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DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR GLYCYRRHIZIN AND ITS APPLICATION FOR HERBAL ANTI-ACNE GEL

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ABSTRACT

Objective: Number of analytical techniques are available for estimation of glycyrrhizin in extracts and herbal formulations. To our knowledge, there is no specific HPTLC (high-performance thin layer chromatography method) for quantification of glycyrrhizin in herbal gel compositions. In the present study HPTLC method was developed, validated and used for fingerprinting of glycyrrhizin in anti-acne formulation. Methods: Physicochemical, phytochemical and antibacterial potential of Glycyrrhiza glabra was assessed. A simple HPTLC method was validated. Method comprised application of 5 µl sample on 60GF254 plate and was placed in twin trough glass chamber (10 cm×10 cm) having solvent system of Pet ether: Benzene: Ethyl Acetate: Glacial Acetic Acid (4:8:2.8:0.2, v/v/v/v) and quantification was done using TLC scanner and in UV chamber at wavelength of 254 nm. Results: This system was found to give compact spots for glycyrrhizin (Rf value: 0.38 ± 0.02). A strong linear relationship between the peak area and concentration of glycyrrhizin was observed with correlation coefficient (r^2 = 0.99021 ± 7.470210). The method was validated for precision, accuracy, recovery and robustness. Amount of glycyrrhizin present in herbal extract and gel formulation was quantified as (7.934 %) and (1.070 %) respectively at 254 nm. Conclusion: It was a simple, sensitive, precise and reproducible method and can be used for quality assurance of the herbal composition containing glycyrrhizin.

KEYWORDS: Glycyrrhiza glabra, glycyrrhizin, HPTLC, validation, Gel.

1. INTRODUCTION

Acne vulgaris is one of the most common inflammatory skin diseases which develop in the sebaceous glands and hair follicles leading to the formation of comedones.[1] A large number of existing topical preparations have been developed by the pharmaceutical industry but these comedolytic preparations also have several side effects.[2] However, the recent escalation in the demand of herbal formulations has motivated us to formulate extract and quantify the active principle playing a key role in the treatment of acne. In this respect roots and stolons of Glycyrrhiza glabra Linn. (Family- Leguminosae) commonly known as liquorice which has been used in medicines for more than 4,000 years now, are of special interest. The active principle of G. glabra particularly glycyrrhizin (GLY) and its hydrolysis product, glycyrrhetinic acid, possess many importantpharmacological activities including anti-inflammatory.[3,5] Antioxidant.[6,7] antibacterial[8] and anti-acne.[9,13] Anti-inflammatory effect of glycyrrhizin, a saponin glycoside is observed on the adrenal gland, as it produces cortisol, human body's own anti-inflammatory adrenal steroid hormone. GLY was also reported (Figure 1) as a permeation enhancer when percutaneous absorption of diclofenac sodium from sodium carboxymethyl cellulose gels.[14] Because of the promising potential exhibited by G. glabra against acne causing bacteria, researchers have focused on the development of various analytical methods to quantify glycyrrhizin in extract and herbal formulations. Recently HPTLC has recently become as a preferred and routine analytical tool for fingerprinting and quantification of bioactive principles in herbal drugs due to its advantages of low operating cost, high sample throughput and need for minimum sample clean-up. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis. Though various techniques like (GC–MS) gas chromatography–mass spectrometry.[15] high-performance liquid chromatography (HPLC) based on UV absorption[16,18] and even high-performance thin-layer chromatography (HPTLC)[19] are available as analytical tool for GLY but attempts to apply these techniques for the profiling of GLY in herbal formulations are not available. HPTLC has become widely accepted routine analytical technique[20] nowadays due to its advantages of quantification of analytes at micro and even in nanogram levels, high sample throughput and need for minimum sample clean-up. With increasing demand for herbal formulations in the treatment of acne vulgaris, there is an urgent need for standardization of formulation. In this paper development and validation of a HPTLC method for the quantitative analysis of GLY as per ICH guidelines (ICH 1994)[21] is reported. The

method was applied for the fingerprinting and quantification of GLY as markers of herbal gel formulations containing G. glabra.

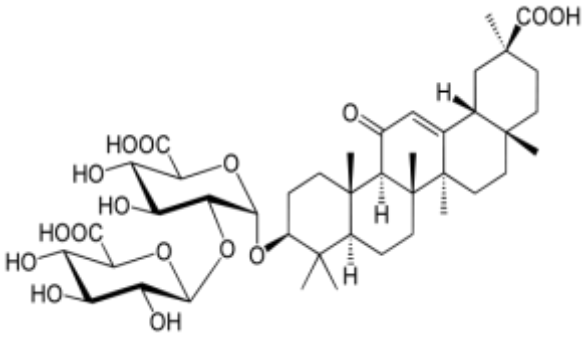

Figure.1. Chemical structure of glycyrrhizin

2. MATERIALS AND METHODS

2.1 Plant material and chemicals

The authentic samples of roots and stolons of Glycyrrhiza glabra were procured from authorized herbal stores in Delhi and authenticated by NISCAIR, Pusa Campus, New Delhi, India with voucher specimen (NISCAIR/RHM/ consult/2008-09/978/09) and have been preserved in our department for the future reference. Glycyrrhizin was obtained Sigma Aldrich Chemicals and was used without further purification, certified to contain 95 % (w/w). Analytical grade methanol, petroleum ether, benzene, ethyl acetate and glacial acetic acid, were obtained from Qualigens Fine Chemicals, Mumbai, India.

2.2 Physicochemical and phytochemical analysis

Physicochemical parameters were done to measure the loss on drying, total ash, acid insoluble ash, water insoluble ash and alcohol soluble extractive as per quality standards of Ayurvedic Pharmacopoeia of India (API 2001). [22] Methanolic extract of roots and stolons of G. glabra was screened for phytochemical screening using standard procedures in order to establish chemical profile.[23]

2.3 Antibacterial potential of Herbal extract and gel formulation

Herbal gel formulation containing methanolic extract (4%) of Glycyrrhiza glabra was prepared using carbopol. Exact composition of formulation is not disclosed.[24a] Antibacterial activity of methanolic extract and gel formulation was performed using clindamycin as positive control by disc diffusion method.[24b]

2.4 Chromatographic analysis

2.4.1 Preparation of standard solutions

Working solutions of analytes were prepared further by appropriate dilutions of the stock solutions of glycyrrhizin (1 mg/mL) with methanol and aliquots were taken for HPTLC analysis.

2.4.2 Chromatographic conditions

The chromatographic estimation was performed by spotting 5 µl of reference compound, GLY (10 mg/mL of methanol) on precoated silica gel aluminum plate 60GF254 (Merck KGA, Germany) using a using Linomate-V applicator (automatic TLC applicator, Camag, Switzerland).[25,27] The samples, in the form of bands of length 6 mm, were spotted leaving a margin of 10 mm from the bottom and left side of the plate and with 14 mm distance between the two bands at a constant application rate of 10 µl/min using nitrogen aspirator. Plates were developed using a mobile phase consisting of Pet ether: Benzene: Ethyl Acetate: Glacial Acetic Acid (4:8:2.8:0.2, v/v/v/v). Chromatogram was developed in 10 cm×10 cm twin trough glass chamber (Camag, Switzerland; Wincats Integrated Software 4.02) with TLC Scanner and equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 15 min at room temperature. Approximately, 10mL of the mobile phase was used for each development, which required 8 min and length of chromatogram run was 7 cm and provided better apparent resolution. Subsequent to the development, TLC plates were air-dried and observed under UV chamber (Camag UV chamber-3, model no. 022.9120). Densitograms were recorded at the wavelength of maximum absorption at 254 nm (Camag TLC Scanner-3, model No. 027.6480). Each concentration of the compounds was spotted two times on the plates and analyzed. Rf value and the percentage of the phytoconstituents chromatographed in extract and gel formulation were determined using peak areas by linear regression method.

2.4.3 Calibration parameters

A stock solution of GLY (1mg/mL) in methanol was prepared. Different volumes of stock solutions were diluted and 5 µl solution was spotted on the TLC plate under the optimized conditions to obtain final concentration range of 50-1000 ng/spot. The analysis was performed in triplicates and mean peak area responses to the concentrations were recorded at 254 nm to establish their correlation.

2.4.4 Precision

Repeatability of the method was validated for instrumental, intraday and interday precisions. The concentrations of gel formulations were selected in such a way that markers were in the experimental range. Instrumental precision was measured for 6 spots of GLY (200 ng/spot) (ISO 1994).[28] Intraday variation was studied by analyzing repeatedly 6 times in the morning and afternoon in the same lab on the same day, using three different concentrations (100, 200,400 ng/spot) of GLY, each of them independently prepared in methanol and each of them applied three times. Inter day precision included analysis of the same three concentrations of each solution analyzed three times for two consecutive days. In both the cases, precision was expressed as the percent relative standard deviation (% R.S.D), coefficient of variation (% C.V.) as per ICH Guidelines (ICH 1994).

2.4.5 Accuracy, recovery and robustness

The accuracy of the method was determined by the standard addition technique. Known amounts of the GLY (0% and 50%) were added externally to the methanolic extract G. glabra and reanalyzed by chromatography under the optimized conditions. Robustness of the method was determined by altering small change in experimental conditions like composition of mobile phase: Pet ether: Benzene: Ethyl Acetate: Glacial Acetic Acid (4:7:2.8:0.2 v/v/v/v).

2.4.6 Analysis of glycyrrhizin in herbal extract and gel formulation

Methanolic extract (100 mg/mL) and gel formulation (100 mg/mL) of Glycyrrhiza glabra were sonicated for 30 min and resulting solution was allowed to settle for about an hour. The extracts were filtered through 0.45µm filter paper and HPTLC was performed under the conditions optimized for the reference compounds. The plates were scanned at 254 nm and the UV-vis spectra of the bands corresponding to GLY were recorded using optimized mobile phase. The analysis was repeated in triplicate and GLY content was calculated from the regression analysis equation obtained from calibration curve.

Statistical analysis

The statistical analysis was performed using Microsoft Excel 2003 and Origin 6.0

3. RESULTS AND DISCUSSION

Demand of herbal drugs is periodically increasing now days due to lesser side effects than synthetic drugs. Simultaneously there is less of availability of the authentic herbal drug resources, coupled with lack of knowledge in the identification of the genuine materials

which has further lead to adulteration. Hence standardization of herbs and their formulations need to assess quality and optimum levels of active principles for their bio-potency. For the quality control of extract and formulation, rapid method of quantification is desirable. Recently, the concept of marker based standardization of herbal drugs is gaining more attention for quality control of herbs. Liquorice (Glycyrrhiza glabra) and its extract have been in used in Indian system of medicine since antiquity and recently there is escalation in the demand for conventional therapy for the treatment of acne. However, no attempt has been made to standardize the herbal gel composition containing Glycyrrhiza glabra for the treatment of acne and glycyrrhizin being main ingredient in terms of its active principle or marker compound.

3.1 Physicochemical analysis and phytochemical screening

In the present study, quality control standards were strictly followed as per the Ayurvedic Pharmacopoeia of India and the results of the physicochemical analysis clearly indicated the range which was found similar as mentioned in API. In the present study, it was found that all the values were within the Pharmacopoeial limits (Table 1). Loss on drying indicates the deterioration time of the plant material because it depends upon the amount of water present in the plant material. If the water content is high, the plant gets easily deteriorated due to fungal attack. Ash content of the drug is the residue after incineration and represents inorganic salts naturally occurring in drug or deliberately added to it in the form of adulteration. Total ash of the drug is inclusive of physiological as well as non-physiological ash. Physiological ash is derived from the plant tissues while the non physiological ash consists of the residue of extraneous matter such as sand and soil, adhering to the herb itself. Total ash usually consists of carbonates, oxides, phosphates, silicates and silica. Acid insoluble ash is a part of the total ash insoluble in diluted HCl (API 2001). [22]Dirt and sand present along with the herb may be determined by acid insoluble ash content. Furthermore, determination of water soluble extractive values are important for the medicinal plants as plants contain water soluble active constituents such as tannins, sugars, plant acids, mucilage and glycosides. For alcohol soluble extractives, 95% ethanol is an ideal solvent for extraction of various constituents like tannins and resins. Based on the above results it can be ascertained that the crude drug is an authentic sample and can be used further for formulation purpose.

Isolation of pure, pharmacologically active constituents from plants continues to be a long and tedious process. Several methods are available for the identification, characterization and screening of the active components of plant extracts. Preliminary phytochemical screening of methanolic extract of G. glabra revealed the presence of carbohydrates, saponins, glycoside, flavonoids, terpenes and tannins.

Table 1. Monographic analysis of selected G. glabra

Medicinal plant	Loss on drying %	Total ash	Acid insoluble ash	Alcohol soluble extractives	Water soluble extractive
G. glabra	4.53 ± 0.043	6.73 ± 0.356	0.32 ± 0.098	21.31 ± 0.32	28.26 ± 0.12

3.2 Antibacterial activity of methanolic extract and its formulation

In this study, In vitro disc diffusion assay was carried out for comparison of antimicrobial potential and it was observed that all the strains were sensitive but methanolic extract of G.glabra indicated maximum zone of inhibition against P.acnes when compared to S.aureus and S.epidermidis. It was then formulated as gel composition and further screened for antibacterial activity. Zone of inhibition of formulation was higher in comparison to extract and highest was observed against S.aureus (Table 2).

Table 2: Mean±SEM (mm) of zone of inhibitions of G. glabra using disc diffusion method

Extract	S. aureus (MTCC 96)	S. epidermidis (MTCC 2639)	P. acnes (MTCC *1951)
G. Glabra extract	11.5±0.28	11.8±0.15	13.9±0.15
G. Glabra Gel formulation	13.5±0.058	13.17±0.065	12.1±0.116

3.3 Validation parameters

HPTLC is a simple, rapid and sensitive method and has been found to be advantageous as it enables monitoring of densitograms at different wavelengths. It facilitates automatic application and scanning in situ and can be used to record the UV–vis spectra of the analytes directly from the plates. It further reduces the time and cost of analysis with better resolution and also minimizes exposure, risks and disposal problems of toxic organic effluents, thereby helps in reducing possibilities of environment pollution.

3.3.1 Optimization of Chromatographic condition

TLC procedure was optimized with a view to develop stable chromatographic conditions so as to quantify the phytoconstituents. Initially, Pet ether: Benzene: (3: 5 v/v) was tried as

mobile phase for the resolution of GLY on silica gel HPTLC plates. Rf value was too low so second level was followed. In the second level solvent strength was increased by adding polar solvents. This mobile phase showed good resolution of GLY and is a quick method as the time needed for development of plate was less than half an hour. Previous report demonstrated simultaneous quantification of GLY, glycyrrhetinic acid, apigenin, kaempferol and quercetin from G. glabra at 215 nm. [19] Validated HPTLC method for GLY using ethyl acetate-methanol water- formic acid (15:2:1:1 v/v/v/v) with Rf value 0.34 was also reported. [27] In the present study, Pet ether: Benzene: Ethyl Acetate: Glacial Acetic Acid (4:8:2.8:0.2v/v/v/v) mobile phase was found to give well defined peaks of GLY with Rf value 0.38.

3.3.2 Linearity

This system was found to give compact spots for glycyrrhizin with (Rf value: 0.38 ± 0.02). In the present study, linearity between the peak area and concentration of GLY was found satisfactory in this experimental range as per the ICH guidelines and least-square linear regression analysis exhibited a strong linear relationship having correlation coefficient for GLY (r^2 = 0.99021 ± 7.470210). The mean value of slope and intercept were 0.19479 ± 0.00785 and 28.61462 ± 4.18523 respectively, for GLY. No significant difference was observed in the slope of standard curves (ANOVA, P > 0.001).

3.3.3 Precision

Results indicated high extraction efficiency of GLY from formulation components and showed high precision % R.S.D in range of 0.4047 - 1.958 for Intraday and 0.8288 - 1.5852 for Interday (Table 3). This confirms that the proposed method can be used for determination of GLY in gel formulation developed in our laboratory.

Table 3. Precision data of HPTLC of glycyrrhizin (n=6)

Parameter	Concentration (ng/spot)	Mean Area ± S.D.	% R.S.D.
Instrument precision	200	71.85 ± 0.59	0.83
Intraday	100	42.68167 ± 0.8359	1.958
	200	72.493 ± 0.746	0.7466
	500	133.78 ± 0.541	0.4047
Interday	100	43.43 ± 0.6884	1.5852
	200	73.32 ± 1.665	1.3376
	500	133.95 ± 1.1102	0.8288

S.D.: Standard deviation; R.S.D.: Relative standard deviation

3.3.4 Accuracy, recovery and robustness

The accuracy was calculated from the test results as the percentage of GLY recovered by the assay and results in terms of % recovery of analyte (Table 4) was expressed using formula: observed concentration x 100/theoretical concentration. The recovery of GLY ranged from 97.75 to 100.63%, average of 99.29 ± 1.03 % after zero addition and 98.92 % to 101.75 %, average of 99.96 ± 1.14 % after 50 % addition in methanolic extract. For robustness, average % R.S.D was found to be 0.907 % which shows the robustness of the method.

Table 4. Results of recovery studies of glycyrrhizin (n=3)

Amount added (µg)	Amount recovered ± S.D.	% Recovery ± S.D.
800 (0 %)	794.333 ± 8.238	99.29 ± 1.03
1200 (50 %)	1199.5 ± 13.66	99.96 ± 1.14

S.D.: Standard deviation

3.3.5 HPTLC Analysis of glycyrrhizin in herbal extract and its formulation

Quantification of glycyrrhizin in methanol extract of Glycyrrhiza glabra and its gel formulation was done at 254 nm by spotting 5 µl on precoated plates using optimized mobile phase (Figure 2). In HPTLC profiles, there were many peaks that were not detectable, but the peak of GLY was identified by comparing Rf and UV spectra with that of reference compound, which is responsible for anti-acne activity was well resolved. In the present study, development of HPTLC method enabled the determination of GLY from Glycyrrhiza glabra extract and gel formulation. We focused on thorough validation as per International Conference on Harmonization (ICH) guidelines which enabled monitoring of densitograms of GLY using TLC scanner III (Camag TLC Scanner–3, model No. 027.6480). There was no interfering peaks confirming the selectivity of the method and total glycyrrhizin present in herbal extract and gel formulation (Figure 2) was found to be (7.934 %) and (1.070 %). The contents of GLY in Glycyrrhiza glabra obtained here by HPTLC method are in accordance with the previous reports. Previous reports revealed that presence of GLY confirms its anti bacterial, anti-inflammatory potential in the treatment of acne [5, 9] having effect on adrenal gland, which is ultimately responsible for production of cortisol, human body's own anti-inflammatory adrenal steroid hormone. Hence fingerprinting of the formulation in the form of densitogram could be useful for marker-based quality assurance of the herbal composition containing glycyrrhizin.

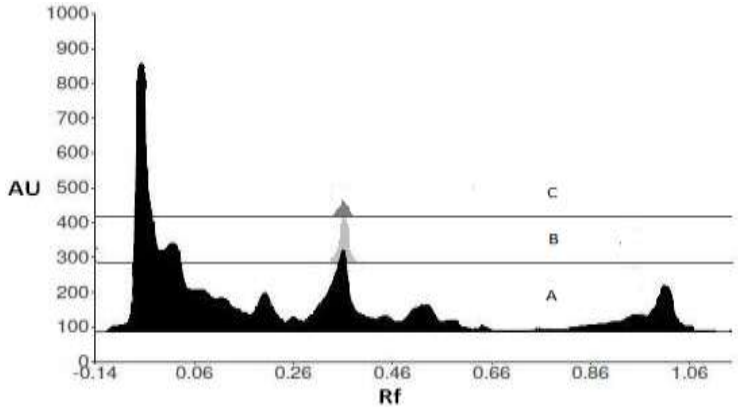

Figure 2. HPTLC densitograms of glycyrrhizin in extract and its gel formulation at 254 nm; AU: Area under the curve; (Rf = 0.38) ; A: chromatogram of extract of G. glabra; B: chromatogram of standard glycyrrhizin; C: chromatogram of herbal gel formulation

4. CONCLUSION

Quantification of bioactives through modern analytical tools is essential for establishing the authenticity of herbal drugs. The present work gives a scientific data for qualitative as well as quantitative analysis or presence of glycyrrhizin by HPTLC method. Glycyrrhizin is a common chemical constituent of G. glabra possessing antibacterial properties and is used as a chemical marker compound in present study. The developed HPTLC method was validated as per ICH guidelines and was found to be linear, accurate and precise Statistical analysis proved that the method is selective for the analysis of glycyrrhizin and reproducible for determination of glycyrrhizin in G. glabra extract and its herbal formulation without any interference from the excipients. It is advantageous being rapid and sensitive method with reduced cost of analysis and better resolution in less time.

CONFLICTS OF INTEREST

All authors have none to declare.

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