Quantification of glycyrrhizin in anti-stress herbal formulations by validate HPTLC method: A rational paradigm towards quality control of herbals

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Abstract: In the present study an analytical method of high-performance thin-layer chromatography (HPTLC) has been developed for quantification of glycyrrhizin for marketed antistress liquorice root capsules (LRC) and herbal tea (HT). Chromatography was performed by using mobile phase ethyl acetate (EA): glacial acetic acid (GAA): Methanol (MeOH): water (H₂O) in proportion of 6:2:2:1, v/v/v/v. The developed plate was scanned and quantified densitometrically at absorption maxima 254nm. The method was validated for various analytical parameters viz. precision, accuracy, recovery, robustness, specificity, detection and quantification limits. The developed system was found to give compact spot for glycyrrhizin (Rf = 0.33±0.001). The linearity relationship was described by the equation Y=6.841X+ 70.428. The limit of detection (34 ng band⁻¹), limit of quantification (101ng band⁻¹), recovery (99.4-99.8%), and precision (≤1.84% and ≤1.62%; intraday and interday, respectively) were found satisfactory for glycyrrhizin. Linearity range for glycyrrhizin was 100-600ng (r²=0.998). The amount of glycyrrhizin was estimated by comparing the peak area of standard and the same was present in crude extract. The content of glycyrrhizin was estimated as 11.4% and 4.7% w/w in sample LRC and HT, respectively. The proposed method will be useful to quantify the therapeutic dose of glycyrrhizin in herbal formulations as well as in bulk drug.

Keywords: Anti-stress herbal capsule, validation, HPTLC, glycyrrhizin, quality control.

INTRODUCTION

Stress is a very commonly used term in our daily life and sometimes it makes the life miserable. Stress plays an important role in exaggeration of many diseases. Stress is one of the major causes of diseases like hypertension, diabetes etc. The term stress was coined by Hans Selye in 1975 (Selye, 1975). The definition of stress may be given as physiological disarray or threat to homeostasis. The positive stress is called ‘Eustress’ and if it is negative known as ‘Distress’. Negative stress causes alteration in the autonomic nervous system, the endocrine system and the immune system of the body (Sumanth and Mustafa, 2007). Stress usually causes activation of HPA (hypothalamic-pituitary-adrenal) axis by some unknown mechanism that leads to stimulation of hypothalamus resulting into discharge of multiple corticotropin (ACTH) secretagogues, corticotropic-releasing hormone (CRH), vasopressin, arginine etc. consequently the enhanced plasma level of cortisol causes ailments such as hypertension, osteoporosis, depression and the development of a wide range of metabolic disorders that may include visceral obesity, insulin resistance and dyslipidemia as well as the kinds of cardiovascular diseases (Rosmond, 2005). There are many herbal drugs and their formulations like Ocimum sanctum leaves, R. damacena root and Panax ginseng roots have been proved their effect as anti-stress (Singh et al., 1991). Some of the herbal drugs are very well known for their adaptogenic, immunomodulatory and anti-stress properties among which Withania somnifera, Panax ginseng, Asparagus racemosus and Picrorhiza kurroa roots etc are prominent (David and Anderson, 2008; Siddiqui et al., 2012).

Glycyrrhizin (GLY), a triterpenoid saponin glycoside from the roots and rhizomes of genus Glycyrrhiza (Licorice, Family-Fabaceae). It is the major bioactive constituent of genus Glycyrrhiza which has been traditionally used in herbal medicine for over 4000 years for curing numerous diseases such as hepatic disorders, stress, allergy, inflammation, spasm, constipation, depression, ulcer, diabetes, dyspepsia, bronchitis, rheumatoid arthritis etc. Glycyrrhiza is widely distributed throughout the certain areas of Asia and Mediterranean region. The ancient literature proves the use of dried rhizomes and roots of liquorice as expectorant and carminative by Egyptian, Greek, Chinese, Indian and Roman civilizations (Afnan et al., 2012; Chandrasekaran et al., 2011; Chan et al., 2011).

It is well known that herbal tea (Camellia sinensis) have many medicinal uses due to their major phytoconstituents i.e., polyphenols. Herbal teas are used as antioxidants,
Quantification of glycyrrhizin in anti-stress herbal formulations by validate HPTLC method

antimicrobials (Johnson et al., 2010) and as chemopreventive agents particularly in case of prostate cancer (Puodziūniene et al., 2004). Now day’s herbal teas are mixed with some other medicinally useful herbs to modulate their effect according to purpose of use. There are many examples of such drinks like throat clearing herbal teas having herbs like glycyrrhiza, sweet fennel, thyme etc. (Stacy, 2011), relaxation drink to relieve the stress which generally contain herbs like valerian, kava etc. that possess tranquilizing, neurocognitive functions and have some role in sleep (Li et al., 2011).

To overcome the side effects of modern stress relieving drugs, herbal are the best choice provided that these herbal formulations should pass the quality control tests. In quality control parameters the actual concentration of biologically active compound is of paramount importance. These days HPTLC is used as conventional analytical technique due to some of its unique features such as high sample throughput, minimum sample requirement, multiple compounds can be analyzed simultaneously by using small volume of mobile phase that makes the process fast and economic as well (Faiyazuddin et al., 2010). The developed HPTLC chromatograms are useful in identification of marker compounds in various herbal formulations by comparing the fingerprints with standard (Siddiqui et al., 2014).

EXPERIMENTAL

Materials
The commercial formulations “Licorice Root Capsules (LRC)” and “Herbal Tea (HT)”, were purchased from Riyadh, Kingdom of Saudi Arabia. These formulations are meant for stress relief and possess glycyrrhizin as major active constituent.

Chemicals and reagents
The standard glycyrrhizin was obtained from Sigma Aldrich, Bayouni Trading Co. Ltd., Al-Khobar, Saudi Arabia. Ethanol, hexane, ethyl acetate, methanol and sulfuric acid were used for performing the experiments. All reagents, chemicals and solvents were of analytical grade, purchased from WINLAB and BDH (U.K.).

Preparation of standard stock solution
Stock solution of glycyrrhizin (1.0mg mL⁻¹) was prepared in methanol and by appropriate dilution it was made to the concentration of 0.1µg/µl. For calibration, glycyrrhizin standard solution (1-6µL) was applied to a HPTLC plate to furnish amounts in the range 100-600ng band⁻¹.

Preparation of Sample from Licorice root capsules (LRC)
Ten capsules of sample weighing 4500mg have been extracted with methanol by ultrasonic method for 1 hour. Thereafter methanol extract was filtered and concentrated under reduced pressure and finally vacuum dried. The yield of the methanol extract was 5.4% w/w.

Preparation of sample from herbal tea (HT)
Herbal tea weighing 4500 mg has been extracted with methanol by ultrasonic method for 1 hour. Thereafter methanol extract was filtered and concentrated under reduced pressure and finally vacuum dried. The yield of the methanol extract was 3.5% w/w.

Instrumentation and chromatographic conditions
Chromatography was performed, as described by researcher (Faiyazuddin et al., 2010) on 10cm × 20 cm glass HPTLC plates precoated with 200µm layers of silica gel 60F254. Samples were applied as 6 mm wide and 8 mm apart bands by means of Camag Linomat IV sample applicator equipped with a 25-µL syringe. The constant application rate was 160nL S⁻¹. Linear ascending development with solvents EA: GAA: MeOH: H₂O (6: 2: 2: 1, v/v/v/v) as mobile phase was performed in a 10 cm × 20 cm twin-trough glass chamber (Camag) previously saturated with mobile phase for 20min at room temperature (25±2°C) and relative humidity 60%±5%. The development distance was 7.2 cm (development time 10 min) and 10mL mobile phase was used. The plates were dried at room temperature and then heated to identify compact bands. Densitometric analysis was performed at 254 nm in absorbance/reflectance mode with a Camag TLC scanner III operated by Win CATS 4 software (Version 1.2.0). The slit dimensions were 5 mm × 0.45 mm and the scanning speed of 20 mm S⁻¹.

Preparation of calibration graphs
Calibration graphs for standard glycyrrhizin were prepared by applying a series of spots of standard with six different volumes so as to get different amount of glycyrrhizin per spot. They were prepared with respect to height and area vs amount per spot.

Validation procedure
Validation of the developed method has been carried out as per ICH guidelines (ICH, 1996) for linearity, precision, accuracy, limits of detection (LOD) and quantification (LOQ), recovery studies and robustness.

Linearity range
For determining the linearity range of standard glycyrrhizin, a series of spots of standard of different volumes (1µl-6µl) were applied so as to get different quantity of glycyrrhizin per spot. The plate was scanned and a curve was prepared with respect to height and area vs amount per spot.

Precision and accuracy
Precision (inter and intraday) and accuracy of the assay were evaluated by performing replicate analyses (n=6) of samples at three quality control levels. Inter-day precision and accuracy were determined by repeating the intra-day
assay on three different days. Precision was expressed as the coefficient of variation (CV, %) of measured concentrations for each calibration level whereas accuracy was expressed as percentage recovery ((Drug found/drug applied) × 100).

**Limit of detection and limit of quantification**

Limit of detection (LOD) and limit of quantification (LOQ) values for glycyrrhizin were determined based on the standard deviation of the response and the slope as per ICH guideline (ICH, 1996). They were calculated based on the following equations:

\[
\text{Detection Limit} = \frac{3.3\sigma}{S},
\]

\[
\text{Quantification Limit} = \frac{10\sigma}{S}.
\]

Where \(\sigma\) = Standard deviation of the response
\(S\) = Slope of the calibration curve
\(\sigma\) was determined from the response of a number of blank samples.

**Recovery studies**

Recovery was studied by applying the method to drug samples to which known amounts of marker corresponding to 50%, 100% and 150% of the glycyrrhizin had been added. Each level was analyzed in triplicates. Recovery of the marker at different levels was determined.

**Robustness**

Robustness was studied in triplicate at 300 ng band\(^{-1}\) by making small changes to mobile phase composition, mobile phase volume, and duration of mobile phase saturation and activation of TLC plates, the effect on the results were examined by calculation of RSD (%) and SE of peak areas. Mobile phases prepared from Ethyl acetate: Glacial acetic acid: Methanol: H\(_2\)O (6:2:2:1, v/v/v/v) in different proportions (6:2:2:0.5, v/v/v/v; 6: 2: 1: 1, v/v/v/v; 6: 3: 2: 1, v/v/v/v) were used for chromatography. Mobile phase volume and duration of saturation investigated were 20±2 mL (18, 20, and 22 mL) and 20±10 min (10, 20, and 30 min), respectively. The plates were activated at 105°C for 30 min before chromatography.

**Assay**

Standard glycyrrhizin and test solutions were spotted on HPTLC plate. The percentage of glycyrrhizin present in LRC and HT test solution was determined by measuring area for the standard and test solutions. Thereby the percentage of glycyrrhizin was calculated for LRC and HT.

**RESULTS**

Chromatogram was developed for standard glycyrrhizin using Ethyl acetate: Glacial acetic acid: Methanol: H\(_2\)O (6:2:2:1, v/v/v/v) as mobile phase. The same mobile phase has been also employed for the separation of components in methanol extracts of sample LRT and HT (fig. 1). UV spectra measured for the spots showed maximum absorbance at 254 nm in the absorbance mode. Compact bands as sharp, symmetrical and with high resolution were obtained at R\(_f\) 0.33±0.001 (fig. 2). The method developed here was found to be quite selective with good baseline resolution. The identity of the bands of marker compound in the sample extracts were confirmed by overlaying their absorption spectra with those of the standard at 254 nm (fig. 3).

![Glycyrrhizin Std](image1)

**Fig. 1:** HPTLC plate with mobile phase Ethyl acetate: Glacial acetic acid: Methanol: Water (6:2:2:1, v/v/v/v) scanned at 254 nm.

![Glycyrrhizin Std](image2)

**Fig. 2:** HPTLC chromatogram of standard glycyrrhizin with mobile phase Ethyl acetate: Glacial acetic acid: Methanol: Water (6:2:2:1, v/v/v/v) scanned at 254 nm.
Quantification of glycyrrhizin in anti-stress herbal formulations by validate HPTLC method

and ≤1.62%; intraday and interday, respectively) were found satisfactory for glycyrrhizin. Both intraday and interday precision expressed in table 2 was determined in terms of percent of coefficient variation (% CV). Intra-day and interday precisions (n=6) for glycyrrhizin were found to be 1.66-1.84% and 1.46-1.62%, respectively, which exhibited good precision of proposed method. However, intra-day and interday accuracy of glycyrrhizin were observed as 99.2-99.7%, and 98.9-99.6%, respectively. These results indicated the accuracy of the proposed method. The standard deviation (SD) and % relative standard deviation (RSD) were also calculated at 300ng band⁻¹ concentration level of glycyrrhizin. The low value of SD and % RSD obtained after making slight intentional changes in the method proves the robustness of the proposed method (table 3).

Fig. 3: Overlaying of absorption spectra of LRC and HT with standard glycyrrhizin with mobile phase Ethyl acetate: Glacial acetic acid: Methanol: Water (6:2:2:1, v/v/v/v) scanned at 254 nm

Table 1: Rₖ, linear regression data for the calibration curve and sensitivity parameter for glycyrrhizin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glycyrrhizin</th>
<th>Rₖ</th>
<th>Correlation coefficient</th>
<th>Slope±sd</th>
<th>Intercept±sd</th>
<th>Standard error of slope</th>
<th>Standard error of intercept</th>
<th>LOD</th>
<th>LOQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (ng band⁻¹)</td>
<td>0.33±0.001</td>
<td>100-1000</td>
<td>r=0.9980</td>
<td>6.841±0.003</td>
<td>70.428±0.005</td>
<td>0.001</td>
<td>0.001</td>
<td>34</td>
<td>101</td>
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<tr>
<td>Regression equation</td>
<td>Y=6.841X+70.428</td>
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<tr>
<td>Mobile phase volume (18, 20 and 22 mL)</td>
<td></td>
<td></td>
<td></td>
<td>3.85</td>
<td>0.011</td>
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<tr>
<td>Duration of saturation (10, 20 and 30 min.)</td>
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<td></td>
<td></td>
<td>4.21</td>
<td>0.016</td>
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<tr>
<td>Activation of TLC plate (2, 5 and 7 min)</td>
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<td></td>
<td>4.48</td>
<td>0.020</td>
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</table>

CONCLUSION

The developed method can be used for the estimation of glycyrrhizin in bulk drug as well as in marketed formulations of liquorice such as Candy, herbal cough mixtures, herbal lozenges etc. Routine analysis for quality control of material can be easily evaluated by the proposed method.

ACKNOWLEDGEMENT

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project No. RG-1435-053.
Table 4: Recovery studies of glycyrrhizin

<table>
<thead>
<tr>
<th>Concentration added to analyte (%)</th>
<th>Theoretical (ng)</th>
<th>Added (ng)</th>
<th>Detected (ng)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
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<tr>
<td>50</td>
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<td>450</td>
<td>447.4</td>
<td>99.4</td>
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<td>100</td>
<td>1000</td>
<td>600</td>
<td>597.6</td>
<td>99.6</td>
<td>1.22</td>
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<td>150</td>
<td>1500</td>
<td>750</td>
<td>748.2</td>
<td>99.8</td>
<td>1.10</td>
</tr>
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REFERENCES


