ESTIMATION OF GUGGULSTERONE-Z IN *GOKSHURADI GUGGULU* USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Objective: A study was aimed to estimate guggulsterone-Z (GZ) in *Gokshuradi Guggulu* (GG).

Methods: An analytical method was developed and validated using Waters Alliance high-performance liquid chromatography system (Empower software), equipped with photodiode array detector. Separation was achieved using Phenomenex, C-18 (250 mm×4.6 mm, 5 µ) column. Mobile phase consisted of acetonitrile:water (70:30,v/v). Flow rate was set to 1 ml/min and detection was performed at 251 nm.

Results and Discussion: Validation parameters such as linearity, precision, accuracy, limit of detection, limit of quantification, and robustness were performed. Amount of GZ was estimated using linearity equation.

Conclusion: GG was found to contain 0.815±0.03 g% w/w GZ. Validated method may be used as one of the parameters to standardize the formulation.

Keywords: *Gokshuradi Guggulu,* Guggulsterone-Z, High-performance liquid chromatography.

INTRODUCTION

Gokshuradi Guggulu (GG) is a solid formulation, prepared using *Guggulu* as basic ingredient. The formulation is official in *Ayurvedic* Formulary of India. *Gokharu* (*Tribulus terrestris*) and *Guggulu* (exudates from *Commiphora wightii*) are the major ingredients of GG. GG is used in the treatment of dysuria, urinary obstruction, excessive vaginal discharge, gout, spermatogenic, and vitiation of semen [1]. Guggulsterone is an active constituent of *Guggulu*, characterized as cis (E) and trans (Z) stereoisomers of 4,17(20)-pregnadiene-3,16-dione [2,3]. Chemical structure of guggulsterone-Z (GZ) is shown in Figure.1. GZ was first isolated from ethyl acetate fraction of gum resin of *Commiphora mukul* (known as *Guggulu*) belongs to family Burseraceae [4-7]. The oleo gum resin is traditionally used in the management of hypercholesterolemia and obesity [8-10]. Apart from this, *Guggulu* is used extensively as base in many *Ayurveda* formulations, commonly known as *Guggulu*.

Various physical parameters were evaluated for shodhit guggul [11]. Several analytical methods are reported for the estimation of GZ in different matrix present in combination with different phytoconstituents. Guggulsterone was estimated by high-performance liquid chromatography (HPLC) from its raw material *C. wightii* [12-14], rabbit plasma [15], and from a marketed herbal formulation [16]. Guggulsterone was also estimated using reversed-phase HPLC (RP-HPLC) in few herbal tablet formulations [17]. GZ with gallic acid and piperine was estimated using HPLC–mass spectrometry (MS) [18]. High-performance thin-layer chromatography (HPTLC) method was developed to estimate guggulsterone from tablets [19], amount of GZ was estimated from *Triphala Guggulu* using HPTLC [20,21]. There has been no report of any chromatographic method for the estimation of GZ in GG; thus, it was planned to develop a chromatographic method for the estimation of GZ in GG.

METHODS

GG was manufactured by Sunder Pharmacy (GMP certified manufacturing unit associated with J and S Ayurveda College, Nadiad,

Gujarat, India) on our request. GZ (>95%) was purchased from Natural Remedies, Bangalore, India. All solvents used were of HPLC grade and procured from Merck India. Water used was double distilled. The solvents were filtered through a 0.45 µm filter (Millipore Bedford, MA, USA) and degassed in an ultrasonic bath (Remi Instruments, Mumbai, India) before use.

Preparation of combined standard stock solution
Accurately weighted, 10 mg GZ was dissolved in 5 ml of methanol using sonication. The solution was diluted up to 10 ml using methanol.

Preparation of working standard solution
Different volumes of standard stock solution were diluted using methanol to produce different dilutions in the range of 20–320 µg/ml, for GZ.

Preparation of sample solution
GG was received in the form of compressed tablets. Tablets were grounded to powder using mortar and pestle. 50 g tablet powder was weighed accurately and transferred quantitatively to a 250 ml round bottom flask and extracted using 50 ml of chloroform for 2 h. Mixture was allowed to cool at room temperature. Chloroform extract was collected. Procedure was repeated twice using 100 ml of chloroform for 1 h each. Chloroform extracts were mixed together, filtered through Whatman filter paper and subjected to evaporation using rotary vacuum evaporator at 40°C. Resulting semisolid mass was stored in a container and analyzed immediately after drying. 4 g of chloroform extract was dissolved in 50 ml of methanol in a volumetric flask. This solution is diluted up to 50 ml using methanol. 1 ml of resultant solution as diluted up to 10 ml using methanol. This solution was filtered using syringe filter to avoid the presence of insoluble extractives in sample solution. This clear solution was used as sample solution for the estimation of GZ and injected directly in HPLC system.

Development and optimization of chromatographic conditions
Initially, reported HPLC-based analytical methods were adopted to separate peak of GZ in sample solution [12-17]. The studies revealed

that none of the adopted analytical methods could separate peak corresponding to GZ from nearby peaks in sample solution. A new
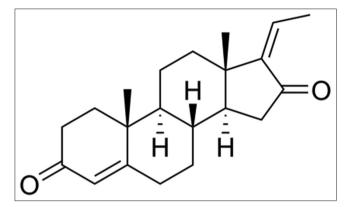

Fig. 1: Chemical structure of Guggulsterone-Z

analytical method, thus, was developed by modifying the reported analytical method. It was kept in consideration that the proposed method should be isocratic and could be able to separate GZ peak at R_T <10 min, without affecting resolution. The peak should be Gaussian in shape and plates were sufficiently high to yield recommended value of repeatability. The results of the studies are shown in Fig. 2a and b, in the form of chromatogram.

Validation of analytical method using HPLC
The developed method was subjected to analytical method validation to evolve value for the parameters as mentioned in ICH guidelines. Details of the procedure adopted to validate the method are mentioned below.

System suitability parameters (SSP)
Repeatability, number of plates, resolution, capacity factor, and tailing were considered as SSP. Repeatability was measured from area of GZ peak from sample solution. Sample solution was subjected to HPLC analysis, by adopting optimized chromatographic conditions. Percentage relative standard deviation (RSD) of peak
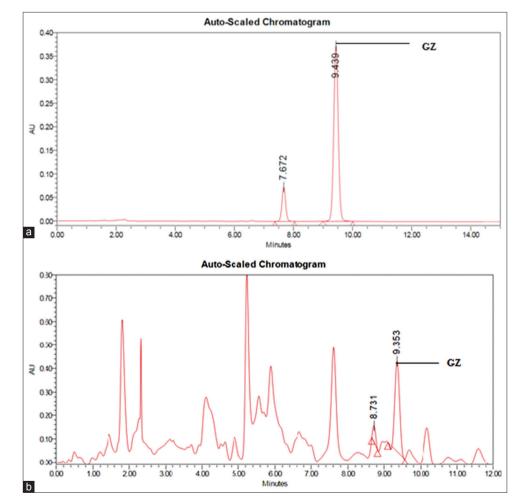

Fig. 2: (a) Chromatogram of guggulsterone-Z (GZ) in standard solution. (b) Chromatogram of GZ in chloroform extract of *Gokshuradi Guggulu*

Table 1: Value for selected SSP

Sr. No	Parameter	Value obtained	Recommended value [20]
1	R_T	9.4	--
2	Repeatability (% RSD)	0.80	RSD<1
3	Number of plates	23059.48	>2000
4	Capacity factor	9.38	>2
5	Resolution	3.06	≥1.5
6	Tailing	1.14	<2

RSD:Relative standard deviation

area was determined. Number of plates, resolution, capacity factor, and tailing factors were determined for peak of GZ from recorded chromatogram of sample solution. The calculations were performed by methods recommended by USP. The results of the studies are shown in Table 1.

Linearity

Calibration range selected for the set of experiments was 20–320 μg/ml. Each concentration was injected for 6 times (n=6). Calibration curve was constructed by plotting average peak area versus concentrations using the least square equation. The average value of regression coefficient (r^2), y-intercept (C), and slope of calibration curve (m) was calculated and shown in Table 2.

Precision

Precision studies were performed by determining interday and intraday variation in area corresponding to GZ for particular concentration of GZ.

Three selected concentrations of GZ standard solution 40, 80, and 160 μg/ml were subjected to intraday and interday precision studies. The fixed volume was injected and chromatogram was recorded by following optimized chromatographic conditions. Each concentration was injected for 6 times in a day for intraday analysis and daily once for 6 days for intraday analysis. The area covered by GZ peak in each chromatogram was recorded. RSD value was determined for peak area corresponds to each concentration. The data for each concentration was paired with the data obtained for a particular concentration by injecting six injections consecutively by following optimized chromatographic conditions and percentage RSD for combined data set was determined as shown in Table 3.

Sensitivity

Sensitivity of developed method for GZ was established in terms of limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ wereinitially estimated through determining S/N ration, when injectingsolutions while establishing linearity for GZ. GZ concentration yielded S/N value for GZ peak, near to three was considered LOD, and more near to 10 was considered as LOQ. Lately, exact value LOD and LOQ were determined using mathematical equation, with the help of SD for y-intercept for linearity equation and average value of slope.

Accuracy

The accuracy of the method was established by performing recovery studies, by adopting standard addition method. The studies were performed at three levels, 50, 100, and 150% of the working concentration of GZ in sample solutions. Known amount of GZ was spiked in pre-analyzed sample solution. Experiments were performed in triplicate and amount of GZ was determined. The percentage recovery was determined in each case and shown as average recovery along with SD for each level (Table 4).

Selectivity

Selectivity of developed method was established by comparing the contours recorded for GZ peak received in sample solution with those obtained for GZ peak received in standard solution, as shown in Fig. 3.

Robustness

The proposed HPLC method was tested for robustness by introducing deliberate but small alteration in critical chromatographic condition. Alteration in acetonitrile proportion in mobile phase (corresponding alteration in water proportion) (±3%), alteration in flow rate (±0.2), and alteration in selected wavelength (±2.0 nm) were selected parameters. One alteration in a factor was introduced and chromatogram was recorded after injecting standard solution of GZ (80 μg/ml). Area and R_T value for GZ peak were noted. Each experiment was performed thrice to generate data sets. Each such dataset was statistically compared with that generated by injecting GZ using optimized conditions, using ANOVA.

RESULTS AND DISCUSSION

The method was developed and validated using Waters Alliance HPLC system (Empower software), equipped with UV detector. Column was Phenomenex, C-18 column (250 mm×4.6 mm, 5 μ). Mobile phase consisted of acetonitrile:water (70:30, v/v). Flow rate was set at 1 ml/min, injection volume was kept constant at 20 μl, and detection was performed at 251 nm. As shown in Fig. 2a, sharp Gaussian peak corresponding to GZ was obtained in sample solution chromatogram. Retention time for GZ was 9.2 min. Fig. 2a and b showed chromatogram of GZ and chromatogram of GZ in chloroform extract of GG, respectively, showing retention time 9.4 min. The chromatogram generated for sample solution might serve as one of the identifying parameters for GG.

Table 2: Linearity data for GZ peak

Analyte	Slope (m) Mean±SD	Confidence interval	y-intercept (C)Mean±SD	Confidence interval	RegressionCoefficient (r^2) Mean±SD
GZ	53089.5±114.1258	52997.83–53181.17	74958.5±9334.35	67460.42–82456.58	0.9975±0.0012

n=6. GZ: Guggulsterone-Z, SD: Standard deviation

Table 3: Precision of analytical method for GZ

GZ concentration	Variation in peak area of GZ determined on single day (intraday) (%RSD)	Variation in peak area of GZ determined on six different days (interday) (%RSD)
40 μg/ml	0.13	0.38
80 μg/ml	0.14	0.28
160 μg/ml	0.08	0.11

GZ: Guggulsterone-Z, RSD: Relative standard deviation

Table 4: Recovery studies of GZ

Sr. No	% GZ spiked	Conc. of GZ determined in solution Avg±SD (μg/ml)	% Amount recovered±SD	% Average recovery
1	0	81.54±0.25	-	-
2	49%	116.55±1.34	95.79±1.11	95.89%
3	98%	155.32±0.16	96.04±0.10	
4	148%	193.40±1.34	95.84±0.67	

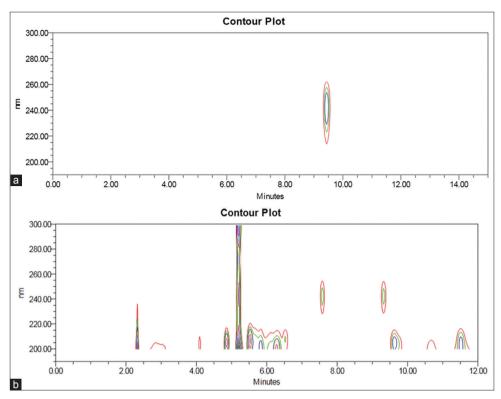n=3. GZ: Guggulsterone-Z, SD: Standard deviation

Fig. 3 (a) Contour of guggulsterone (GZ) in standard solution. (b) Contour of GZ in chloroform extract of *Gokshuradi Guggulu*

SSP parameters were also evolved as shown in Table 1. Percentage RSD value <1.0 for GZ peak confirmed that the variation in the peak area was within acceptable limit. The higher values of plats suggested that the peak obtained was Gaussian as well as sharp. The capacity factor value, more than 5.0 ensured that appropriate time was provided for interaction to happen with stationary phase. Resolution higher than 1.5 confirmed that the peak of GZ in sample solution was separated property from nearby peak, to be analyzed quantitatively. Tailing value near to 1.0 showed that the column packing was intact. As shown in figure, the studies ensured that the developed method could be able to resolute GZ peak.

The correlation coefficient value for plot of concentration of GZ and corresponding peak area was found approaching 1.0, determined using the least square regression analysis. The C value was also comparatively low. These two ensured the linearity of area for the concentration range selected for the studies.

Precision of the developed method was established in the form of variation in peaks of GZ while performing intraday and interday analysis. The results, in Table 3, showed that percentage RSD value for all three selected concentrations was within the recommended limit. Intraday precision and interday precision were found to be 0.11–0.38% RSD and 0.08–0.13% RSD, respectively, as shown in Table 3. The low values of percentage RSD confirmed that the method was precise enough to carry out routine analysis of GZ. It was also noted that there was comparatively higher variation noted in case of interday, which suggested the probable degradation of GZ in solution, though acceptable, it should be kept in consideration.

LOD and LOQ values were determined to establish sensitivity of the developed analytical method for GZ. LOD and LOQ were found to be 1.4 μg/ml and 4.4 μg/ml, respectively, for GZ.

Selectivity of the developed method for GZ was ensured by matching the shape of contour produced for GZ peak in sample chromatogram with that in chromatogram of standard GZ solution. As shown in Fig. 2, the contours were matching, as well as UV spectra of peak were

also matched. It ensured that the peak made up of spectroscopically homogenous material.

Accuracy of the developed method was assessed by performing recovery studies using standard addition method. The pre-analyzed sample solution was spiked with known concentration of GZ, and the sample solution was reanalyzed. Percentage recovery was calculated, found near to 95.89%, as shown in Table 4. The closeness of recovery nearly to 100% with low percentage RSD (i.e., <2) within acceptance range ensured accuracy. The studies confirmed that the developed analytical method was accurate and free from the interference of other material used in formulation.

Selectivity of the analytical method was the ability to assess only the analyte, GZ in the presence of other components which were present. Sample solution of GG contained many different related or unrelated (chemically) phytoconstituents. Selectivity was ensured by recording contour plots, for the peaks. These contour plots corresponding to GZ peak in chromatogram of sample solution were compared with that recorded for GZ peak in chromatogram of GZ standard. The contour plots are shown in Fig. 3a and b.

Robustness was determined initially, by incorporating deliberate small alteration in selected chromatographic parameters, one at a time. The studies showed that within the ranges selected the data sets of peak area for GZ and R_T were not statistically different from those obtained after receiving the peak and R_T, after following optimized chromatographic conditions. This confirmed that robustness of analytical method within the selected ranges. It was revealed from robustness studies that when acetonitrile proportion alteration was ± 3% v/v, alteration in flow rate was ± 0.2 ml/min, and wavelength was within ± 2 nm of reported value, there was no statistically significant alteration in R_T and peak area of GZ.

The estimation of GZ in GG sample solution was performed 3 times, each sample solution was prepared from an independent experiment and area for GZ peak was noted. GZ was, then, estimated from the equation obtained from linearity study. The amount of GZ found in GG was 0.815±0.03 g% w/w.

CONCLUSION

A validated HPLC method was developed for the quantification of GZ from an *Ayurvedic* formulation GG and validated. The developed analytical method could separate GZ from sample matrix without introducing gradient elution or without introducing pH adjustment. The validation studies confirmed that the developed method had linear response in terms of area of GZ peak, for the selected range of GZ concentrations. The developed method was precise, accurate, and sensitive enough for the purpose. The developed method could tolerate alterations in chromatographic parameters, without affecting the performance. This method, thus, may be introduced as one of the standardization parameters for the assessment of quality of GG.

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AUTHOR'S CONTRIBUTION

Kanan Gamit involved throughout the research work in the laboratory. Manan Raval provided his knowledge about chromatography and supported to evaluate data. Nishit Patel and Niraj Vyas supported to carry out experiments on HPLC.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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