Research Journal of Pharmacognosy (RJP) 9(1), 2022: 111–117
Received: 29 Sep 2021
Accepted: 7 Dec 2021
Published online: 12 Dec 2021
DOI: 10.22127/RJP.2021.294636.1739

Original article

Licorice Compound Traditional Syrup: Formulation and Development of Analysis Method

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Abstract

Background and objective: *Glycyrrhiza glabra* L. (licorice) is one of the most important medicinal plants for respiratory disorders. It is used alone or in combination with other species. In the present investigation, an herbal syrup containing licorice, fennel, and fig was formulated according to Iranian traditional medicine prescriptions and glycyrrhizic acid content of the syrup was quantified using a validated HPLC method. **Method:** Traditional syrup was prepared by decocting the mixture of licorice: fennel: fig (20, 8, 62.5 g in 100 mL). It was filtered and concentrated. Sugar was used in the syrup (40%). Quality control tests were performed and glycyrrhizic acid content of the syrup was determined using an HPLC method. The method was verified according to verification parameters, as well. Accelerated stability tests were carried out during 6 months in 40 °C. **Results:** The prepared syrup was brown colored with fennel odor and sweet taste. The pH, viscosity, dry residue and density were 5.13, 134.8 cP, 51.43%, 1.10 g/cm^3, respectively. Glycyrrhizic acid content was 1.99 mg/mL. The HPLC method was valid according to specificity, linearity (9.3-27.9 µg/mL, r^2: 0.9972), intra-day precision (RSD≤1.71%), inter-day precision (RSD: 3.31%), instrument precision (RSD: 0.82%), recovery (95.6%), LOD (1.48 µg/mL) and LOQ (4.49 µg/mL). **Conclusion:** The prepared syrup with suitable physicochemical and microbial characteristics is a proper candidate for producing at industrial scale after further invivo and clinical studies. Moreover, the HPLC method can be used as a validated method for quality control of the syrup.

Keywords: *Glycyrrhiza glabra;* HPLC; quality control; respiratory disorders; syrup; validation study

Citation: Keramatian B, Ara L, Hajimehdipoor H. Licorice-compound traditional syrup: formulation and development of analysis method. Res J Pharmacogn. 2022; 9(1): 111–117.

Introduction

Respiratory disorders are common due to air pollution in many industrial cities. Lots of chemical and herbal drugs are used to alleviate this situation among them preparations containing *Glycyrrhiza glabra* L. rhizome (licorice) are important [1]. The species is used alone or in combination with other plants in variety of respiratory disorders. Usage of licorice has been approved by Commission E as expectorant in cough and bronchial catarrh [2]. This plant contains glycyrrhizic acid with several biological properties including considerable anti-inflammatory effects which is very critical in treatment of respiratory situations [3]. In Iranian traditional medicine (ITM), licorice is considered among the important remedies for treatment of several diseases including respiratory disorders and asthma [4]. In one prescription in ITM, licorice along with *Foeniculum vulgare* Mill. fruits (fennel), *Ficus carica* L. fruits (fig) and sugar has been used in form of syrup [5]. In order to obtain suitable and stable syrup and for better

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acceptance by patients, this preparation should be formulated as a modern pharmaceutical dosage form with appropriate physicochemical and microbial characteristics. Syrups are common dosage forms and many herbal preparations could be formulated as syrup, but adjustment of the taste and stability are the key points [6]. Moreover, in order to assess the stability of the syrup, its standardization by valid methods is necessary. In this investigation, an herbal syrup containing licorice, fennel, and fig has been formulated according to ITM and quality control tests have been carried out. In addition, an HPLC method for quantization of glycyrrhizic acid as the marker of the syrup has been developed and verified. This syrup is going to be used during an in vivo study for alleviating ovalbumin-induced asthma in a mice model (the ethical approval is mentioned in ethical considerations).

Material and Methods

Ethical consideration

Ethical Committee of Shahid Beheshti University of Medical Sciences approved this study with the code of IR.SBMU.RETECH.REC.1399.462

Chemicals

Potassium sorbate was purchased from Merck (Germany). Acetonitril was from Duksun (South Korea). Glycyrrhizic acid ammonium salt was from Fluca (Germany). All other solvents were prepared from Merck Co. (Germany).

Instrumentation

HPLC analysis was performed using a Shimadzu system equipped with a vacuum degasser, quaternary solvent mixing and a photo diode array detector. UV spectra were collected across the range of 200–900 nm, extracting 254 nm for chromatograms. Lab solution software was utilized for instrument control, data collection and data processing. The column used was C_{18} (Shim-pack VP-ODS) 15 cm×4.6 mm, 5 µm. The mobile phase was an isocratic combination of acetonitrile: H_2O: glacial acetic acid (380:614:6) with a flow rate of 1 mL/min. Injection volume for all samples and standard solutions was 20 µL. Column temperature was 30 °C.

Plant material

Glycyrrhiza glabra rhizome, *Foeniculum vulgare* and *Ficus carica* fruits were purchased from local herbal market in Tehran in July 2020. The samples were identified at the Herbarium of Traditional Medicine and Materia Medica Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran (TMRC) with cods of HMS-555, HMS-553 and HMS-554 for licorice, fennel, and fig, respectively.

Syrup formulation

Different formulations were prepared according to an ITM manuscript [5]. Licorice: fennel: fig (20:8:62.5) were powdered coarsely and extracted with distilled water by decoction method. The final mixture was filtered and concentrated by heating. To achieve a suitable viscosity, different proportions of plants: solvent (water) were prepared and examined; to improve the taste, sugar (different proportions: 10-50%) was added to the mixture, shaken, and heated for 5 min. Physical characteristics of the prepared syrups were evaluated and the best formulations were selected for assessment in accelerated stability tests. Potassium sorbate (0.2%) was used as the microbial preservative in the final formulation. The syrup was packed in 100 mL amber bottles.

Quality control of the syrup [6]

Macroscopic characteristics

Color, odor, taste, and appearance of the syrup were checked.

Crystallization evaluation

Three bottles of the syrup were placed in a refrigerator (4 °C) for 14 days and then they were checked for any crystallization.

Cap locking

Three 100 mL bottles of the syrup were placed upside down at room temperature. The opening behavior was checked after a week. Cap locking would be approved if the cap could not be opened easily.

Dry residue

Five mL of the syrup was placed in the oven (110 °C). After two hours and cooling in a desiccator, the sample was weighed. The process was repeated as described earlier to achieve a constant weight, and dry residue was calculated. This procedure was repeated three times.

Sedimentation

Three samples were centrifuged at 3500 rpm for 15 min and the sedimentation was evaluated.

pH
pH of the formulated syrup was measured at room temperature.

Density
Density of the syrup was measured in triplicate at room temperature by using a 10 mL pycnometer.

Viscosity
Viscosity was calculated by placing 600 mL of the syrup in a Brookfield viscometer, Spindle No.2 with 100 rpm speed at room temperature for three times.

Temperature cycle test
Three bottles of the syrup were placed at 4 °C and three other bottles were set at 40 °C for one week. Then, they were replaced and remained for another one week; then, macroscopic characteristics of the syrups were evaluated.

Accelerated stability test
Stability study for optimized formulations were performed at accelerated stability conditions according to ICH guidelines [7]. Three bottles of the syrup were placed at 40 ±2.0 °C in a stability chamber for six months. Then, samples were checked every three months for any changes in physicochemical characteristics and microbial levels, including total aerobic microbial count, total mold and yeast count, bile-tolerant gram-negative bacteria count, and the presence of *E. coli*, *Staphylococcus aureus*, and *Salmonella* spp.

Quantitative determination of glycyrrhizic acid in the syrup
For standardization of the syrup, glycyrrhizic acid, as the syrup marker, was quantified by using HPLC method according to glycyrrhizic acid monograph in BP pharmacopoeia [8].

Sample preparation
Five milliliters of Licorice-compound syrup was added to a volumetric flask and diluted to 50 mL with acetonitrile: H_2O: glacial acetic acid (380:614:6). Then 2.5 mL of the solution was diluted to 25 mL with the same solvent.

Standard preparation
Stock solution of glycyrrhizic acid (as ammonium salt) (0.093 mg/mL) was prepared in acetonitrile: H_2O: glacial acetic acid (380:614:6). Different concentrations (9.3–27.9 µg/mL) were made from stock solution to plot the calibration curve of glycyrrhizic acid.

Procedure
Sample and standard solutions were injected to HPLC system and area under the curve (AUC) of sample and standard peaks were determined. According to AUCs, the glycyrrhizic acid concentration was calculated in the syrup.

Verification procedure
The HPLC method for quantization of glycyrrhizic acid was according to glycyrrhizic acid raw material monograph in British pharmacopoeia [8]. This method was used for glycyrrhizic acid in the herbal compound syrup and verified through its linearity, specificity, precision, recovery, LOD and LOQ.

Specificity
According to the official guidelines for method validation, specificity is the ability to assess unequivocally the analyte in the presence of other components which may exist along with the analyte [9]. In the present investigation, specificity of the method for glycyrrhizic acid was assessed through injection of diluent to HPLC system. Then the chromatogram was compared with sample chromatogram to explore the presence of any interaction with glycyrrhizic acid. Moreover, glycyrrhizic acid peak was evaluated for peak purity and resolution from the nearest eluting peak.

Linearity
Linearity is the mathematical relationship between concentration of a compound and the detector response. Linearity of the method was assessed in about 50-150% of the glycyrrhizic acid concentration in the syrup. Five concentrations of the standard material (9.3-27.9 µg/mL) were prepared and injected to HPLC instrument and AUC of each concentration was recorded. The calibration curve was obtained through two replications of each glycyrrhizic acid concentration, and the average AUC of each concentration was used to prepare the final calibration curve. The coefficient of determination (r^2) was calculated as well [10,11].

Limit of detection (LOD) and limit of quantization (LOQ)
LOD and LOQ were calculated using the expressions 3.3σ/s and 10σ/s, respectively, in

which σ is intercept standard deviation and s is the slope of calibration curve [12].

Precision

The precision of a technique determines the amount of dispersion within a series of determinations of the same sample. In the present study, instrument precision, inter-day and intra-day precisions were determined. In order to assess instrument precision, one sample was analyzed for six times and the relative standard deviation (RSD) was calculated. For determination of intra- and inter-day precisions, three samples were analyzed on the same day and for three consecutive days, respectively and RSD was calculated. For each process, three samples were prepared and each one was injected to HPLC three times [10,11].

Recovery

This parameter shows the proximity between the real values and the experimental ones. It ensures that no loss or uptake has occurred during the process. The determination of this parameter was performed for the method by studying the recovery after a standard addition procedure with two additional levels (15&30%). The concentrations of glycyrrhizic acid added to the sample were 300 and 600 µg/mL. In each additional level, three determinations were carried out and the recovery percentage was calculated in every case. Each sample was injected to HPLC three times [10,11].

Results and Discussion

This research has been focused on the formulation and quality control of an herbal syrup. In addition, the method of quantization of glycyrrhizic acid in the syrup has been verified.

The syrup was prepared according to traditional manuscripts. Different percentages of sugar were added to the syrup to gain good taste, among them the syrup with 40% sugar was found to be the best for covering the unsuitable plants taste. Among different formulations with constant plant ratio and different volumes of solvent, the syrup contains licorice 26.7 g, fennel 10.7 g and fig 83.3 g in 100 mL was the choice. However, this formulation failed during accelerated stability tests and formed a concentrated and non-uniform syrup after 3 months at 40 °C. Therefore, the syrup was diluted. The final concentrations of the herbals in the syrup were: licorice 20 g, fennel 8 g and fig 62.5 g in 100 mL. This syrup was stable during 6 months at 40 °C. No crystallization, cap locking, and sedimentation were observed in the syrup. It was stable during temperature cycle test as well. The physicochemical characteristics of the syrup has been shown in Table 1. Microbial levels of the syrup were in agreement with pharmacopoeia requirements during 6 months [8].

The results of quantitative determination of glycyrrhizic acid in licorice-compound syrup by HPLC method demonstrated that glycyrrhizic acid showed an excellent peak in the sample chromatogram as seen in Figure 1. By using HPLC method, the concentration of glycyrrhizic acid in the syrup was found to be1.99 mg/mL.

The results obtained from method verification for glycyrrhizic acid assay according to linearity, specificity, precision and recovery showed that the proposed method was reliable. Excellent linearity was obtained for glycyrrhizic acid between peak areas and concentrations 9.3–27.9 µg/mL with r^2=0.9972, Y=9574.1X-147.1.

Glycyrrhizic acid in the sample and the standard chromatographs were spectrally similar and pure. No interaction between chromatogram of diluent with sample chromatogram was observed. The three-dimensional and contour plot views of the chromatograms also confirmed the complete separation. LOD and LOQ were calculated as 1.48 and 4.49 µg/mL for glycyrrhizic acid. RSD% for the instrument precision was 0.82 (Table 2).

Table 1. Physicochemical characteristics of Licorice compound syrup during 6 months at 40°C

Test	Time		
	0	3rd Month	6th Month
Appearance	Brown and viscose liquid with fennel odor	Brown and viscose liquid with fennel odor	Brown and viscose liquid with fennel odor
pH	5.13±0.03	5.10±0.03	5.01±0.01
Density (g/cm^3)	1.10±0.00	1.10±0.00	1.10±0.00
Dry residue (%)	51.43±0.23	52.20±0.12	53.21±0.20
Viscosity (cp)	134.80±3.50	127.60±4.80	130.40±2.90
Assay of glycyrrhizic acid (mg/mL)	1.99±0.06	1.95±0.11	1.93±0.09

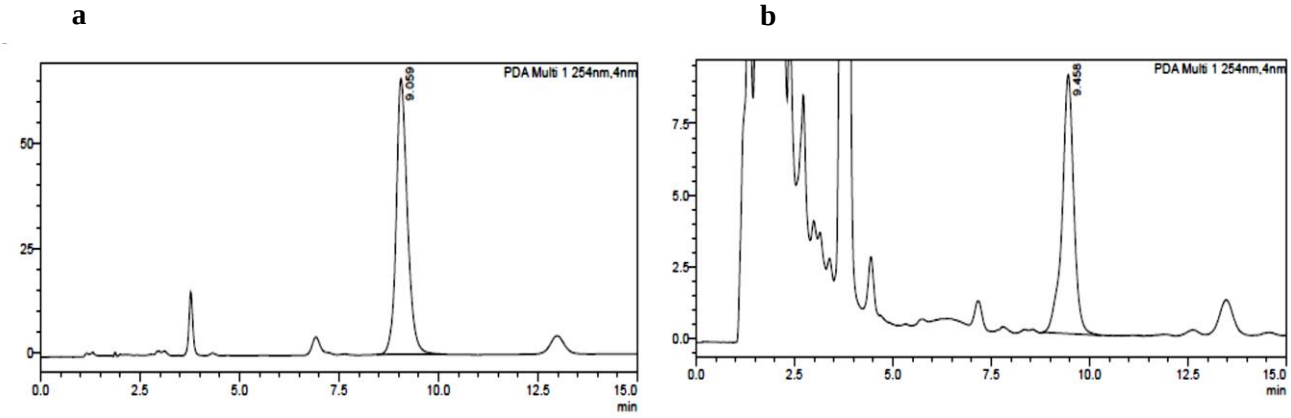

Figure 1. HPLC chromatograms; a) glycyrrhizic acid standard; b) Llcorice-compound syrup

Table 2. Results of Instrument precision of glycyrrhizic acid quantization in licorice-compound syrup

No.	Glycyrrhizic acid content (mg/mL)	Mean±SD (RSD%)
1	1.919	1.932±0.016 (0.82)
2	1.930	
3	1.939	
4	1.921	
5	1.960	
6	1.921	

The precision results for analysis of glycyrrhizic acid showed RSD≤ 1.71% intra-day and RSD 3.31% for inter-day precision which are reasonable for herbal preparations (Table 3). Accuracy, which was evaluated as recovery after spiking the syrup with glycyrrhizic acid at two levels of 15% and 30%, was found to be 94.03% and 97.17%, respectively (Table 4).

Licorice is one of the most important plants in traditional medicine and phytotherapy. It is famous for anti-inflammatory effects due to glycyrrhizic acid which is the sweet components of the species [13]. Different investigations have proved the beneficial properties of licorice in respiratory disorders especially asthma due to anti-inflammatory and anti-allergic activities. Patal et al. showed that the saponin fraction of licorice demonstrated anti-asthmatic activity and could inhibited mast cell degradation [1]. During a clinical trial on licorice capsules (500 mg twice daily) it was established that licorice improved forced vital capacity% and forced expiratory volume 1% in asthmatic patients [14]. In another study, respiratory effects of licorice were compared with olibanum and it was proved that licorice extract was more efficient in alleviating the asthmatic symptoms [15]. Fennel is a widespread medicinal plant with various biological effects. According to ITM, it is useful for respiratory diseases and its fruit decoction has mucolytic effects and is beneficial for catarrh [16]. According to recent investigations, extract and essential oil of the plant exhibited bronchodilatory activity in guinea pig with probable mechanism of potassium channel opening [17].

Table 3. Intra- and inter-day precisions results of glycyrrhizic acid quantization in licorice-compound syrup

Day	Sample no.	Glycyrrhizic acid content (mg/mL)	Mean±SD (RSD%)	
			Intra-day	Inter-day
1	1	1.912	1.928±0.017 (0.86)	1.991±0.066 (3.31)
	2	1.928		
	3	1.945		
2	4	1.955	1.986±0.034 (1.71)	
	5	1.980		
	6	2.022		
3	7	2.066	2.060±0.005 (0.25)	
	8	2.055		
	9	2.059		

Table 4. Recovery results of glycyrrhizic acid quantization in Licorice-compound syrup

Glycyrrhizic acid added	Found (mg/mL)	Recovery (%)	Mean±SD
0	1.99	-	-
15%	2.08	90.83	94.03
	2.19	95.63	
	2.19	95.63	
30%	2.48	95.75	97.17
	2.55	98.46	
	2.52	97.30	

Anethol, as the marker of the plant, is responsible for the sympathomimetic activity [18]. Moreover, fennel has anti-inflammatory effects against acute and sub-acute inflammatory diseases which is effective in asthma [19]. Fig is one of the first plants that was cultivated by human. The fruit is

a source of vitamins, minerals, carbohydrates, sugar, organic acids and phenolics [20]. According to ITM, it is useful for respiratory illnesses such as cough and asthma [4]. In ITM, sugar is a useful material in lung disturbances. It dissolves mucosa and is used for cough and hoarseness [4]. Taking together, all components of the prepared syrup have beneficial effects in respiratory complications. These properties can be raised due to synergistic effect of the compounds in the herbal syrup.

Conclusion

Considering the effects of the licorice-compound syrup components in respiratory disturbances, it could be concluded that prepared product may alleviate respiratory disorders including asthma with synergistic effects of species; but, in-vivo tests and clinical trials are necessary in the future. The syrup with suitable physicochemical and microbial characteristics is a good candidate for production in industrial scale after further investigations. Moreover, the validated HPLC method which is a specific, precise and accurate method for the quantitative analysis of glycyrrhizic acid, as a major components of licorice, could be used for quality control of the prepared herbal syrup.

Acknowledgments

The project was supported by Traditional Medicine and Materia Medica Research Center (grant no: 00-22601), Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Author contributions

Homa Hajimehdipoor supervised the project; Leila Ara performed formulation part; Behnaz Keramatian was involved in method verification.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

HPLC: high performance liquid chromatography; LOD; limit of detection; LOQ: limit of quantization; RSD: relative standard deviation; SD: standard deviation; ITM: Iranian traditional medicine; AUC: area under the curve; σ: intercept standard deviation; s: slope of calibration curve