Z*) were found to be 0.89 μg/mL, 1.05 μg/mL, 1.13 μg/mL, 1.25 μg/mL, respectively, as depicted in Table 1.

Precision

The Precision of the method was examined by performing the intra- and inter-day assays of six replicate injections of the mixture of standard solution at three concentration levels. The intra-day assay precision was performed with the interval of 4 h in 1 day while the inter-day assay precision was performed over 6 days.

The results of the intra-day and inter-day precision experiments are shown in Table 2. The developed method was found to be precise as the relative standard deviation (RSD) values for repeatability and intermediate precision studies, respectively, were around <2%.

Specificity

The specificity of the method was determined by analyzing the standard and test samples. The peaks for gallic acid, piperine and guggulsterones test samples were confirmed by comparing the room temperature value and the UV-spectrum of the peak with that of the standard. Furthermore, the adequate selectivity and separation power was given by MS. The mass spectrum acquired in

Table 1: LOD and LOQ

Compound	Linearity range (ng/mL)	Calibration curve	R^2	LOD (μg/mL)	LOQ (μg/mL)
Gallic acid	10-800	Y=3.113X−5.502	0.997	0.32	0.89
Piperine	10-200	Y=3.148X−2.677	0.999	0.41	1.05
Gugulasterone *E*	10-200	Y=4.196X+18.88	0.998	0.64	1.13
Gugulasterone *Z*	10-200	Y=4.391X+19.65	0.997	0.59	1.25

LOD: Limit of detection; LOQ: Limit of quantitation

the m/z range from 0 to 600 showed [M-H]- ion at m/z 168.8 for gallic acid [Figure 2a] and [M+H]+ ions at m/z 286.1 [Figure 2b] and 313.2 [Figure 2c and d] for piperine and guggulsterones respectively. Thus, the identity of the peaks and an adequate chromatographic selectivity were confirmed.

Table 2: Precision studies (*n*=6)

Drug	Amount (µg/mL)	Repeatability		Intermediate precision	
		Mean area (AU) ±SD	% RSD	Mean area (AU) ±SD	% RSD
Gallic acid	40	130.1±1.95	1.49	139.5±2.21	1.58
	100	324.9±2.81	0.86	343.0±3.24	0.94
	200	581.2±4.15	0.71	610.1±5.25	0.71
	20	46.60±1.35	2.89	51.35±1.95	3.79
Piperine	40	123.7±3.54	2.86	138.4±3.71	2.68
	100	363.1±4.80	1.32	389.1±3.75	0.96
	20	104.3±3.25	3.11	113.5±2.75	2.42
Gugulasterone *E*	40	225.8±3.54	1.58	243.2±4.60	1.89
	100	574.1±4.25	0.74	590.2±4.57	0.77
	20	106.3±3.65	3.41	110.5±3.15	2.86
Gugulasterone *Z*	40	220.8±3.44	1.54	248.2±4.70	1.88
	100	564.1±4.05	0.71	580.2±4.47	0.77

SD: Standard deviation; AU: Adult unit; RSD: Relative standard deviation

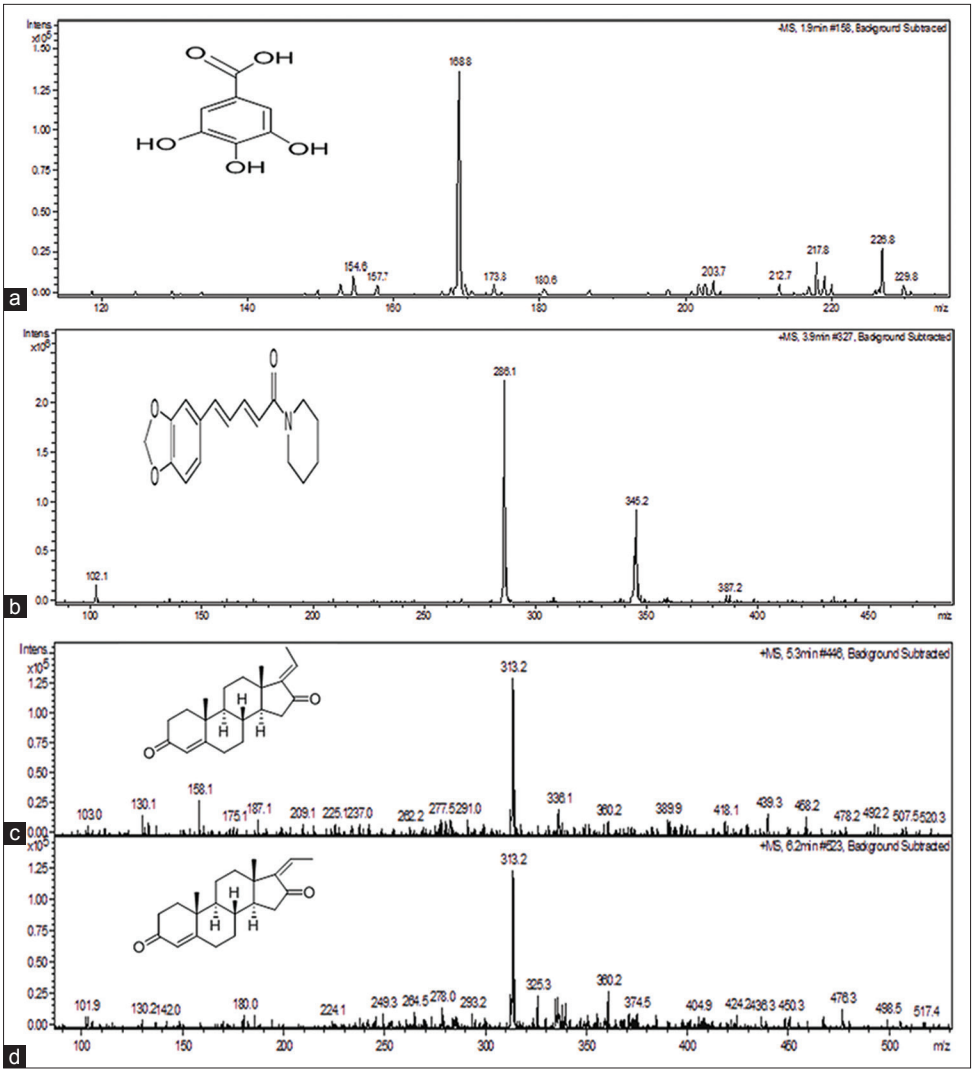

Figure 2: (a) Liquid chromatography-electron spray ionization/mass spectrometry mass spectra of gallic acid, (b) piperine, (c and d) guggulusterone *E* and *Z*

Table 3: Recovery data (*n*=6)

Sample name	Gallic acid			Piperine			Gugulasterone E			Gugulasterone Z		
	Concentration added	Concentration found	RSD %	Concentration added	Concentration found	RSD %	Concentration added	Concentration found	RSD %	Concentration added	Concentration found	RSD %
TGE-B	40	38.95±1.56	4.00	20	21.53±0.95	4.41	20	19.51±0.78	3.99	20	18.63±0.62	3.32
TGE-D	100	102.54±2.98	2.90	40	39.21±1.14	2.90	40	41.25±1.85	4.48	40	42.65±1.98	4.63
TGE-IH	200	198.35±3.64	1.83	100	102.65±2.27	2.21	100	98.85±2.09	2.11	100	99.25±2.15	2.15

RSD: Relative standard deviation, TGE: *Triphalaguggulu* extract

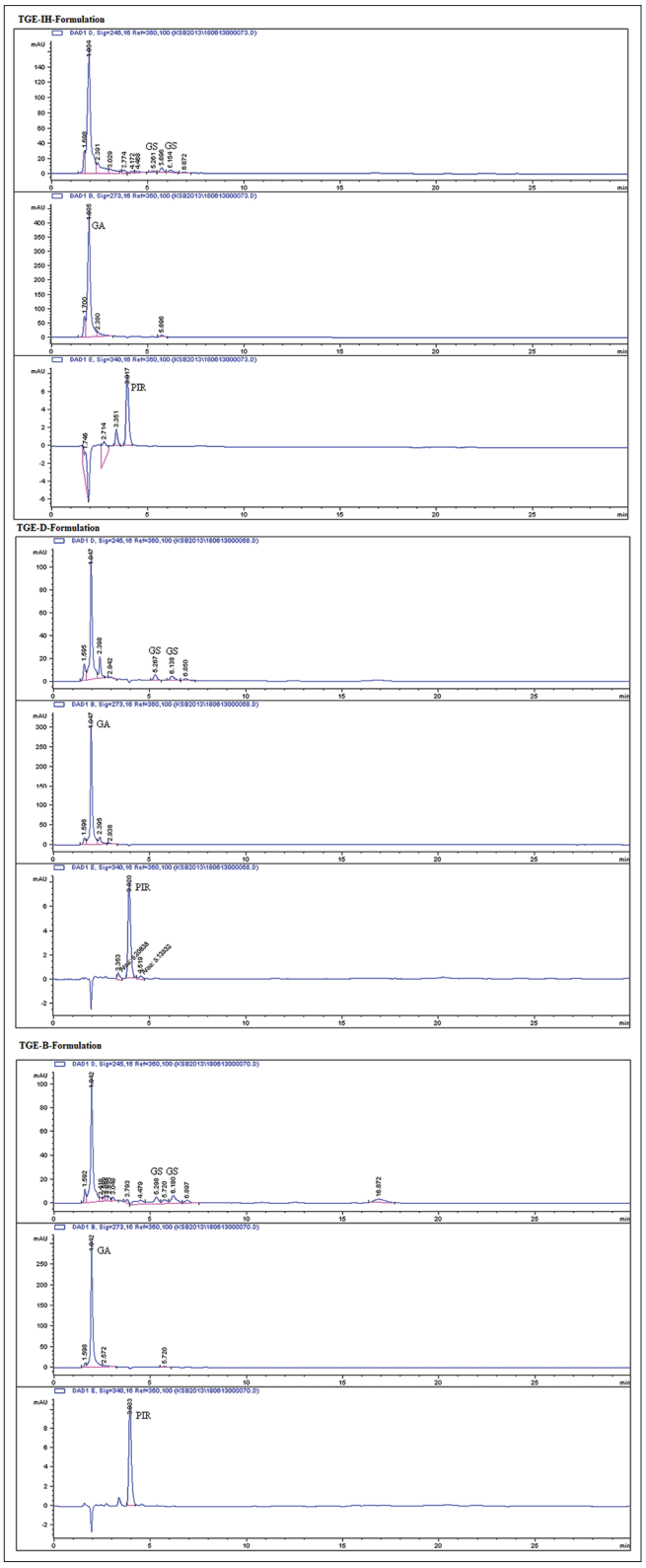

Figure 3: High-performance liquid chromatography-diode array detector chromatograms are corresponding to three formulations. (1) Gallic acid at 273 nm, (2) piperine at 340 nm, (3) guggulsterones at 245 nm

Table 4: Estimation of standards in the formulation

Sample name (powder formulation) (10 mg/mL)	Gallic acid (µg)	Piperine (µg)	Gugulasterone *E* (µg)	Gugulasterone *Z* (µg)
TGE-B	794.9489	31.6087	116.5264	110.3485
TGE-D	781.5465	23.7529	37.4019	39.5160
TGE-IH	1532.6960	24.2981	20.3880	25.3696

TGE: *Triphalaguggulu* extract

Accuracy

The accuracy of the method was confirmed by the recovery studies. The standard addition method was used for determining the recoveries of standards understudy. The recovery experiments of these compounds were performed by adding gallic acid, piperine and guggulsterones in different concentrations to the preanalyzed sample solutions. The results of the recovery study ranged from 98% to 102% with RSD ranging 1.83–4.48. Results of recovery studies are reported in Table 3.

Application of the method

The newly developed and validated RP-HPLC method was applied for the analysis of studied marker compounds in different *Triphalaguggulu* samples. The peak areas of triplicate samples were analyzed by the regression equation obtained from the calibration plot to determine the content of these markers in samples. No interference was found at the retention time of the studied analytes in samples [Figure 3]. The analytical results for each component identified are summarized in Table 4. Therefore, the simultaneous determination of these active components in herbal preparations could be successfully applied to improve the safety and quality control of the formulations available in the market.

CONCLUSIONS

Traditional medicines are easily available to masses but are complex mixtures of natural substances and are prone for variation and adulteration. *Triphala guggulu* is one of the well-known Ayurvedic polyherbal preparation, which contains *guggulu* as one of the ingredient, which is in high demand and also listed as an endangered medicinal plant by IUCN.[17] In the present communication, we are reporting successful development and validation of RP-HPLC-PDA method for simultaneous determination of four biologically active markers representing the majority of the constitution of this polyherbal formulation. In the present study simultaneous quantification of gallic acid, piperine and guggulsterones has been achieved using RP-HPLC with multiple wavelength monitoring.

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