ORIGINAL ARTICLE

Quantitative analysis of Glycyrrhizic acid from a polyherbal preparation using liquid chromatographic technique

Amit K. De, Sriparna Datta, Arup Mukherjee

Department of Chemical Technology, University College of Science and Technology, University of Calcutta, Kolkata, West Bengal, India

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ABSTRACT

Glycyrrhizic acid has been used in Indian traditional medicine for ages. It is obtained from the root extract of *Glycyrrhizaglabra*. There is seasonal variation of Glycyrrhizic acid content in the roots of the plant. So a proper method for quantification of the same is necessary from the polyherbal preparation available in the market. A simple, rapid, sensitive and specific reverse phase high performance liquid chromatographic method have been developed for the quantitative estimation of glycyrrhizic acid from polyherbal preparation containing aqueous root extract of *Glycyrrhizaglabra* using a photodiode array detector. The identity confirmation was carried out using mass spectrometry. Baseline resolution of the glycyrrhizic acid peak was achieved on a reverse phase C18 column (125 mm × 4.0 mm, 5 μ) using an isocratic mobile phase consisting of 5.3 mM phosphate buffer and acetonitrile in the ratio 65:35 v/v. Chromatograms were monitored at 252 nm.5.3 mM phosphate buffer was replaced with 0.5mM ammonium acetate buffer in the mobile phase when MS detector was used. The method was found to be linear in the concentration range of 12.4 to124 μg/ml with a correlation co-efficient of 0.999. The limit of detection and the limit of quantitation were 3.08 μg/ml and 10.27 μg/ml respectively. The average recovery from three spike levels was 99.93 ± 0.26%. Identity confirmation of the chromatographic peak was achieved by electrospray ionization mass spectrometry and similar molecular ion peak was obtained for both sample and standard. The developed method is suitable for the routine analysis, stability testing and assay of glycyrrhizic acid from polyherbal preparations containing aqueous extracts of *Glycyrrhizaglabra*.

Key words: Glycyrrhizaglabra, Glycyrrhizic acid, LC-DAD, LC-MS/MS

INTRODUCTION

Yastimadhu (*Glycyrrhizaglabra*) is being used as expectorant, emollient, anti-inflammatory, anti-hepatotoxic and anti-bacterial from ages in Indian *Ayurveda*,[1,2] Chinese and Japanese system of medicine. It is used in ayurvedic preparations like churnas, avaleh, asav and arista.[3] It is also used as cosmetic ingredient for its skin whitening properties. The bioactive components reported from *G. glabra* include liquiritin, isoliquiritin, liquiritinapioside, glabridin, glycyrrhetic acid and glycyrrhizic acid (GA).[4-7]

GA is a major triterpene saponin found mainly from the roots of *Glycyrrhizaglabra* [Figure 1]. It has wide spectrum of medicinal applications which includes hepato-protective, anti-inflammatory, antidotal, anti-allergic, immunomodulatory and antiviral and all these have been substantiated by modern pharmacological analysis.[8-13] It has been found to be effective in the amelioration of peptic ulcer diseases, inflammatory bowel disease[14-16] in human immunodeficiency virus infection[17-19] and anti-angiogenic properties in tumor progression.[20] GA is commonly used as sweetening and flavoring agent in food, tobacco and confectionary products and is about 170 times sweeter than sucrose.[21]

Address for correspondence:
Mr. Amit Kumar De,
92, A.P.C. Road, Kolkata, West Bengal, India.
E-mail: amitkumarde200401@gmail.com

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Extensive literature survey revealed only one analytical method for the estimation of GA from extract that has been properly substantiated by a mass spectroscopic analysis.[22] However, several chromatographic method of estimation has been reported where an UV detector has been used and the eluent is monitored at 254 nm following an isocratic elution. A C18 column is used with a mobile phase consisting of either methanol or acetonitrile as organic component along with an aqueous phase containing either an acid modifier like acetic acid or phosphoric acid.[23-36] GA being a triterpinesaponin, its content in the plant tissue varies significantly at different time periods of the year. Thus a proper quantification of the same in the marketed polyherbal preparation is essential. In our study we have developed a simple, rapid and sensitive method for the estimation of GA from a polyherbal preparation containing extract of *Glycyrrhizaglabra*. We have used LC- DAD method for quantification and LC MS-MS system with gradient elution for identity confirmation. The earlier methods [37-39] are much more tedious and time consuming compared to the newly developed method which is rapid and reproducible and has been validated as per ICH guidelines.[40]

MATERIALS AND METHODS

Materials

The solvents used were of analytical reagent grade and HPLC grade (for chromatography) and were purchased from Spectrochem India limited. Phosphoric acid of HPLC grade (Spectrochem, India) was used for pH adjustment of the eluent. Glycyrrhizic acid mono-ammonium salt (purity = 98.1%) was purchased from Sigma Aldrich India Ltd. The polyherbal formulation (cough syrup) containing *Glycyrrhizaglabra* extract as one of the principle component was purchased from market with batch number HD514, manufacturing date January 2012 and expiry date December 2014.

Methods

Instrumentation and chromatographic conditions for LC-DAD and LC-MS/MS analysis (API mode)

The chromatographic determinations were carried out on a Waters (Waters, USA) binary gradient system equipped with Waters 515 pump (two numbers), a manual Rheodyne injector port attached with a 20 µl loop and Waters 2996 PDA detector. The system control and data acquisition was carried out using Empower 2 software (Waters, USA). The separation was carried out in reverse phase Kromasil C18 column (125 mm × 4.0 mm, 5 µ; Akzonobel, USA). The mobile phase was a mixture of 65%, 5.3 mM phosphate buffer (pH = 3.0) and 35% acetonitrile which was filtered through 0.45 µm Millipore filter paper. The flow rate was 1.0 ml/minute and the column was maintained at ambient temperature. The column effluent was monitored at 252 nm with PDA detector. Prior to chromatographic separation both the standard and the sample were filtered through 0.2 µm membrane filter (Pall Life science, India).

For identity confirmation the analysis was carried out on a LC-MS/MS equipped with Shimadzu SILHTC autosampler-pump module (Shimadzu, Japan) and Mass detector with Atmospheric Pressure Ionization -ESI (API2000, Applied Biosystems, California). For LC-MS/MS analysis a different chromatographic condition was maintained. Waters X-bridge column (Waters X-Bridge, C18, 4.6 mm × 50 mm) was used. The 5.3 mM phosphate buffer (pH = 3.0) was replaced with 0.5 mM ammonium acetate buffer and a linear gradient elution at a flow rate 1.2 ml/minutes was used. The LC MS/MS system was operated at source gas pressure 100 psi, exhaust gas pressure 50 psi and curtain gas pressure 30 psi. The ESI was operated at Ion spray voltage 5500 V. The source temperature was 200°C. The cone voltage was 50 V, focusing potential 200 V and entrance potential 10 V.

Preparation of standard solution and sample solution

The GA stock solution (620 µg/ml) was prepared by dissolving 15.5 mg of glycerrhyzic acid mono-ammonium salt in 25 ml of hot water. The stock solution was diluted to the range 124 µg/ml to 12.4 µg/ml of GA for analysis. A six point calibration curve [Figure 2] was drawn for linearity study and for quantification purpose. Each dilution of the stock was injected in triplicates. The least square method was used. The sample solution was prepared by diluting the sample cough syrup to a dilution range 100 µg/ml.

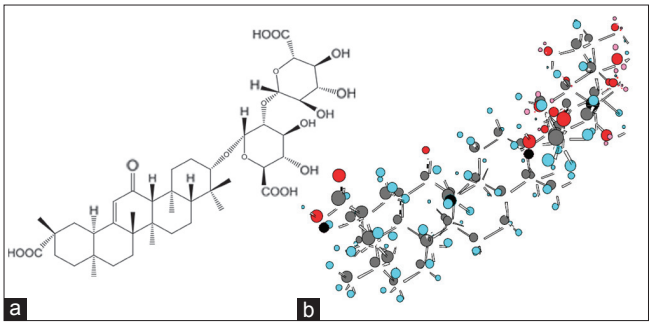

Figure 1: (a) Structure of GA and (b) 3-D structure of GA

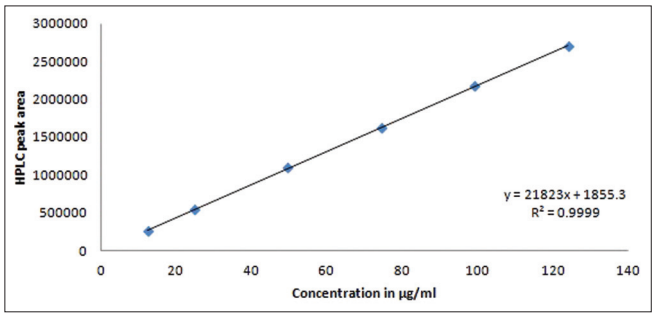

Figure 2: Calibration curve for GA

Validation of the Developed Method

The analytical method was validated as per USP and ICH guidelines. The studied parameters were accuracy, precision, linearity, range, ruggedness and robustness. To ensure reliability and accuracy of the proposed method, recovery studies were carried out by mixing a known quantity of the standard drug with the sample at three different concentration levels (10, 20 and 30% above assay value labeled as A, B and C). Six injections of the standard solutions were done to study the precision of the method. The linearity of the method was established by triplicate injections of standard solution in the concentration range of 12.4 to124 µg/ml. The intra-day precision was calculated using six injections at the higher concentration range (124 µg/ml) on the same day. These studies were repeated with solutions of different concentration on different days to obtain the inter-day precision. The specificity of the method was studied from purity plot of PDA detector. The limit of detection (LOD) and the limit of quantitation (LOQ) were determined by injecting progressively low concentrations of standard solution under optimized chromatographic conditions.[41] Ruggedness of the method was studied by carrying out experiment on instruments of different make. The robustness of the method was determined by making slight changes in chromatographic conditions like composition (±5%) and pH (±0.1%) of mobile phase.

Statistical Analysis

The statistical analysis was carried out on Sigma plot software (Version 8.02 SPSS Inc., USA) and MS Excel 2007. The results were represented as mean ± S.D. values for three to six replicate injections.

RESULTS AND DISCUSSIONS

LC-DAD and LC MS/MS identity confirmation

The newly developed simple method was more specific

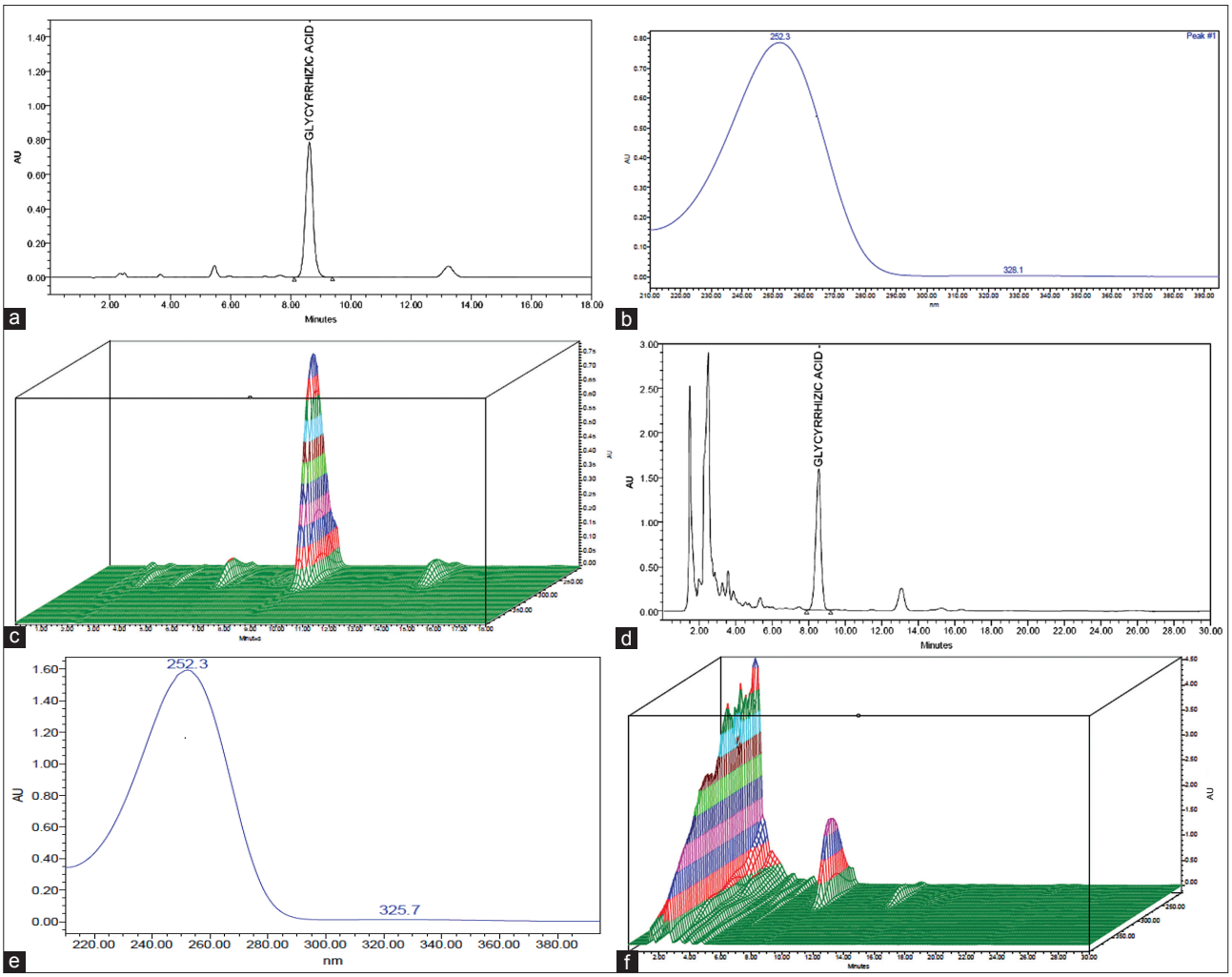

Figure 3: HPLC chromatogram of pure GA (a), UV spectrum of GA obtained from LC-DAD analysis (b), 3-dimentional photodiode array chromatogram of pure GA (c), HPLC chromatogram of sample syrup containing extract of *Glycyrrhizaglabra* root (d), UV spectrum of GA isolated from sample obtained from LC-DAD analysis (e) and 3-dimentional photodiode array chromatogram of sample cough syrup containing extract of *Glycyrrhizaglabra* root (f)

and less time consuming compared to the previously reported methods.[37-39,42,43] The maxima in case of LC-DAD analysis was observed at 252 nm. The chromatogram was subjected to peak purity analysis in order to study any co-elution. The LC-DAD analysis revealed the peak to be pure and spectrally homogenous with peak purity angle 0.357. This was less than the peak purity threshold 0.438. A reasonable resolution between the GA peak and the closely eluting peaks was observed in the sample [Figure 3].

The average retention time of GA peak was 8.5 ± 0.09 minutes (± S.D.; *n*=3). The representative chromatograms presented in Figure 3 showed the analyte peaks to be symmetrical and well resolved from the closely eluting peaks. In case LC-MS/MS analysis the mass spectrum represented a high degree of identity confirmation. The presence of molecular ion peak at m/z = 839 for both the sample and the standard spectrum was taken as confirmation that the peak observed was of GA in both sample and standard solutions [Figure 4].

Validation of the Developed Method

The liquid chromatographic method was validated as per USP and ICH guidelines and the various parameters were evaluated which are presented as follows.

Specificity

A HPLC method was considered to be specific if it ensures that the measured peak was only due to the substance being analyzed and it was free from potential impurities or co-eluting components. The specificity with regard to other co-eluting components of GA was investigated. Sufficient resolution between GA peak and the closely eluting peaks was observed under optimized chromatographic conditions [Figure 3d]. In all cases the purity angle was less than the peak purity threshold indicating the absence of any co-eluting peaks. The resolution factor between the GA peak and closely eluting peak in the chromatogram of polyherbal preparation of *G. glabra* was 1.9.

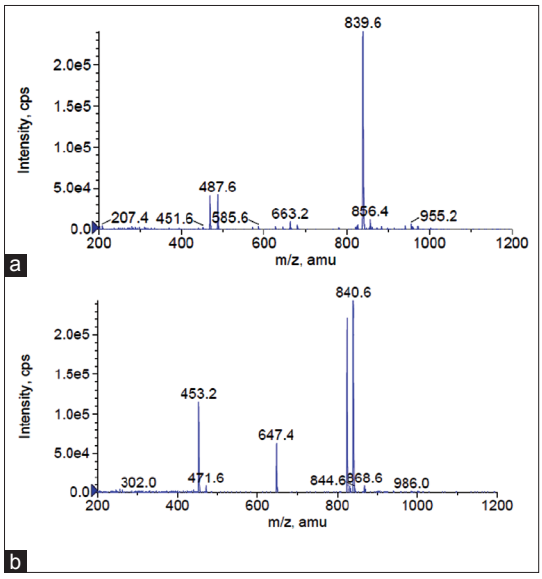

Figure 4: LC-MS/MS spectrum of standard (a) and sample (b)

Linearity and Sensitivity (LOD and LOQ)

LOD denotes the lowest amount of analyte in a sample could be detected but not necessarily quantified and LOQ denotes the lowest amount of analyte that could be quantified with precision and accuracy.[44] Reference standard solutions of six different concentrations ranging between 12.4 µg/ml to 124 µg/ml were injected into the chromatographic system and the area of the major peak was recorded separately (six replicates each). An area verses concentration curve was drawn with the concentration on X-axis and the peak area on the Y-axis. The linear regression parameters were slope 21823 ± 33.4103 and Y- intercept 1855.3 ± 8168.0187 ($P < 0.0001$). The correlation coefficient for GA was $r^2 = 0.9999$. The residual plot analysis demonstrated that the residual values were randomly distributed around zero. This confirms the choice of linear model. The sensitivity of the method was evaluated on the basis of LOD and LOQ values which were 3.08 µg/ml and 10.27 µg/ml respectively.

Precision

The precision was measured on the basis of repeatability and intermediate precision. The repeatability was measured on the basis of six replicate injection of the 124 µg/ml GA solution on the same day. The intermediate precision was obtained by triplicate injection of the sample solution on three different days. The intra-day and inter-day precision values were calculated from the linearity curve and the observed % RSD value was found to be < 2.0 [Table 1].

Table 1: Validation data for the estimation of GA from polyherbal preparation based on LC-DAD analysis

Parameters	Glycyrrhizic acid
System suitability	
Retention time (minutes)	8.5
Capacity factor	2.3
Resolution	1.9
USP tailing factor	0.1
USP theoretical plates (N)	11791
Sensitivity	
Limit of detection (LOD) (µg/ml)	3.08
Limit of quantitation (LOQ) (µg/ml)	10.27
Precision	
Intra-day	
Repeatability (mg /5 ml) (*n* = 6)	199.02
%RSD	0.29
Inter-day	
Mean glycyrrhizic acid content from polyherbal preparation(mg/5 ml) (day 1/day 2/day 3) (*n* = 3)	199.01/199.07/199.18
(%RSD) (day 1/day 2/day 3) (*n* = 6)	0.35/0.99/1.35

Table 2: Table for accuracy study

Average actual concentration of sample solution (μg/ml)	Solution	Theoretical concentration from spiked solution (μg/ml)	Theoretical excess amount added (μg/ml)	Average actual assay from sample solution (mg/5ml)	Assay from spiked solution (mg/5ml)	Observed excess amount (μg/ml)	Recovery difference %	Accuracy %	%RSD
99.51	A	110.46	11.00	199.02	220.89	10.99	0.01	99.99	0.26
	B	120.41	21.00		240.50	20.84	0.16	99.84	
	C	128.36	28.99		256.77	29.02	0.03	99.97	

Accuracy

The accuracy of a method was defined as a percentage of systematic error and was calculated as the deviation agreement between the measured value and the true value. The acceptable value for deviation was 15% of the actual value.[45] The sample solution was spiked at three levels and the recovery was 99.99%, 99.84% and 99.97% respectively [Table 2]. The average recovery was 99.93 ± 0.26%.

Robustness

The robustness was tested in order to evaluate the variation of analytical result due to deliberate changes in analytical conditions.[46] The changes in operational parameters (change in person) did not lead to significant changes in the performance of chromatographic analysis. The observed variation was only 0.98%. Thus the robustness of the study could be established.

System Suitability

System suitability was evaluated to verify if the chromatographic system was adequate for performing the analysis. The approximate results were theoretical plates (N = 11791), capacity factor (k = 2.30), peak asymmetry, or tailing factor (t = 0.10). The values for these parameters were satisfactory in accordance with the literature[47] [Table 1].

Solution Stability

The prepared solution was found to be stable for seven days for evaluation purpose only when kept at 4°C.

CONCLUSION

The developed RP-HPLC method was validated in terms of linearity and precision in the studied concentration range. The retention time of GA was only 8.5 minutes. Thus this rapid, simple, precise and accurate method could be used for routine analysis, stability testing and estimation of GA from herbal formulations available in the market.

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