A Fast Isolation Method for Glycyrrhizic Acid, the Bioactive Marker of *Glycyrrhiza glabra,* and Its Quantitative Evaluation in Some Single and Multiherbal Formulations Using High-Performance Thin-Layer Chromatography

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Key Words:

Glycyrrhiza glabra
Glycyrrhizic acid
Combiflash chromatography
High-performance thin-layer chromatography
Quantification

Summary

Glycyrrhizic acid, the major metabolite of *Glycyrrhiza glabra,* is a 'Hot Molecule' in view of its sweet tasting properties and potent anti-viral and anti-allergic activities. A fast chromatographic method for its isolation and a rapid, sensitive reproducible high-performance thin-layer chromatography (HPTLC) method for its quantification have been developed. Glycyrrhizic acid was isolated in 6.9% yield from the 50% aqueous ethanolic extract of *G. glabra* roots by employing CombiFlash chromatography. For its quantitative studies, HPTLC silica gel F_{254} pre-coated plates were used with *n*-butanol–acetic acid–water (7:2:1) as the mobile phase. The method was validated for limit of detection, limit of quantification, linearity, specificity, precession, and recovery. The results indicate that the glycyrrhizic acid was present in *G. glabra* roots (0.88%) and in Yastimadhu Churna (0.78%). Surprisingly, the marker compound could not be detected in Artin capsule, which contains more than 10 herbal ingredients including *G. glabra*. The absence of a marker in this formulation might be due to its loss during the manufacturing process or different plant parts used in this formulation. The developed methods can be used successfully for fast isolation of glycyrrhizic acid and for the quality control and quality assurance of *G. glabra* formulations, where the root part is used.

1 Introduction

Glycyrrhizic acid, also known as glycyrrhizinic acid, is an oleanane class of pentacyclic triterpene saponin with 2 glucuronic acid units linked at 3β–hydroxyl position. It is chemically known as 18β-glycyrrhetinic acid-3-O-β-D-glucuronopyranosyl-(1→2)-β-D-glucuronide [1]. Glycyrrhizic acid is considered as the major bioactive metabolite of *Glycyrrhiza glabra,* which is popularly known as licorice and traditionally known as Yashtimadhu or Mullethi in the Indian system of medicine [2]. Glycyrrhizic acid is a natural sweetener with sweetness ~50 times higher than sucrose and most importantly has no calorific value [3]. It has been used clinically for more than 20 years in patients with chronic hepatitis in China and Japan and shows a satisfactory therapeutic effect in many other diseases [4]. It is reported to exhibit a wide range of therapeutic activities like anti-inflammatory, anti-ulcer, anti-allergic, antioxidant, anti-tumor, antidiabetic, and hepatoprotective features [5]. *In vitro* studies have demonstrated that glycyrrhizic acid inhibits cyclooxygenase activity and prostaglandin formulation as well as platelet aggregation indirectly [6, 7]. Glycyrrhizic acid has also been shown to inhibit growth and cytopathology of numerous RNA and DNA viruses, including hepatitis A and C, herpes zoster, herpes simplex, human immunodeficiency virus (HIV), and cytomegalovirus (CMV) [8]. Interestingly, in controlled clinical trials, the antiulcer potential of glycyrrhizic acid, its aglycone, corresponding extract, and root powder of its source *G. glabra* plant material was established [9]. In view of these interesting therapeutic and sweetener properties, glycyrrhizic acid has become a 'Hot Molecule' and attracted the attention of biologists and chemists all over the world. In order to carry out detailed and systematic research and developmental studies on glycyrrhizic acid, its availability on a large scale is highly warranted. Literature search reveals that glycyrrhizic acid was isolated earlier by a number of research groups from different *Glycyrrhiza* species at the milligram to multigram level [10]. The isolation of glycyrrhizic acid involves two major steps, such as extraction of the source plant material and separation, followed by purification of the desired compound from the crude extract. For the extraction of *Glycyrrhiza* species, various polar solvents

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like alcohol, hydro alcohol, and water using conventional maceration, hot Soxhlet, sonication, and microwave methods were employed [11]. *Xuejun et al.* reported the extraction of licorice with EtOH, EtOH–water, water, ammonia solution, and ethanol–water–ammonia using a microwave-assisted extractor, followed by high-performance liquid chromatography (HPLC) analysis [12]. *Jiang et al.* extracted the licorice roots with 70% aqueous methanol followed by filtration, precipitation with HCl, freeze-drying of the resultant precipitate, and separation by high-speed counter-current chromatography [13]. *Boqiang et al.* used sonication method for the extraction of licorice, followed by separation using macro-porous resins [14]. *Shen et al.* reported the extraction of glycyrrhizic acid from dried licorice slices with aqueous ammonia solution (0.5% vol%) under sonication followed by 3 liquid phase systems containing 4 components, such as organic solvent, inorganic salt, polymer, and the treated licorice extract [15]. *Tian et al.* have reported that the mixture of ethanol–water (70:30) and the extraction time of 60 min at 50°C are optimum conditions for the extraction of glycyrrhizic acid from licorice [16]. The above listed methods have drawbacks, such as the use of either specialized extraction equipment or acid–base media. Further, all these methods are mostly to improve the efficiency of the extraction of *Glycyrrhiza* species, but not for the physical separation or purification of glycyrrhizic acid.

Although glycyrrhizic acid is the major bioactive molecule of *G. glabra* and attracted the attention all over the world, very few reports are available on its quantitative evaluation in *G. glabra* formulations using liquid chromatography (LC) methods. However, some LC, high-performance thin-layer chromatography (HPTLC), and capillary electrophoresis methods are available for its calcium or ammonium salt (glycyrrhizin) or its aglycone (glycyrrhetinic acid) [17–19].

As the demand for glycyrrhizic acid is increasing considerably in developing new chemical entities to develop potent therapeutic agents and *G. glabra* for various traditional preparations, there is an urgent need to develop fast isolation and rapid quantitative methods for glycyrrhizic acid from *G. glabra*. Recently, the CombiFlash chromatographic system has been found to be highly useful in isolating ursolic acid from *Diospyros melanoxylon* leaves in 5.5 h with an overall yield of 0.6% [20]. This method has several advantages, such as low solvent consumption, short isolation time, and high yields of the desired compounds. Hence, this method can be extended to glycyrrhizic acid isolation also. For quantitative evaluation, HPTLC is the most suitable choice of analytical tool as we have successfully quantified several botanical and traditional preparations [21, 22]. In this connection, we have carried out detailed chromatographic and chemical studies on *G. glabra* and the results are presented in this paper.

2 Experimental

2.1 Reagents and Materials

For extraction and isolation of the marker compound, commercial grade solvents such as *n*-hexane, ethyl acetate, acetone, and methanol were used after drying and distillation, following standard purification procedures. Silica gel G was used for thin-layer chromatography. Pre-coated HPTLC silica gel 60 F_{254} (E. Merck, Darmstadt, Germany) aluminum-backed plates were used. The 50% aqueous ethanol extract of *G. glabra* roots was procured from the Council of Scientific & Industrial Research-Indian Institute of Integrative Medicine (CSIR-IIIM) (formerly Regional Research Laboratory [RRL]), Jammu, India. The roots of *G. glabra* were also collected through Sri Baidyanath Ayurved Pharmacy, Patna, India. The 2 commercial formulations of *G. glabra,* such as Yastimadhu Churna and Artin capsules of M/s Ganga Pharmaceuticals and Imis Pharmacy, respectively, were procured from local market.

2.2 Instruments

The IR spectrum was recorded using a Thermo Nicolet Nexus 670 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) using a KBr pellet. NMR spectra were recorded with Bruker Avance HD (Billerica, MA, USA) 400 MHz for ^{1}H and 100 MHz for ^{13}C in DMSO-d6. The mass spectrum was recorded using Shimadzu Biotech AXIMA Performance (Kyoto, Japan) matrix-assisted laser desorption/ionization–tandem time of flight (MALDI–TOF/TOF) instrument.

2.3 Isolation of the Marker Compound, Glycyrrhizic Acid

The 50% aqueous ethanol extract (4 g) was absorbed on silica gel (6 g) and subjected to fractionation using an ISCO CombiFlash chromatographic system Sg 100c. A glass column of 350 × 25 mm ($l \times d$) with Teflon connectors at both ends was packed with silica gel (30 g) of 230–400 mesh size. Then, the adsorbed material was poured into this column slowly and packed uniformly. The column was then eluted initially with chloroform–methanol (1:1), followed by *n*-butanol at a flow rate of 20 mL min^{-1} at a 15 psi pressure, using an ultraviolet (UV) detector at a wavelength of 215 nm. The whole process is controlled by a PC-based Peak Track software.

The *n*-butanol washings were combined and evaporated to get a brown-colored residue (504 mg), which was again subjected to CombiFlash chromatography with 100 test tubes using chloroform, chloroform–methanol (1:1), methanol, and *n*-butanol **(Figure 1).** Concentration of *n*-butanol fractions (56–83) under reduced pressure afforded the marker compound, glycyrrhizic acid **(Figure 2).**

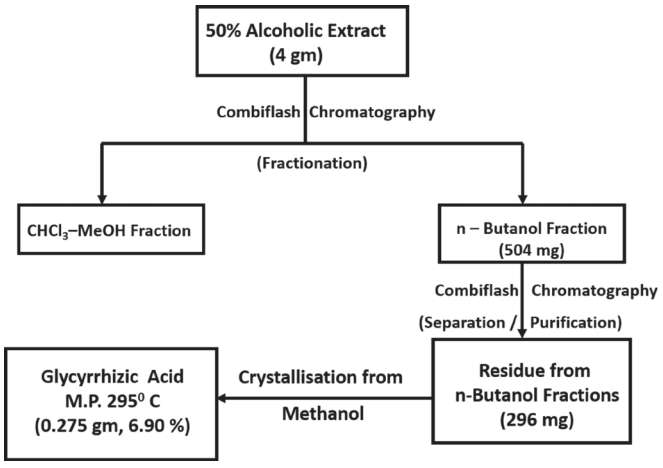


Figure 1

Isolation process of *Glycyrrhiza glabra* marker compound.

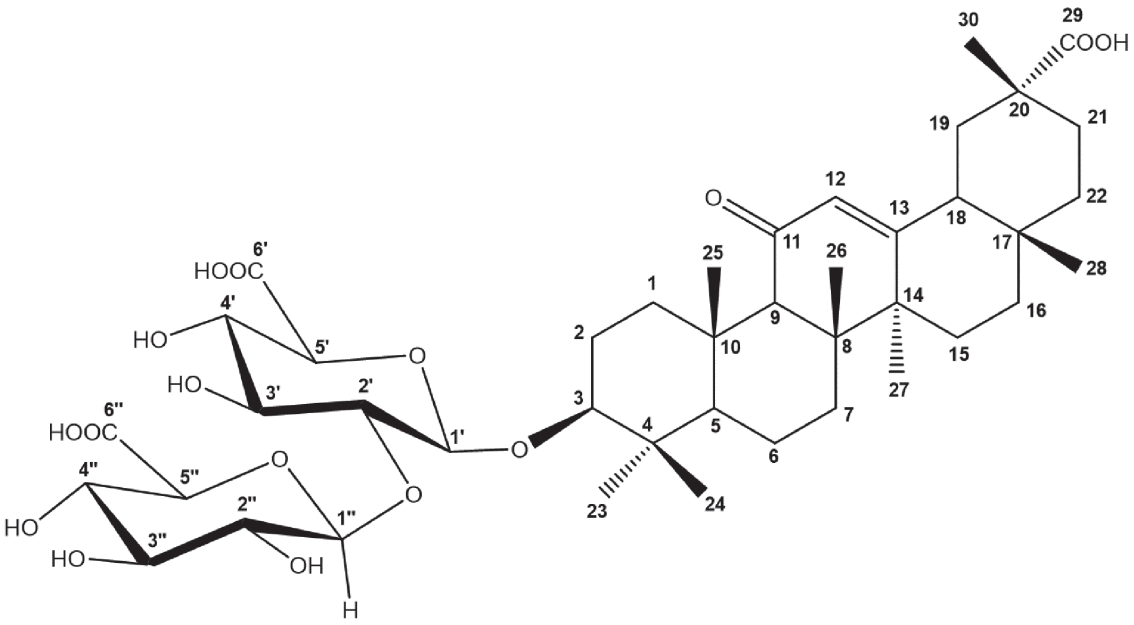


Figure 2

The chemical structure of glycyrrhizic acid.

It was obtained as pale brown amorphous powder (275 mg, 6.9%). m.p. 295°C, R_F: 0.33 [*n*-butanol–acetic acid–water (7:2:1)]. It gave positive tests for glycosides (Molisch's) and triterpenes (Liebermann–Burchard). IR (KBr, cm^{-1}): 3447 (hydroxyl), 1731, 1698, and 1654 (–COOH); ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.50 (s, 1H, 12-H), 5.40 (br.s, 2H, H-1',1''), 4.47 (b.d, 2H, d, J = 7.70 Hz, H-2',2''), 4.31 (b.d, 2H, d, J = 7.50 Hz, H-3',3''), 3.41–3.13 (m, H-3. 4',4''.5'.5''), 1.34–0.72 (6xs CH_3-23,24,25,26,27,28,&30). ^{13}C NMR (125 MHz, DMSO): 198.5 (C-11), 177.9 (C-29), 171.3 (C-6'), 171.0 (C-6''), 170.5 (C-13), 126.8 (C-12), 107.5 (C-1'), 102.3 (C-1''), 87.6 (C-2'), 87.5 (C-3), 78.9 (C-4'), 78.3 (C-2''), 75.8 (C-3'), 75.3 (C-4''), 75.2 (C-5'), 74.5 (C-3''), 71.5 (C-5''). 71.3 (C-6'), 60.5 (C-9), 55.9 (C-5), 47.5 (C-18), 44.6 (C-14), 44.3 (C-20), 42.6 (C-8), 42.4 (C-19), 40.2 (C-4), 38.1 (C-22 & C-1), 37.0 (C-10), 33.4 (C-7), 31.9 (C-17), 31.0 (C-21), 30.0 (C-28), 29.3 (C-30), 27.9 (C-23), 27.8 (C-2), 27.3 (C-15 & C-16), 25.0 (C-27), 18.1 (C-26 & C-6), 15.8 (C-25), 15.5 (C-24). MALDI: 845.36 $[M + Na]^+$ corresponding to $C_{42}H_{62}O_{16}Na$. Based on the above spectral data and by comparing its values with the reported values, it was identified as glycyrrhizic acid [23].

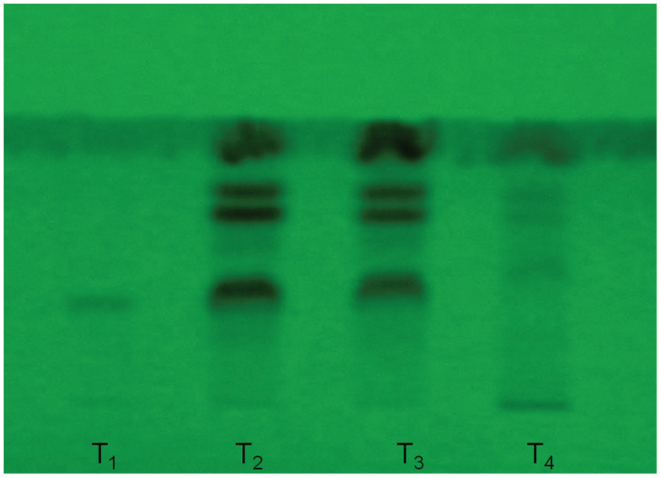
2.4 HPTLC Instrumentation and Chromatographic Conditions

2.4.1 Extraction and Sample Preparation

The powdered materials (1.00 g each) were extracted using Soxhlet extractors for 7 h in ethanol. These extracts were further dried in a freeze drier to get the respective extracts. One-hundred milligrams of each extract have been dissolved in 10-mL volumetric flasks.

2.4.2 Visualization

Visualization of the TLC plate is being done at 254 nm **(Figure 3)** and after derivatization with a freshly prepared anisaldehyde spray reagent.



T_1 = Glycirrhizin, T_2 = Extract, T_3 = Yastimadhu churna, T_4 = Artin capsule

Figure 3

TLC chromatogram of *G. glabra* and its commercial formulations.

2.4.3 HPTLC Instrumentation

The chromatographic estimation was performed by spotting standards and extracted samples of glycyrrhizin on pre-coated silica gel aluminum sheet plate 60 F_{254} (20 cm × 10 cm with 250-µm thickness, E. Merck, Darmstadt, Germany) using a CAMAG Linomat V sample applicator (CAMAG, Muttenz, Switzerland) and a 100-µL syringe. The samples, in the form of bands of length 6 mm, were spotted (5 µL each), 10 mm from the bottom, 35 mm from the left margin of the plate, and 13 mm apart, at a constant application rate of 80 nL s^{-1} using a nitrogen aspirator. Plates were developed using a mobile phase consisting of *n*-butanol–acetic acid–water (7:2:1, *v*/*v*). Linear ascending development was carried out in a 20 cm × 10 cm twin-trough glass chamber (CAMAG) equilibrated with the mobile phase. The optimized chamber saturation time for mobile phase was 10 min at room temperature. The length of

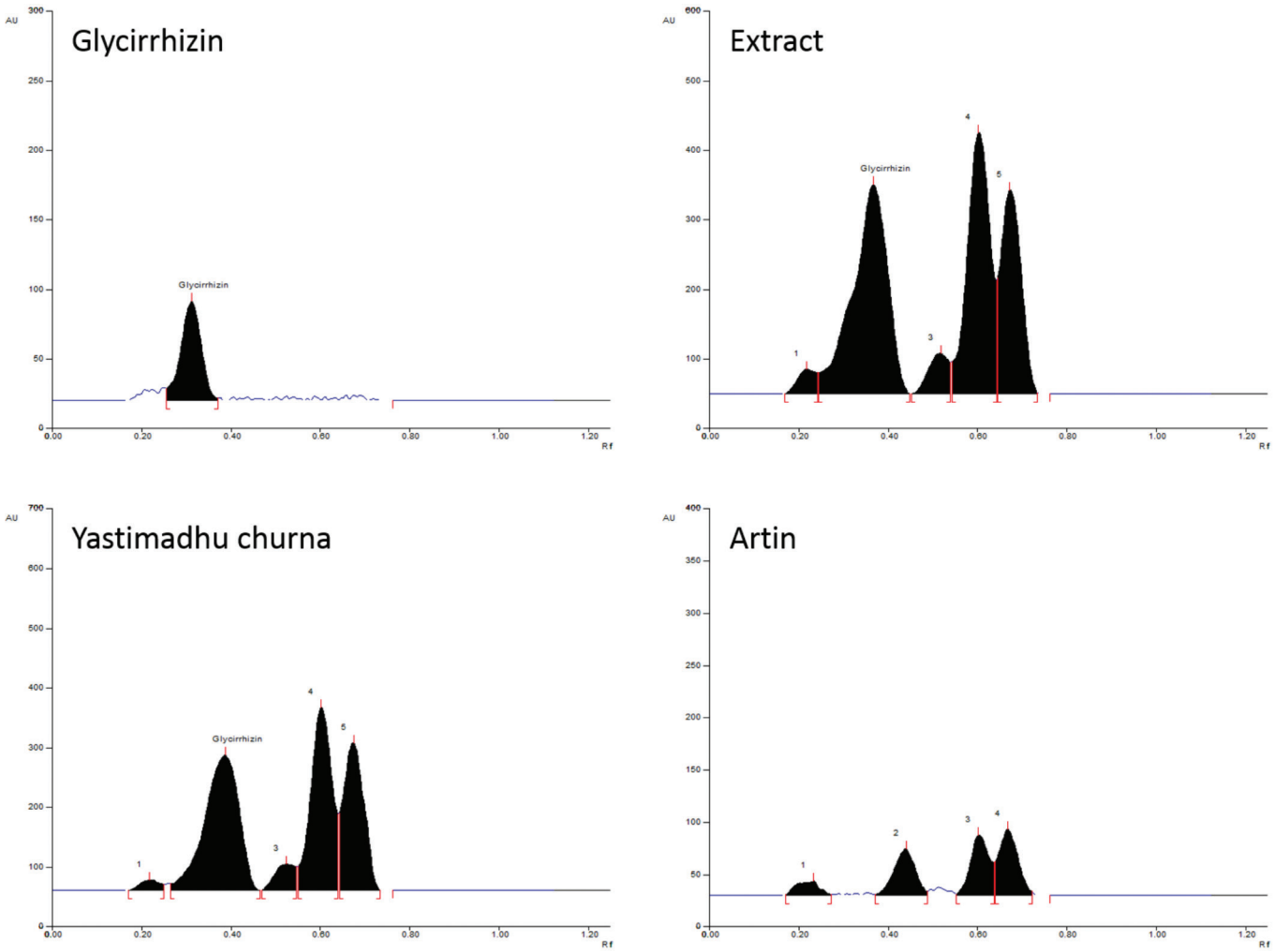


Figure 4

HPTLC chromatograms of the marker compound, *G. glabra,* and its commercial formulations.

chromatogram run was 5.0 cm. Approximately an amout of 20 mL of the mobile phase (10 mL in a trough containing the plate and 10 mL in the other trough) was used for each development, which required 15 min. It results in better apparent resolution with more convenient capability of the detecting device to perform integration of peak area. Subsequent to the development, TLC plates were dried in a current of air with the help of an air-dryer for 5 min. The slit dimension settings of length 5.00 mm and width 0.45 mm, and a scanning rate of 20 mm s^{-1} were employed. The monochromator band width was set at 20 nm.

Densitometric scanning was performed using a CAMAG TLC Scanner III in the absorbance mode at 260 nm and operated by winCATS planar chromatography version 1.1.3.0. The sources of radiation utilized were deuterium and tungsten lamps. Concentrations of the compound in sample chromatograms **(Figure 4)** were determined from the intensity of absorbance. Evaluation was made *via* peak areas *versus* glycyrrhizic acid amount in linear regression.

2.4.4 Calibration Curves of Standard

Calibration curves of standard in ethanol-containing concentrations of 0.02 mg mL^{-1} to 0.25 mg mL^{-1} were prepared from the stock solution of 1.0 mg mL^{-1} **(Figure 5).** An amount of 10 µL

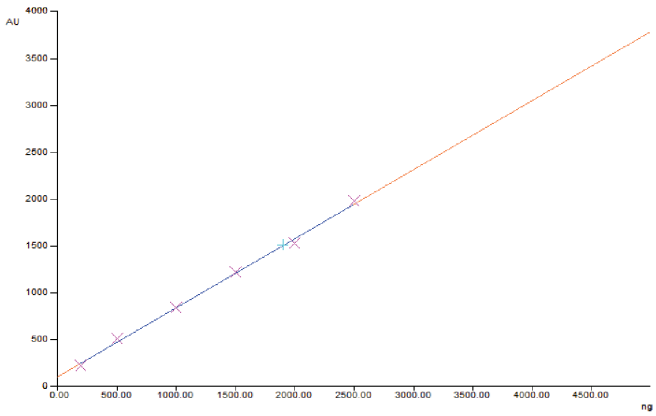


Figure 5

Calibration curve of the marker compound and the *G. glabra* samples.

from each solution was spotted on the TLC plate to obtain final concentration range of 0.2–2.5 µg per spot. Each concentration was spotted two times on the TLC plate. The data of peak area *versus* sample concentration were treated by linear regression analysis.

2.4.5 Method Validation

The HPTLC method developed was validated for the following parameters.

2.4.5.1 Sensitivity

The sensitivity of the method was determined with respect to limit of detection (LOD), limit of quantification (LOQ), linearity range, and correlation coefficient. Solutions containing 0.2–2.5 µg of the standard were spotted on the TLC plate. LOD was calculated to be 160 ng (3.3 times the noise level), and LOQ was calculated to be 487 ng (10 times the noise level). The regression data for the 4 samples are given in **Table 1,** which shows a good linear relationship in that studied range.

Table 1

Results of linearity.

Linearity range	r^2	Slope	Intercept
0.2–2.5 µg	0.99867	0.737	101.387

2.4.5.2 Recovery Study

Recovery of the standard was calculated by spiking 1.0 mg to the formulation, Artin capsule (1.00 g), extracted in ethanol and analyzed three times as described in Section 2.2.2. The recovery was calculated by comparing the resultant peak areas with the standard **(Table 2).**

Table 2

Recovery study.

Sample	Amount spotted [ng]	Amount detected [ng] (mean ± SD, *n* = 3)	RSD [%]	Recovery [%]
Artin	500 (10 µl)	494.19 ± 5.27	1.06	98.83

2.4.5.3 Precision and Accuracy

Different amounts of Artin (spiked) samples are spotted on TLC plate. These spots were analyzed by using the above described HPTLC method. Precision was expressed as the percent of relative standard deviation (*%RSD*) **(Table 3).**

Table 3

Results of precision and accuracy.

Actual amount of standard spotted [µg]	Amount detected [µg ± SD] (*n* = 5)	RSD [%]
0.5	0.486 ± 0.013	2.67
1.0	0.979 ± 0.036	3.76
3.0	2.884 ± 0.112	3.88

2.4.5.4 Reproducibility

The repeatability was evaluated by analyzing the known amounts of samples spotted on the TLC plate in replicates (*n* = 5). The inter-day and intra-day precision were evaluated by analyzing the same amount of analyte over a period of 3 days (*n* = 5) and expressed in terms of *%RSD* **(Table 4).**

Table 4

Results of reproducibility.

Amount spotted [µg]	Amount detected [µg ± SD]	RSD [%]
Inter-day (*n* = 3)		
0.5	0.483 ± 0.023	4.76
1.0	0.971 ± 0.050	5.14
3.0	2.868 ± 0.164	5.71
Intra-day (*n* = 3)		
0.5	0.491 ± 0.012	2.44
1.0	0.983 ± 0.031	3.15
3.0	2.881 ± 0.124	4.30

3 Results and Discussion

The 50% aqueous ethanolic extract of the roots of *G. glabra* was subjected to a novel CombiFlash chromatography over Sigel with the initial elution with chloroform–methanol (1:1) followed by *n*-butanol, which resulted in the isolation of the bioactive marker compound as colorless amorphous powder. Preliminary physical and chemical tests identified the compound as triterpene glycoside. Detailed spectroscopic analysis (IR, ^{1}H and ^{13}C NMR, and mass spectroscopy) confirmed its structure as glycyrrhizic acid. Detailed thin-layer chromatographic studies on this compound optimized the solvent system as *n*-butanol–acetic acid–water (7:2:1), which showed a single spot at R_F = 0.33 (Figure 3). This compound was taken as a marker compound and quantitatively evaluated *G. glabra* roots along with two of its commercial samples such as Yastimadhu Churna (Ganga Pharmaceuticals) and Artin capsules (Imis Pharma) using HPTLC.

3.1 Method Validation

3.1.1 Sensitivity

The sensitivity of the method was determined with respect to LOD, LOQ, linearity range, and correlation coefficient. Solutions containing 0.2–2.5 µg of the standard were spotted on the TLC plate. LOD was calculated to be 160 ng (3 times the noise level), and LOQ was calculated to be 487 ng (10 times the noise level). The regression data for the 4 samples are given in the Table 1, which shows a good linear relationship in that range studied (Figure 5). The above analytical method was found to be specific by comparing the spectrum of the analyte and its presence in the samples **(Figure 6).**

3.1.2 Recovery Study

Recovery of standard was calculated by spiking 1.0 mg to the formulation, Artin (1.00 g), extracted in ethanol, and analyzed three times as described in Section 2.3.3. The recovery was calculated by comparing the resultant peak areas with the standard (Table 2).

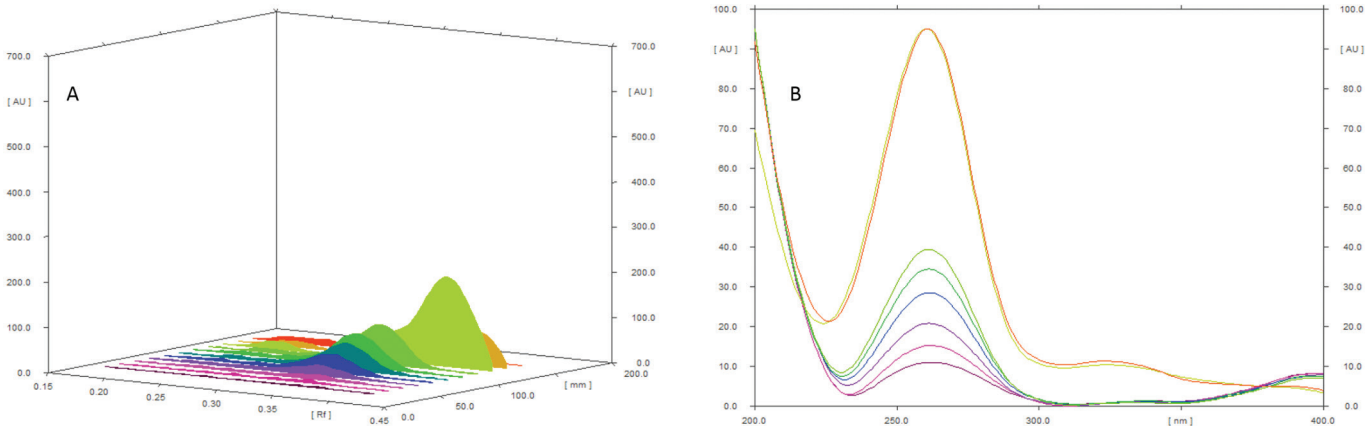


Figure 6

(A) 3D overlaid chromatograms and (B) UV spectra of the standard and *G. glabra* samples.

Table 5

Accumulation of the marker in various *G. glabra* samples.

S. No	Samples	Glycyrrhizic acid [%]
1	Root	0.88
2	Yastimadhu Churna	0.78
3	Artin capsule	Not detected

3.1.3 Precision and Accuracy

Different amounts of the Punarnava Mandur (spiked) samples were spotted on the TLC plate. These spots were analyzed by using the above described HPTLC method (Table 3). Precision was expressed as the percent relative standard deviation (*%RSD*).

3.1.4 Reproducibility

The repeatability was evaluated by analyzing the known amounts of spiked samples spotted on the TLC plate in replicates ($n = 3$). The inter-day and intra-day precision were evaluated by analyzing the same amount of analyte over a period of 3 days ($n = 3$) and expressed in terms of *%RSD* (Table 4).

3.2 Sample Analysis

The validated HPTLC method was applied for the quantitative determination of the marker, glycyrrhizic acid in *G. glabra* root sample and two of its commercial formulations such as Yastimadhu Churna and Artin capsules, and the results are presented in **Table 5.** The marker compound was found to accumulate (0.88%) in the *G. glabra* root sample. The developed HPTLC method was found working very well in the case of one of the commercial formulations, Yastimadhu Churna, where the marker compound was found to be present (0.78%). Surprisingly, the marker compound could not be detected even in traces in Artin capsule, which contains more than 10 herbal ingredients including *G. glabra*. The absence of the marker in this formulation might be due to its loss during the manufacturing process or different plant parts used in this formulation. The developed methods can be used successfully for its fast isolation and quality control and quality assurance of *G. glabra* formulations, where the root part is used. Hence, the marker compound in these 2 formulations might be lost during their manufacturing process or due to different plant part used. But, marker compound could be detected accurately in the whole plant samples. The developed method can be used successfully for the quality control and quality assurance of *G. glabra* formulations, where the root part is used.

4 Conclusion

In the present study, a novel two-step CombiFlash chromatographic method was developed for the fast isolation of glycyrrhizic acid, the bioactive marker of *G. glabra*. Further, glycyrrhizic acid was quantitatively estimated in the root part of *G. glabra* and 2 of its commercial samples, namely, Yastimadhu Churna and Artin, by employing a rapid and validated HPTLC method. The method is rapid and reproducible. The marker compound was found to be present in the roots of *G. glabra* (0.88%) and in Yastimadhu Churna (0.78%). But surprisingly, it could not be detected in Artin capsules. The developed method can be successfully employed for the quality control and quality assurance of *G. glabra,* where the root part is used.

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