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 (H_2O) 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 (R_f = 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 (r^2=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.

INTRODUTION

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 disharmony 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, corticotropin-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,

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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, herbals 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: H_2O (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:

$$\text{Detection Limit} = \left(\frac{3.3\sigma}{S}\right)$$

$$\text{Quantification Limit} = \left(\frac{10\,\sigma}{S}\right)$$

Where σ = Standard deviation of the response
S = Slope of the calibration curve
σ 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 300ng 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_2O (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±2mL (18, 20, and 22mL) and 20±10 min (10, 20, and 30 min), respectively. The plates were activated at 105ºC 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_2O (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).

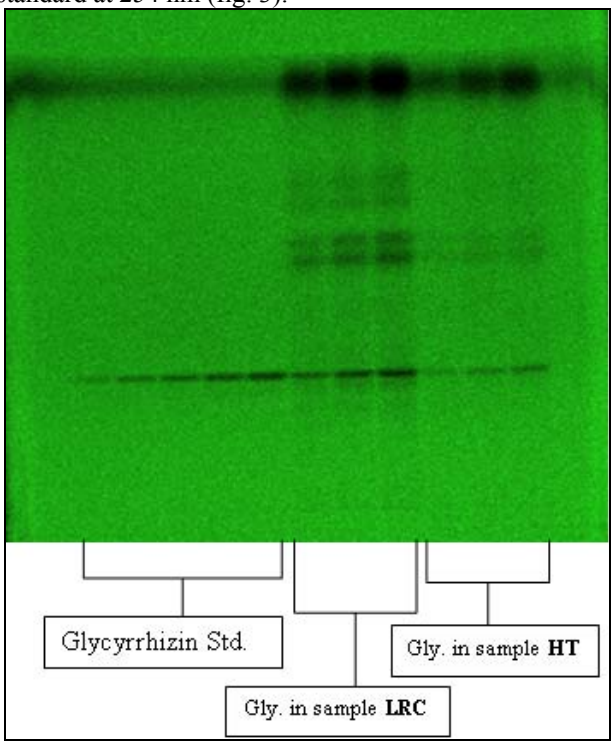

Fig. 1: HPTLC plate with mobile phase Ethyl acetate: Glacial acetic acid: Methanol: Water (6:2:2:1, v/v/v/v) scanned at 254 nm.

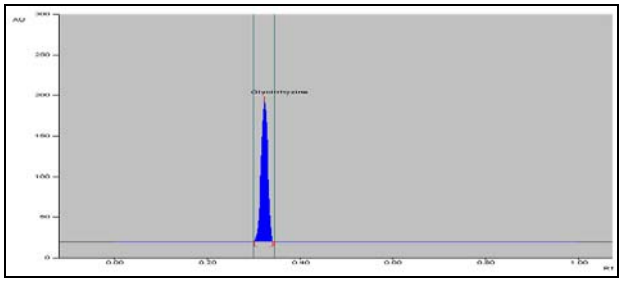

Fig. 2: HPTLC chromatogram of standard glycyrrhizin with mobile phase Ethyl acetate: Glacial acetic acid: Methanol: Water (6:2:2:1, v/v/v/v) scanned at 254 nm

The linearity range for glycyrrhizin was found to be between 100-600ng/spot. Calibration curve was described by the equation Y=6.841X+ 70.428 with r^2=0.998 and a standard deviation of 1.11% (table 1). The limit of detection (34ng 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. 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).

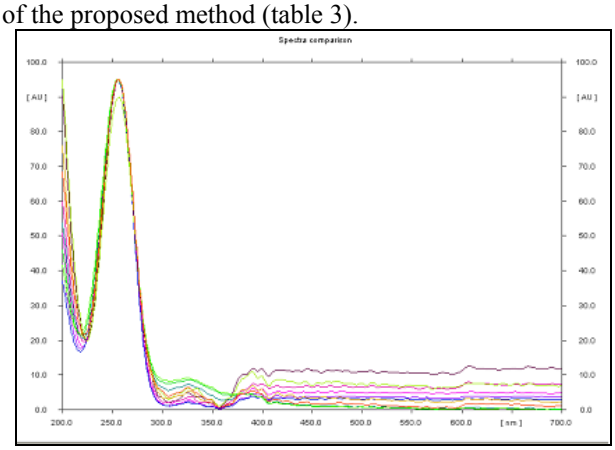

Fig. 3: Overlaying of absorption spectra of LRC and HT with standard glycyrrhizin with mobile phase Ethyl acetate: Glacial acetic acid: Methanol: Water (6:2:2:1, v/v/v/v) scanned at 254 nm

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

Parameter	Glycyrrhizin
R_f	0.33±0.001
Linearity range (ng band^{-1})	100-1000
Regression equation	Y=6.841X+70.428
Correlation coefficient	r^2=0.9980
Slope±sd	6.841±0.003
Intercept±sd	70.428±0.005
Standard error of slope	0.001
Standard error of intercept	0.001
LOD	34 ng
LOQ	101 ng

It is evident from the results that the percent recoveries for glycyrrhizin after sample processing and applying were in the range of 99.4-99.8% as shown in table 4.

DISCUSSION

The content of glycyrrhizin was estimated in the methanol extract samples of LRC and HT by the proposed method was 11.4% w/w and 4.7% w/w respectively. It is a very sensitive HPTLC method, which will be very useful for the quantification of bioactive marker glycyrrhizin in different anti-stress herbal formulations and other formulation containing glycyrrhizin as active constituent. In this study we have attempted to develop and validate a cost effective simple HPTLC technique to quantify bioactive marker components in the above-mentioned marketed formulations. The difference of glycyrrhizin content in two samples indicates the dose of glycyrrhizin required in our body for two different purposes. LRC can be used for moderate to severe state of depression while HT can be taken as refreshing drink.

Table 2: Intraday and interday precision and accuracy

Nominal Concentration (a)	Glycyrrhizin Obtained (b)	Precision (c)	Accuracy (d)
Intraday batch			
150	148.8	1.84	99.2
300	298.2	1.77	99.4
600	598.3	1.66	99.7
Interday batch			
150	148.4	1.62	98.9
300	297.8	1.52	99.2
600	597.6	1.46	99.6

Table 3: Robustness of the method

Optimization condition	Glycyrrhizin	
	SD	%RSD
Mobile phase EA: GAA: MeOH: H_2O (6:2:2:1, v/v/v/v; 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)	4.52	0.020
Mobile phase volume (18, 20 and 22 mL)	3.85	0.011
Duration of saturation (10, 20 and 30 min.)	4.21	0.016
Activation of TLC plate (2, 5 and 7 min)	4.48	0.020

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.

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Table 4: Recovery studies of glycyrrhizin

Concentration added to analyte (%)	Theoretical (ng)	Added (ng)	Detected (ng)	Recovery (%)	RSD (%)
	300				
50		450	447.4	99.4	1.48
100		600	597.6	99.6	1.22
150		750	748.2	99.8	1.10

REFERENCES

Afnan Q, Adil MD, Nissar-Ul A, Rafiq AR, Amir HF, Kaiser P, Gupta VK, Vishwakarma R and Tasduq SA (2012). Glycyrrhizic acid (GA), a triterpenoid saponin glycoside alleviates ultraviolet-B irradiation-induced photoaging in human dermal fibroblasts. *Phytomedicine,* **19**(7): 658-664.

Chan EW, Soh EY, Tie PP and Law YP (2011). Antioxidant and antibacterial properties of green, black, and herbal teas of Camellia sinensis. *Pharmacognosy Res.,* **3**(4): 266-272.

Chandrasekaran CV, Sundarajan K, Gupta A, Srikanth HS, Edwin J and Agarwal A (2011). Evaluation of the genotoxic potential of standardized extract of Glycyrrhiza glabra (GutGard™). *Regul. Toxicol. Pharmacol*., **61**(3): 373-380.

David C and Anderson DC (2008). Assessment and nutraceutical management of stress-induced adrenal dysfunction. *Integrative Medicine*, **7**(5): 18-25.

Faiyazuddin M, Ahmad N, Baboota S, Ali J, Ahmad S and Akhtar J (2010a). Chromatographic analysis of trans and cis-Citral in lemongrass oil and in a topical Phytonanocosmeceutical formulation and validation of the Method. *J. Planar Chrom*., **23**: 233-236.

ICH Harmonised Tripartite Guideline (2005). Validation of analytical procedures: Text and methodology, Q2 (R1). International conference on harmonization of technical requirements for registration of pharmaceuticals for human use, Geneva. Pp. 6-13.

Johnson JJ, Bailey HH and Mukhtar H (2010). Green tea polyphenols for prostate cancer chemo prevention: A translational perspective. *Phytomedicine*, **17**(1): 3-13.

Li YJ, Chen J, Li Y, Li Q, Zheng YF, Fu Y and Li P (2011). Screening and characterization of natural antioxidants in four *Glycyrrhiza* species by liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry *J. Chromatogr. A.*, **1218**(45): 8181-81 91.

Puodziūniene G, Janulis V, Milasius A and Budnikas M (2004). Development of throat clearing herbal teas. *Medicina (Kaunas)*, **40**(8): 762-767.

Rosmond R (2005). Role of stress in the pathogenesis of the metabolic syndrome. *Psychoneuroendocrinology*, **30**(1): 1-10.

Selye H (1975). Confusion and controversy in the stress Field. *J. Human Stress*, **1**(2): 37-44.

Siddiqui NA, Alam P, Khan AA, Ahmad A, Al Rehaily AJ and Alanazi AM (2014). Quantification of physiologically available glycyrrhizin in anti-stress herbal formulations by validated HPTLC method. *Asian J. of Chem*., **26**(3): 874-878.

Siddiqui NA**,** Singh S, Siddiquei MM and Khan TH (2012). Immunomodulatory effect of *Withania somnifera, Asparagus racemosus* and *Picrorhiza kurroa* roots. *Int. J. of Pharmacol.,* **8**(2): 108-114.

Singh V, Singh A, Nath R, Mishra N, Dixit KS and Singh N (1991). Effect of some Anti-stress plant drugs on the intestinal transit. *J. Biol. Chem. Res*., **10**(4): 601-602.

Stacy S (2011). Relaxation drinks and their use in adolescents. *J. Child Adolesc. Psychopharmacol.*, **21**(6): 605-610.

Sumanth M and Mustafa SS (2007). Antistress, adoptogenic and immunopotentiating activity roots of *Boerhaavia diffusa* in Mice. *Int. J. Pharmacol*., **3**(5): 416-420.