

# Simultaneous determination of 18 $\alpha$ -glycyrrhetic acid and 18 $\beta$ -glycyrrhetic acid in *Glycyrrhiza glabra* root by reversed phase high-performance liquid chromatography

## Abstract

**Background:** The aim of the present research work is to develop a high-performance liquid chromatography (HPLC) method for simultaneous analysis of 18 $\alpha$ -glycyrrhetic acid (18 $\alpha$ -GA) and 18 $\beta$ -GA (18 $\beta$ -GA) of *Glycyrrhiza glabra*. **Materials and Methods:** About 20  $\mu$ L aliquots of each 18 $\alpha$ -GA and 18 $\beta$ -GA were analyzed using reversed-phase C-18 column. The mobile phase was acetonitrile:tetrahydrofuran:water (10:80:10, v/v/v). The run time was 10 min at flow rate of 1 ml/min. Ultraviolet detection was carried out at 254 nm. **Results:** 18 $\alpha$ -GA and 18 $\beta$ -GA were well resolved in reversed phase C-18 column using mobile phase acetonitrile: tetrahydrofuran: water (10:80:10, v/v/v, pH 7.9). The  $R_f$  of 18 $\alpha$ -GA and 18 $\beta$ -GA was detected at 2.091 and 2.377 min, respectively. **Conclusion:** The developed chromatography method could be extended for potential quantification or simultaneous determination of these markers in plant as well as in herbal formulation.

### Key words:

18 $\alpha$ -glycyrrhetic acid, 18 $\beta$ -glycyrrhetic acid, *Glycyrrhiza glabra*, high-performance liquid chromatography

## Introduction

*Glycyrrhiza glabra* is very well-known medicinal plants of diverse pharmacological applications. The most important phytoconstituent present is glycone glycyrrhizin (GL).<sup>[1]</sup> On acid, alkali or enzymatic hydrolysis, GL gives two molecules of glucuronic acid and one molecule of glycyrrhetic acid (GA).<sup>[2]</sup> The GL and GA are the most used liquorice saponins in pharmaceuticals and cosmeceuticals owing to their biological properties such as anti-inflammatory,<sup>[3]</sup> anti-allergic,<sup>[4]</sup> and anti-ulcerative activity.<sup>[5]</sup> Its tumor protective activity has also been reported.<sup>[6]</sup> Due to structural similarity to steroids, their mineralocorticoid-like effects and inhibition of metabolic enzymes for adrenocorticosteroids have been reported.<sup>[7]</sup> Naturally occurring GL is  $\beta$ -isomer. The  $\beta$  isomers of GL (18 $\beta$ -GA, 18 $\beta$ -GL) and GA can be

isomerized to their  $\alpha$ -isomers (18 $\alpha$ -GA, 18 $\alpha$ -GL) under alkaline conditions.<sup>[8]</sup> Since 18 $\alpha$ -GA and 18 $\beta$ -GA showed different biological activities and physicochemical properties,<sup>[9]</sup> their different stereochemistry is important for biological properties. Therefore, an accurate, simple,

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and efficient method is required to assess the optical purity of GA.

In the present paper, an accurate and precise reversed-phase high-performance liquid chromatography (RP-HPLC) method has been described for simultaneous determination of 18 $\alpha$ -GA and 18 $\beta$ -GA present in biotransformed *G. glabra* extract.

## Materials and Methods

### Plant materials, microorganism, and chemicals

*G. glabra* root was procured from Global Herbs, New Delhi, and authenticated in NISCAIR, New Delhi, India. The bacterial strain *Escherichia coli* MTCC 1652 was obtained from the Institute of Microbial Technology, Chandigarh, India. It was grown in nutrient agar slants and maintained at 4°C. Standard 18 $\alpha$ -GA (18 $\alpha$ -GA) and 18 $\beta$ -GA (18 $\beta$ -GA) were received from Sigma Aldrich, Mumbai, India. HPLC grade acetonitrile, tetrahydrofuran, and water were procured from Merck, Mumbai, India.

### Preparation of *Glycyrrhiza glabra* root extract

*G. glabra* root extract was prepared by using Soxhlet apparatus. Air-dried root of *G. glabra* (25 g) was coarsely powdered and extracted with water (100%) for 72 h. The extract was concentrated under reduced pressure in rotary evaporator at 40°C.

### Biotransformation of glycone glycyrrhizin to 18 $\alpha$ -glycyrrhetic acid and 18 $\beta$ -glycyrrhetic acid by *Escherichia coli*

To 50 mL of synthetic media, 5.0 mL of bacterial seed culture was inoculated and fermented at 37°C, 150 rpm for 24 h.

### Extraction and analysis of glycone glycyrrhizin, 18 $\alpha$ -glycyrrhetic acid, and 18 $\beta$ -glycyrrhetic acid

The fermented broth was subjected to sonication for 10 min. The disrupted cell was then centrifuged at 5000 rpm for 10 min. The supernatant was filtered, and the amount of 18 $\alpha$ -GA and 18 $\beta$ -GA formed were analyzed.

### High-performance liquid chromatography instrumentation and conditions

Chromatographic separation was achieved using analyticals technologies HPLC system (E67 and E68, Ravi Park, Vasna Road, Vadodara, Gujarat 390015) equipped with Model P3000A pump. The eluates were monitored by ultraviolet (UV) detector (Model UV3000) at 254 nm. Reversed phase C-18 column (LiChroCART® 250) was purchased from Merck, Mumbai, India.

A volume of 20  $\mu$ L aliquots of each 18 $\alpha$ -GA and 18 $\beta$ -GA were prepared and analyzed. The analysis was carried out in mobile phase used acetonitrile:tetrahydrofuran: Water (10:80:10, v/v/v). The total run time was 10 min at

flow rate of 1.0 ml/min and UV detection was carried out at 254 nm.

### Method validation

The method was validated for following parameters: Linearity, limit of quantitation (LOQ), limit of detection (LOD), and system suitability.<sup>[10]</sup>

Following equations were used for calculating LOD and LOQ:

$$\text{LOD} = (3 \times \text{SD}/\text{slope}) \text{ and}$$

$$\text{LOQ} = (10 \times \text{SD}/\text{slope})$$

Linearity test solution for assay method was prepared from stock solution at different concentrations, and 25  $\mu$ g/ml of each solution was analyzed and peak area of chromatograms was noted.

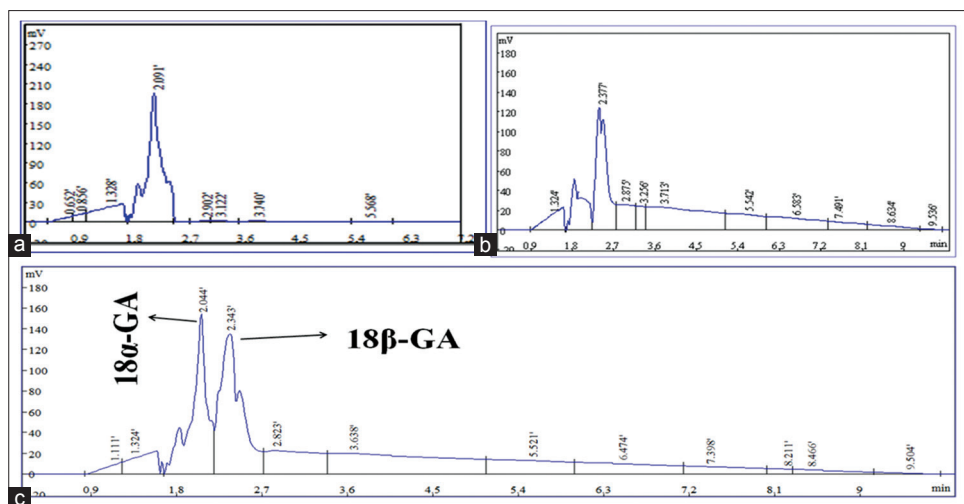
The interday precision of assay method was evaluated at four concentration (25, 50, 75, and 100  $\mu$ g/ml) ( $n = 3$ ). The interday precision was performed on three different, i.e., day 1<sup>st</sup>, day 2<sup>nd</sup>, and day 3<sup>rd</sup> at four different concentration (25, 50, 75, and 100  $\mu$ g/ml) ( $n = 3$ ). The relative standard deviation (%RSD) of the obtained assay values at four different concentrations was calculated.

The LOQ and LOD were based on standard deviation of response and slope of the constructed calibration curve ( $n = 3$ ).<sup>[10]</sup>

## Results

### Chromatographic separation of 18 $\alpha$ -glycyrrhetic acid and 18 $\beta$ -glycyrrhetic acid

18 $\alpha$ -GA and 18 $\beta$ -GA were well resolved in RP C-18 column. The mobile phase was different solvents with different concentrations to get sharp and well-separated peaks optimized. The different mobile phases were A (methanol: water 85:15, v/v), B (acetonitrile:methanol:water 25:15:60, v/v/v), C (acetonitrile:water 30:70, v/v), D (acetonitrile:tetrahydrofuran 10:90, v/v), and E (acetonitrile: tetrahydrofuran:water 10: 80: 10, v/v/v) were tested to get sharp chromatograms. When mobile phases A, B, C, D, and E at different flow rate (0.5 ml/min, 1.0 ml/min, and 1.5 ml/min) and different run time (10 min, 20 min, and 30 min) were run, the chromatograms obtained were overlapped at different  $R_t$ . However, sharp chromatograms were only obtained in mobile phase (E) containing acetonitrile: Tetrahydrofuran: water (10:80:10, v/v/v, pH 7.9) when the flow rate was maintained at 1.0 ml/min with total run time of 10 min. The resolution of both the molecules was strongly affected by pH of the mobile phase. At lower pH (4.5), the chromatograms were overlapped at 4.9 min of run time. When pH was



**Figure 1:** High-performance liquid chromatography chromatogram of standard 18 $\alpha$ -glycyrrhetic acid (a), 18 $\beta$ -glycyrrhetic acid (b), and their mixture (c)

increased to 7.2, decrease in retention time was observed and well-resolved chromatograms were detected. The  $R_t$  of 18 $\alpha$ -GA and 18 $\beta$ -GA was detected at 2.091 and 2.377 min, respectively [Figure 1]. The biotransformation of GL to 18 $\alpha$ -GA and 18 $\beta$ -GA by *E. coli* was studied and found that the sonicated *E. coli* has produced a maximum concentration of 18 $\alpha$ -GA (705.985  $\mu\text{g/ml}$ ) and 18 $\beta$ -GA (133.036  $\mu\text{g/ml}$ ) since  $\beta$ -glucuronidase present in *E. coli* is an intracellular enzyme.

The calibration curve was linear over the concentration range of 25–100  $\mu\text{g/ml}$  [Table 1] and regression equation was found to be  $y = 2577.5x + 2,000,000$  with correlation coefficient of 0.9923 for 18 $\alpha$ -GA and  $y = 31779x + 605206$  with correlation coefficient of 0.9944 for 18 $\beta$ -GA [Figure 2].

The RSD was found to be 0.001–0.447 (intraday) and 0.001–0.004 (interday) for 18 $\alpha$ -GA [Table 2] and 0.001–0.006 (intraday) and 0.002–0.004 (interday) for 18 $\beta$ -GA [Table 3]. The LOQ and LOD for 18 $\alpha$ -GA were found to be 0.052 and 0.015  $\mu\text{g/ml}$ , respectively. The LOQ and LOD for 18 $\beta$ -GA were found to be 0.377 and 0.113  $\mu\text{g/ml}$ , respectively. The proposed method was applied for the determination of 18 $\alpha$ -GA and 18 $\beta$ -GA content in the fermented broth. RSD (%) of 18 $\alpha$ -GA and 18 $\beta$ -GA was found to be 0.268 and 0.080  $\mu\text{g/ml}$ , respectively. The LOQ and LOD for 18 $\alpha$ -GA were calculated as 1.112 and 0.333  $\mu\text{g/ml}$ , respectively. The LOQ and LOD for 18 $\beta$ -GA were 0.456 and 0.136  $\mu\text{g/ml}$ , respectively.

## Discussion

The present development of RP-HPLC method could be used as potential quantification method for the simultaneous determination of plant terpenoids isomers. These isomers

**Table 1: Linearity of 18 $\alpha$ -glycyrrhetic acid and 18 $\beta$ -glycyrrhetic acid**

Concentration ( $\mu\text{g/ml}$ )	18 $\alpha$ -glycyrrhetic acid		18 $\beta$ -glycyrrhetic acid	
	Mean peak area $\pm$ SD	RSD (%)	Mean peak area $\pm$ SD	RSD (%)
25	1,579,925 $\pm$ 494.974	0.031	1,387,924 $\pm$ 6505.382	0.467
50	1,648,865 $\pm$ 569.220	0.034	2,156,151 $\pm$ 102.645	0.004
75	1,722,576 $\pm$ 749.533	0.043	3,099,961 $\pm$ 43.133	0.013
100	1,770,149 $\pm$ 13.435	0.007	3,721,588 $\pm$ 1198.382	0.032

SD – Standard deviation; RSD – Relative standard deviation

**Table 2: Intra- and inter-day precision studies of 18 $\alpha$ -glycyrrhetic acid**

Concentration ( $\mu\text{g/ml}$ )	Intra-day precision		Inter-day precision	
	Mean peak area $\pm$ SD	RSD (%)	Mean peak area $\pm$ SD	RSD (%)
25	1,578,920 $\pm$ 7071.067	0.447	1,576,920 $\pm$ 74.953	0.004
50	1,666,865 $\pm$ 53.740	0.003	1,666,065 $\pm$ 35.355	0.002
75	1,799,576 $\pm$ 25.455	0.001	1,799,076 $\pm$ 32.526	0.001

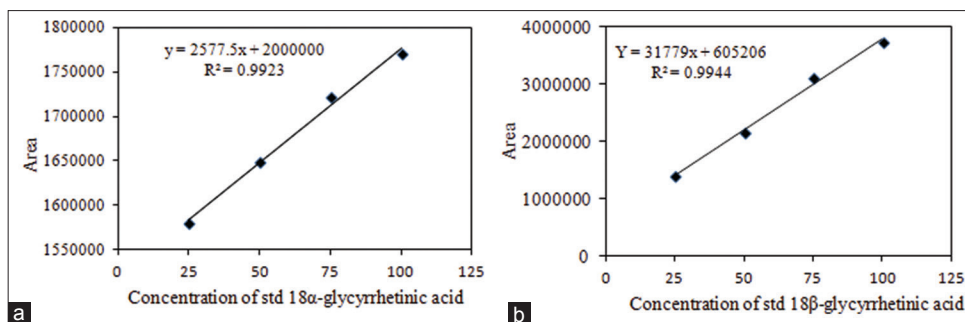
SD – Standard deviation; RSD – Relative standard deviation

**Table 3: Intra- and inter-day precision studies of 18 $\beta$ -glycyrrhetic acid**

Concentration ( $\mu\text{g/ml}$ )	Intra-day precision		Inter-day precision	
	Mean peak area $\pm$ SD	RSD (%)	Mean peak area $\pm$ SD	RSD (%)
25	1,387,942 $\pm$ 91.923	0.006	1,387,902 $\pm$ 67.882	0.004
50	2,156,511 $\pm$ 77.781	0.003	2,156,311 $\pm$ 79.781	0.003
75	3,099,916 $\pm$ 58.689	0.001	3,099,9216 $\pm$ 707	0.002

SD – Standard deviation; RSD – Relative standard deviation

are generally well resolved in the specific chiral column. Moreover, the present HPLC method was developed in RP



**Figure 2:** Regression plot of peak area against concentration of standard 18α-GA (a) and 18β-GA (b)

C-18 column. Hence, this may reduce the cost and economy of the process.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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