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Abstract

Background: A simple and sensitive thin-layer chromatographic method has been established for quantification of glycyrrhizin in *Glycyrrhiza glabra* rhizome and baby herbal formulations by validated Reverse Phase HPTLC method.

Materials and Methods: RP-HPTLC Method was carried out using glass coated with RP-18 silica gel 60 F254S HPTLC plates using methanol-water (7: 3 v/v) as mobile phase.

Results: The developed plate was scanned and quantified densitometrically at 256 nm. Glycyrrhizin peaks from *Glycyrrhiza glabra* rhizome and baby herbal formulations were identified by comparing their single spot at $R_f = 0.63 \pm 0.01$. Linear regression analysis revealed a good linear relationship between peak area and amount of glycyrrhizin in the range of 2000-7000 ng/band.

Conclusion: The method was validated, in accordance with ICH guidelines for precision, accuracy, and robustness. The proposed method will be useful to enumerate the therapeutic dose of glycyrrhizin in herbal formulations as well as in bulk drug.

Key words: Glycyrrhizin; HPTLC densitometry; ICH guidelines; Qualitative; Quantitative

Introduction

The name of the Genus *Glycyrrhiza* derives from the Greek *glykys*, for "sweet", and *rhiza*, for "root" (Henry George Liddell, Robert Scott, *A Greek-English Lexicon*, on Perseus). Licorice rhizomes were used during the time of the Roman Empire and were also used in the Chinese herbal medicine. The ancient greek scientist 'Theophrastus' describes the plant for asthma and wound healing. Medicinally, it has been used for the treatment of dropsy; fever; menstrual cramps; menopause symptoms; irritated urinary, bowel, or respiratory passages; influenza; and hypoglycemia. It has also been used as a diuretic, demulcent, expectorant, emollient, antispasmodic, mild laxative, and cough remedy (Kowalchik and Hylton, 1987). The main ingredient in the rhizome of *G. glabra* is glycyrrhizin. Glycyrrhizin (or glycyrrhizic acid or glycyrrhizinic acid) is the chief sweet-tasting constituent of *Glycyrrhiza glabra* (liquorice) rhizome. Structurally it is a saponin and has been used as an emulsifier and gel-forming agent in food stuff and cosmetics. It is more than fifty times sweeter than sugar.

The anti-inflammatory effect of glycyrrhizin may be due to its anti-thrombin properties (Francischetti et al., 1997; Hasanzadeh et al., 2010). The main side effects of licorice include: oedema, hypokalaemia, and hypertension (Olukogo et al., 2000; Armanini et al., 2002) make its use for elder is very limited. However, these side effects are not expected to occur in infants. In the market, many baby relief and antispasmodic products contain mixtures of herbs including fennel, caraway, dill, anise as well as licorice due to its effect and sweet taste (Khalil et al., 2000).

High performance thin layer chromatography (HPTLC) was emerged as analytical approach for the standardization of herbal drugs as it is less expensive and highly efficient (Alqasoumi et al., 2011). In addition to HPLC methods (Yang et al., 2011; Yang et al., 2012; Gupta et al., 2012; Seo et al., 2011; Seo et al., 2012)) there are so many HPTLC methods were developed using normal phase Silica gel plate (Gantait et al, 2010; Alam et al, 2014; Chauhan et al., 1998; Singh et al., 2009; Varsha et al., 2009; Gantait et al., 2010). In most of the methods used of mobile phase containing glacial acetic acid a solvent not handy in use. In HPTLC, many samples could be run simultaneously with less expensive mobile phase consequently leads to time and cost saving (Faisal et al., 2009; Alam et al., 2011).

To the best of our knowledge, no reports on quantification of glycyrrhizin in *Glycyrrhiza glabra* rhizome and baby herbal formulations utilizing the reversed-phase silica gel plates have been mentioned in the literature. In this work,

we developed a validated precise and accurate HPTLC method for the analyses of licorice rhizomes extract, baby herbal mixtures containing Licorice extracts or powders utilizing RP18 silica gel plates. The proposed method was validated as per ICH guidelines (1996).

Experimental

Standard and chemicals

Standard glycyrrhizin was purchased from Sigma-Aldrich, St. Louis, MO, USA. All the solvents were of HPLC grade and other chemicals used were of analytical reagent (AR) grade.

Preparation of standard solutions

Accurately weighed 10 mg of standard glycyrrhizin was initially dissolved in sufficient amount of distilled water and volume was made up to 10 ml with methanol in a volumetric flask to gives concentration of 1000 µg/mL. This solution was used as a reference solution for glycyrrhizin.

Plant material and baby herbal formulations

The *Glycyrrhiza glabra* rhizome (Crude drug) and baby herbal formulation were purchased from the local market at Al-Kharj city, Saudi Arabia and Alexandria, Egypt. The plant was identified by Taxonomist at the Research Center of Medicinal, Aromatic and Poisonous plants, a voucher specimen (#15618) was deposited in the Herbarium of the Department of Pharmacognosy, College of Pharmacy, Prince Sattam bin Abdulaziz University for future reference.

Extractions procedure

The dried powdered licorice (10 g) was extracted by percolation at room temperature with MeOH (4X70 ml) till exhaustion. The solvents were evaporated under reduced pressure and the residues were dissolved in methanol using 50 mL volumetric flask. These solutions were used as the test solutions in the TLC densitometric analysis.

Sample preparation for analysis of glycyrrhizin in baby drink herbal formulations

Accurately weighed 10 g from each product was separately extracted with methanol (3×70 mL) for 30 min. The methanol extracts from each sample were combined and separately concentrated to dryness under reduced pressure using a rotary vacuum evaporator. The residues of each sample were separately reconstituted in accurately measured 50 mL of methanol and stored under refrigeration until TLC analysis.

Chromatographic conditions

HPTLC densitometric analysis was performed on 10×20 cm glass-backed plates coated with 0.2 mm layers of RP-18 silica gel 60 F254S (E-Merck, Germany). Samples were applied to the TLC plates as 6 mm bands using a Camag Automatic TLC Sampler 4 (ATS4) sample applicator (Switzerland) fitted with a Camag microlitre syringe. A constant application rate of 150 nl/s was used. Linear ascending development of the plates to a distance of 80 mm was performed with methanol-water 7:3 (%), v/v as mobile phase in a Camag Automatic Developing Chamber 2 (ADC2) previously saturated with mobile phase vapour for 30 min at 22 °C.

Method Validation

The proposed HPTLC method was validated according to the guidelines of international conference on harmonization (ICH) [19]. The linearity of the method for glycyrrhizin was checked between 2000 and 7000 ng/spot and concentration was plotted against peak area.

Accuracy

Accuracy, as recovery, was determined by the standard addition method. Pre-analyzed samples of glycyrrhizin (3000 ng/spot) were spiked with extra glycyrrhizin standard (0, 50, 100, and 150%) and the mixtures were reanalyzed. Percentage recovery and relative standard deviation (RSD, %) were calculated for each concentration level.

Precision

Precision was assessed by determination of repeatability and intermediate precision. Repeatability of sample was determined as intra-day variation whereas intermediate precision was determined by assessment of inter-day variation for analysis of glycyrrhizin at three different amounts (300, 400, and 500 ng/spot) in six replicate.

Robustness

Robustness of the proposed TLC densitometric method was determined to evaluate the influence of small deliberate changes in the chromatographic conditions like small changes to mobile phase composition, mobile phase volume, duration of mobile phase saturation and activation of HPTLC plates during the determination of glycyrrhizin.

Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection (LOD) and limit of quantification (LOQ) were determined by standard deviation (SD) method. They were determined from the slope of the calibration (S) curve and SD of the blank sample using following equations:

$$\text{LOD} = 3.3 \times \text{SD} / S$$

$$\text{LOQ} = 10 \times \text{SD} / S$$

The standard deviation of the response was determined based on the standard deviation of y-intercepts of regression lines.
Specificity

Specificity of the proposed TLC densitometric was confirmed by analyzing and comparing the R_f values and spectra of the spot for glycyrrhizin in the samples with that of the standards.

Quantification of glycyrrhizin in *Glycyrrhiza glabra* rhizome and baby herbal formulations

The test samples were applied and chromatograms were obtained under the same conditions as for analysis of standard glycyrrhizin. The area of the peak corresponding to the R_f value of glycyrrhizin standard was recorded and the amount present was calculated from the regression equation obtained from the calibration plot.

Results and Discussion

Method development

The mobile phase composition was optimized to establish a suitable and accurate densitometric HPTLC method for analysis of glycyrrhizin. The mobile phase methanol-water 7:3 (% v/v) resulted in a compact, symmetrical, and well resolved peak at R_f value of 0.63 ± 0.01 (Figure 2). UV spectra measured for the bands showed maximum absorbance at approximately 256 nm.

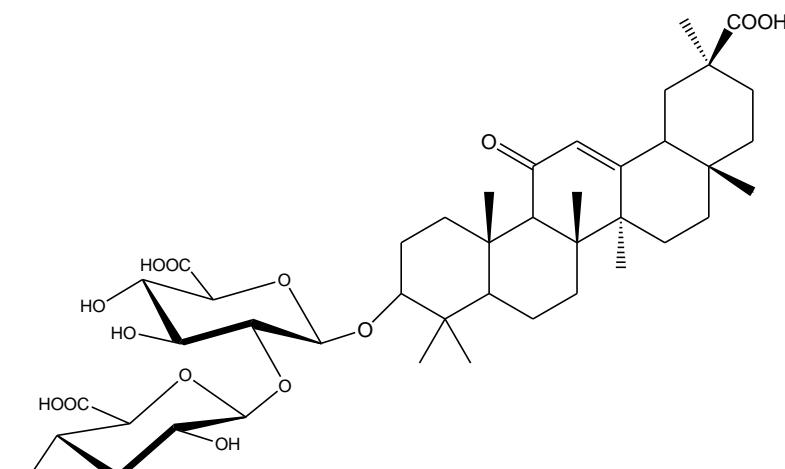


Figure 1: Structure of Glycyrrhizin

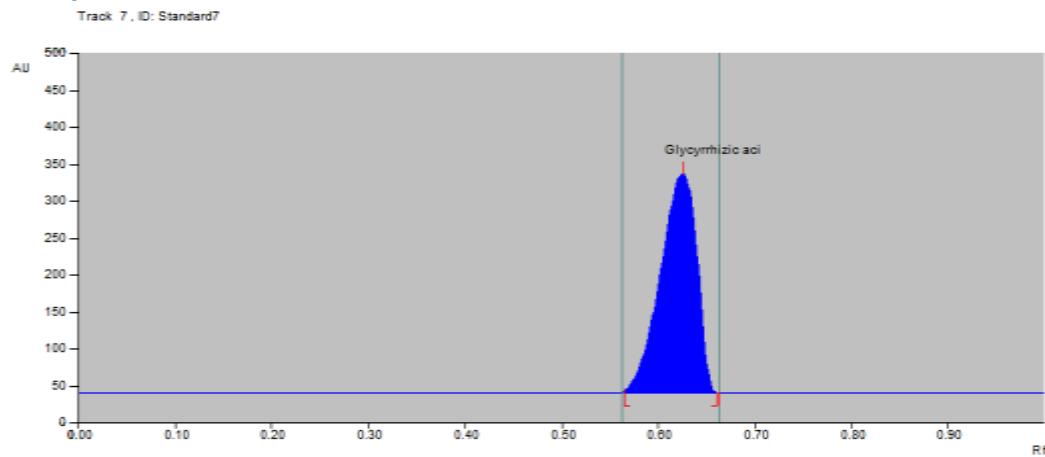


Figure 2: HPTLC densitogram of standard Glycyrrhizin

Method validation

The calibration plot of peak area against amount of glycyrrhizin was linear in the range 2000-7000 ng/spot. Linear regression data for the plot confirmed the good linear relationship (Table 1). The correlation coefficient (R^2) was 0.9986 which was highly significant ($P<0.05$). The linear regression equation was $Y = 1.1722x + 1252.2$, where Y represents the UV absorption while X is the concentration of glycyrrhizin (Figure 2). Accuracy was expressed as percentage recovery. The accuracy of the method, as recovery, was 98.87-99.50%, with RSD values in the range 0.84-1.37. These results indicated the accuracy of the method (Table 2). Precision was expressed as percentage coefficient of variation (% CV) of measured concentrations for each calibration level. Results from determination of repeatability and intermediate precision, expressed as SD (%) are shown in Table 3. RSD was in the range 0.40-0.54 for repeatability and 0.47-0.61 for intermediate precision. These low values indicated that the method is precise. Results of robustness are shown in Table 4. Low values of % RSD (0.55-0.81) were obtained after introducing small deliberate change into the densitometric TLC procedure proved the robustness of the proposed HPTLC method. LOD and LOQ of the proposed method was found to be 7.89 and 21.25 ng/spot, for glycyrrhizin, which indicated that the proposed method can be used in wide range for detection and quantification of glycyrrhizin effectively. The peak purity of glycyrrhizin was assessed by comparing the overlaid spectra at peak start, peak apex and peak end position of the spot. The overlaid spectra of glycyrrhizin standards and baby herbal formulations were given in Figure 7.

Table 1: Linear regression data for the calibration curve of glycyrrhizin (n=6)

Linearity range (ng/spot)	2000-7000
Regression equation	$Y = 1.1722x + 1262.2$
Correlation coefficient	0.9986
Slope \pm SD	1.1722 ± 0.0529
Intercept \pm SD	1657.5 ± 254.69
Standard error of slope	0.02162
Standard error of intercept	104
95% confidence interval of slope	1.113 – 1.234
95% confidence interval of intercept	960- 1538

Table 2: Accuracy of the proposed method (n=6)

Excess drug added to analyte (%)	Theoretical content (ng)	Conc. found (ng) \pm SD	% Recovery	% RSD
0	3000	2966.00 ± 40.60	98.87	1.37
50	4500	4477.50 ± 42.08	99.50	0.94
100	6000	5943.05 ± 56.83	99.05	0.96
150	7500	7441.50 ± 62.49	99.22	0.84

Table 3: Precision of the proposed method

Conc. (ng/spot)	Repeatability (Intraday precision)			Intermediate precision (Interday)		
	Area ± SD (n = 6)	Standard error	% RSD	Area ± SD (n = 6)	Standard error	% RSD
3000	4823 ± 19	7.96	0.40	4823 ± 23	9.30	0.47
4000	5942 ± 25	10.35	0.43	5949 ± 30	12.24	0.50
5000	7145 ± 38	15.63	0.54	7142 ± 44	17.77	0.61

Table 4: Robustness of the proposed HPTLC method

Conc. (ng/spot)	Mobile phase composition (methanol: water)		Results			
	Original	Used	Area ± SD (n = 6)	% RSD	R _f	
4000	7:3	6.9:3.1	+0.1	5947 ± 33	0.55	0.62
		7:3	0.0	5944 ± 37	0.62	0.63
		7.1:2.9	-0.1	5945 ± 48	0.81	0.64

Table 5: Contents of glycyrrhizin in *Glycyrrhiza glabra* rhizome and baby herbal formulations

Samples	Contents Mean ± SD (% w/w)	% RSD
<i>Glycyrrhiza glabra</i> rhizome	0.729 ± 2.34	4.11
Baby drink	0.065 ± 2.56	5.56
Baby cute	0.053 ± 2.53	3.67
Sekum cough	0.025 ± 1.78	5.87

Quantification of glycyrrhizin in *Glycyrrhiza glabra* rhizomes and baby herbal formulations

Glycyrrhizin peaks from *Glycyrrhiza glabra* rhizome and baby herbal formulations were identified by comparing their single spot at $R_f = 0.63 \pm 0.01$ (Figure 3-6) values with those obtained by chromatography of the standard under the same conditions. The glycyrrhizin content in methanol extracts of *Glycyrrhiza glabra* rhizome and baby herbal formulations was quantified by use of the linear regression equation and concentrations are presented in Table 5. The amount of glycyrrhizin in the three formulations reflects the percentage of licorice. Baby drink contained the highest amount of licorice (8.9%) followed by baby cute (7.3%), while Sekum cough has the lowest amount of licorice (3.4%).

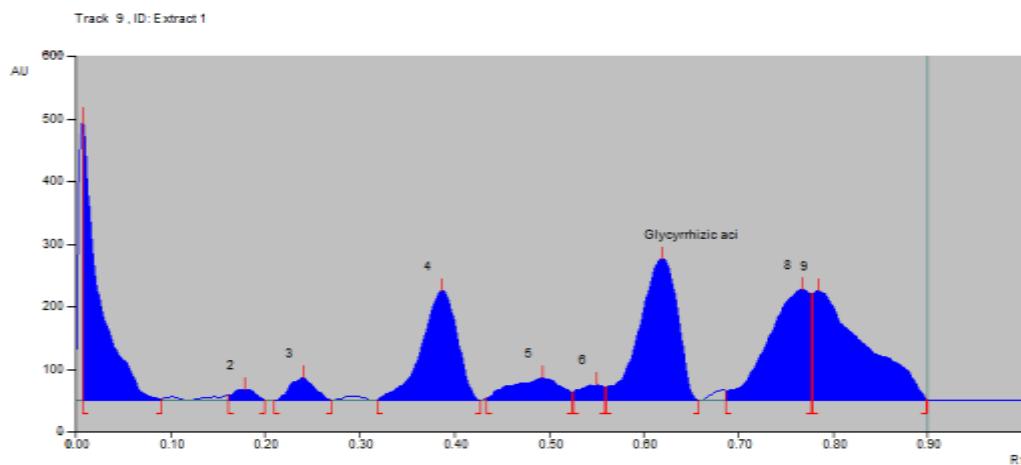


Figure 3: HPTLC densitogram of *Glycyrrhiza glabra* rhizome extract.

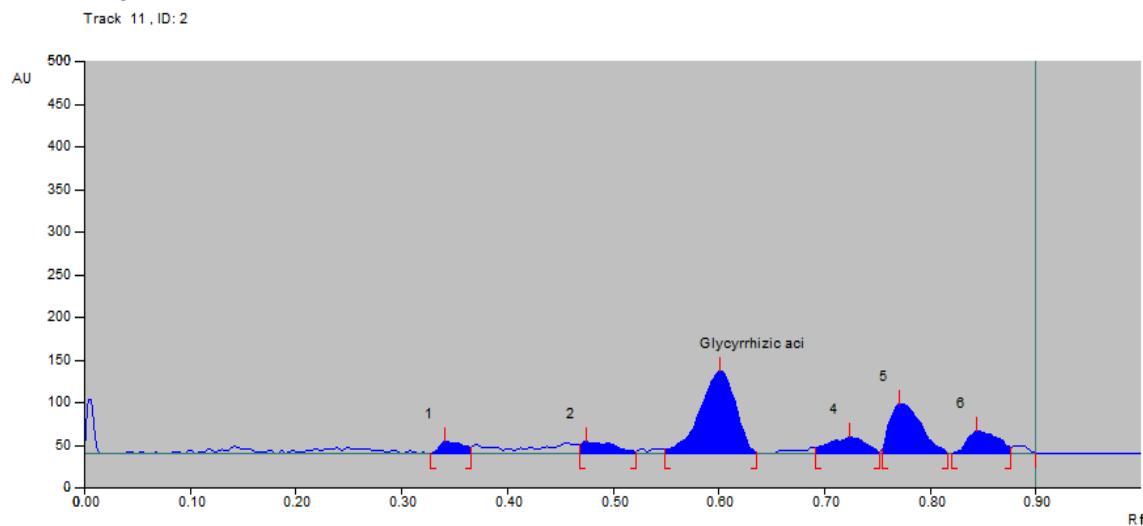


Figure 4: HPTLC densitogram of formulation baby drink

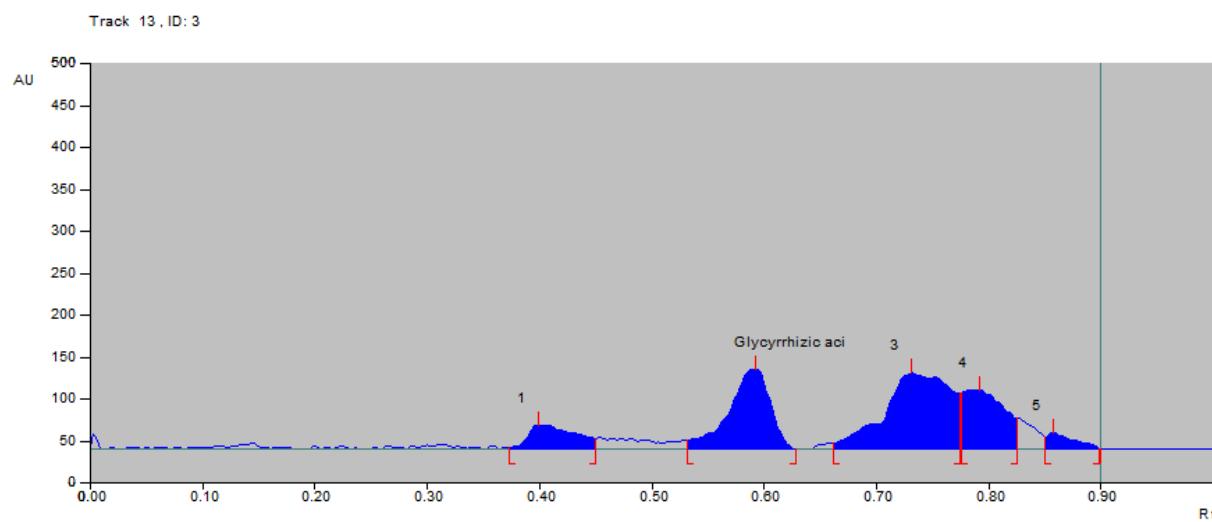


Figure 5: HPTLC densitogram of formulation baby corte

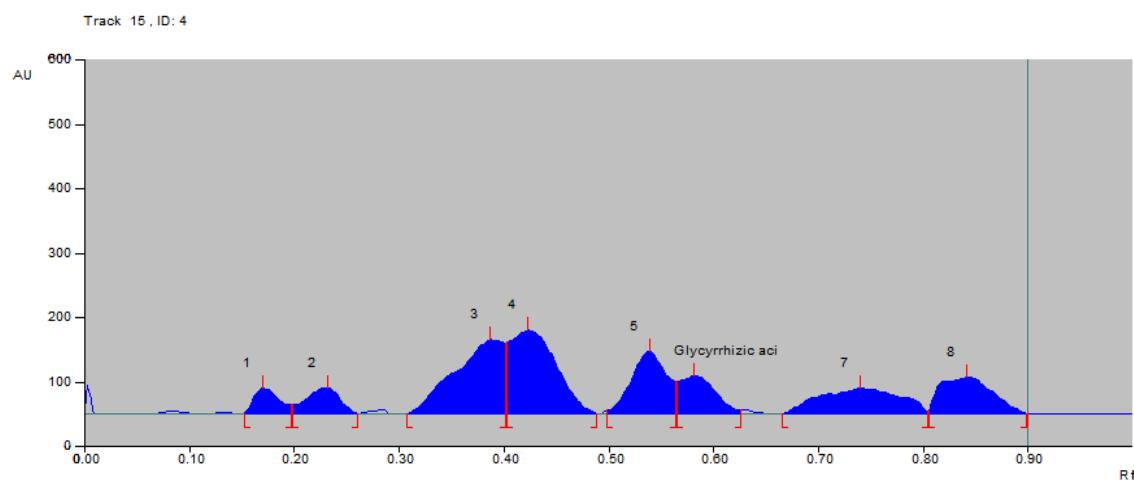


Figure 6: HPTLC densitogram of formulation sekum cough

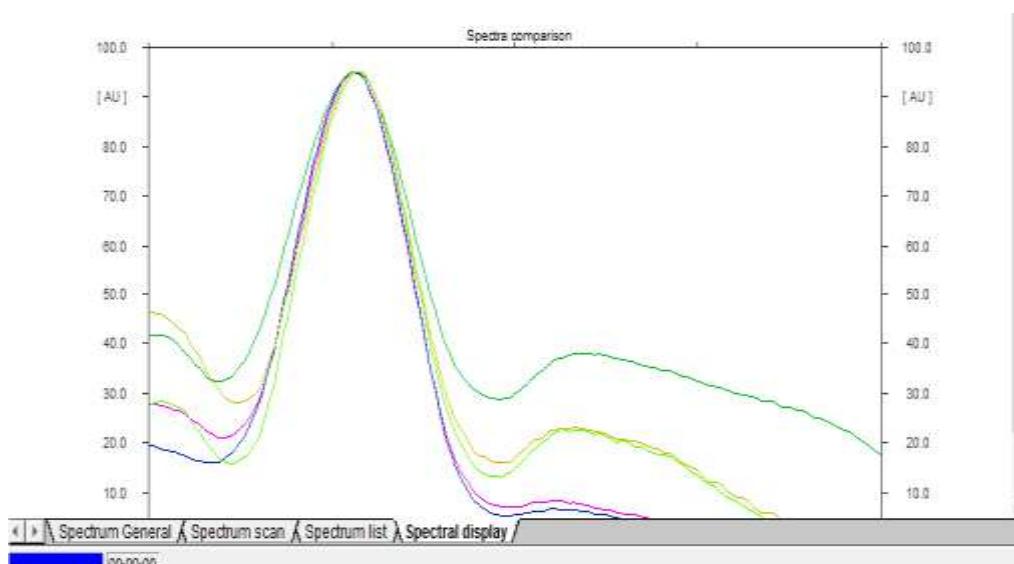


Figure 7: Overlay UV absorption spectra of standard Glycyrrhizin and their baby herbal formulations.

Discussion

According to literature survey, there is no HPTLC densitometric method reported to quantify glycyrrhizin in *Glycyrrhiza glabra* rhizome and baby herbal formulations by reverse phase HPTLC method which was collected from Kingdom of Saudi Arabia. Therefore, attempts were made to develop and validate a cost effective, because in RP-HPTLC technique the mobile phase was prepared by simply mixture of water and methanol as compared to normal phase HPTLC technique. The adaptation of reverse phase methodology over normal phase helped in avoiding the non-polar fractions from the sample in the TLC, which gives a very clear elution pattern. More over it helped in avoiding the interference due to impurities in the chromatograms, formation of compact spot and detection clarity. Glycyrrhizin has a sweetness value about 50 times greater than that of sucrose and one of the sweetest chemical processed commercially that is found in nature. It serves as a flavouring agent and an efficient replacement for sugar especially in baby formulation and other pharmaceutical products. Our study clearly gave a very good analytical technique for the quantitation of the bioactive compound glycyrrhizin in *Glycyrrhiza glabra* rhizome and baby herbal formulations. This method can be successfully employed in the quality control of many pharmaceutical formulations which contain glycyrrhizin as flavoring agent.

Conclusion

HPTLC proved to be an important tool for qualitative and quantitative analyses. The HPTLC method developed for quantification of glycyrrhizin was found to be simple, accurate, reproducible, sensitive, and is applicable to the analysis of a wide variety of glycyrrhizin containing products. This proposed method is the first validated HPTLC method for quantification of the *Glycyrrhiza glabra* rhizome and baby herbal formulations using RP-18 Silica gel. Due to the acidic nature and relatively high polarity of glycyrrhizin the HPTLC developed methods using normal phase silica gel used acidic mobile phase to generate homogenous spots. In the developed HPTLC method using RP-18 a simple mobile phase composed of methanol-water was used. This advantage makes the method handier and safer to personnel and environment. Statistical data prove that the method is reproducible and selective for the analysis of glycyrrhizin with added advantages of short time, minimal sample preparation, in addition to the low cost.

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