



Determination of Glycyrrhetic Acid in Rat Plasma by HPLC After Oral Administration of Licorice Aqueous Extract

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Abstract

Licorice is obtained from *Glycyrrhiza glabra* (*G. glabra*) in Iran. Glycyrrhizin is the main constituent of *G. glabra* and has several pharmacological effects. It is hydrolyzed to glycyrrhetic acid (GA) in the intestine after oral administration. In this study, a novel HPLC method was used to determine the concentration of GA in rat plasma after oral administration of licorice aqueous extract. The method was linear ($r^2 > 0.999$) in the range of 0.1-5.0 $\mu\text{g/ml}$ for GA. Maximum plasma concentration of GA was achieved 8 h after oral administration of licorice aqueous extract. The developed method was suitable for determination of GA in rat plasma.

Keywords: Glycyrrhetic acid; Glycyrrhizin; HPLC; Licorice; Plasma.

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1. Introduction

Licorice is one of the most popular and widely consumed herbs in the world. Worldwide, it is used primarily as flavoring and sweetening additive in food products. Licorice is the rhizome and root of *Glycyrrhiza* species. *Glycyrrhiza glabra* L. (Leguminosae) is native to the Mediterranean and certain areas of Asia [1]. The roots and rhizomes of *G. glabra* were used for their anti-ulcer, expectorant, diuretic, laxative, sedative, antipyretic, antimicrobial and anxiolytic effects [2].

Glycyrrhizin (GL) (glycyrrhizic acid), the

glycoside form of glycyrrhetic acid (GA), is a triterpenoid compound which accounts for the sweet taste of licorice root, and it is the main constituent of *G. glabra*. GL has antiviral [3], anti-inflammatory [4] and antioxidant activities [5]. Activity of flavonoids from the extract of licorice against *Helicobacter pylori* has also been reported [6].

After oral administration of licorice in human, GL is hydrolyzed to GA by intestinal bacteria and no more GL is detectable in the plasma [7]. GA is 200-1000 times more potent than GL in inhibition of 11- β -hydroxysteroid dehydrogenase, which is involved in the corticosteroid metabolism. Excessive consumption of GL results in aldosteronism (an adverse effect) which seems to be dose-dependent, but with a wide inter-individual

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variation [8]. Probably due variability in side effects and the medical application of GL in modern medicine, many researchers have developed various analytical methods for measuring GA in human and rat plasma after oral administration of licorice aqueous extract [9-13].

In the present study, a new HPLC method was applied for determining GA in rat plasma after oral administration of Iranian licorice extract from *G. glabra*.

2. Materials and methods

2.1. Chemicals

Licorice was purchased from a local herbal medicinal plants drugstore. Glycyrrhizic acid and glycyrrhetic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and acetonitrile were of HPLC grade and were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical grade and used without any further purification.

2.2. Animals

Male Wistar rats (200-300 g) were obtained from Razi Institute (Hesarak, Karaj, Iran) and housed in a 12 h light-dark cycle. The rats were fasted for 12 h before drug administration. They had free access to water and food prior to the experiments.

2.3. Instrumentation

The HPLC system was consisted of a 510 pump, 710 plus autosampler and a variable 480 UV detector, all from Waters (Milford, MA, USA). The data processing system was a multichannel Chrom and Spec software for chromatography, version 1.5 x.

2.4. Standard solution

Stock standard solution of GA was prepared by dissolving appropriate amounts of the compound in methanol to give a final concentration of 200 µg/ml. Standard solutions of GA (1-50 µg/ml) were prepared by subsequent dilution. A 12.5 µg/ml solution of nandrolone phenylpropionate (as internal standard) in methanol was prepared, too. These solutions were stored at 4 °C.

2.5. Chromatographic conditions

Analysis of glycyrrhizin was achieved using a Nova-Pak® C8 column (4.6 250 mm, Waters, Milford, MA, USA). The isocratic mobile phase which was consisted of methanol-acetonitrile-water-acetic acid (25:48:26:1, v/v) was prepared daily and degassed by passing through a 0.45 µm filter. It was pumped at a flow rate of 1 ml/min. with detection at 254 nm. Determination of GA was performed on a Nova-Pak® C8 cartridge column (4.6 mm × 250 mm, 4 µm, Waters, Milford, MA, USA) using a mixture of

Table 1. Precision and accuracy of the developed HPLC method for determining glycyrrhetic acid in spiked rat plasma (n=9, three sets for 3 days).

Concentration added (µg.ml ⁻¹)	Concentration found (mean ± SD)(µg.ml ⁻¹)	CV (%)	Error (%)	Accuracy (%)
Within-day (n=3)				
0.1	0.090±0.030	3.33	-10.00	90.0
1.0	1.010±0.043	4.26	1.00	99.0
5.0	4.990±0.036	0.72	-0.20	99.8
Between-days (n=9)				
0.1	0.088±0.000	4.55	-13.00	87.0
1.0	1.010±0.042	4.16	1.00	99.0
5.0	5.000±0.065	1.30	0.00	100.0

methanol-acetonitrile-water-acetic acid (25:48:26:1, v/v) as the mobile phase pumped at a flow rate of 1.2 ml/min. Detection was performed at 254 nm. All separations were performed at the room temperature.

2.6. Sample preparation

To a 500 μ l portion of rat plasma sample, in a test tube, 50 μ l of standard solution of GA, 50 μ l of internal standard (12.5 μ g/ml of

nandrolone phenylpropionate) and 10 μ l acetic acid were added and vortex-mixed for 5 sec. Then 5 ml of 1.5 % isoamyl alcohol in hexane was added and mixed for 2 min. After centrifugation at 4000 rpm for 10 min., the organic layer was separated and evaporated under nitrogen flow at 40 °C. The residue was reconstituted in 100 μ l of the mobile phase and 50 μ l of it was injected in the HPLC system.

2.7. Validation

Six series of standard calibration solutions were prepared in the blank plasma in the range of 0.1-5.0 μ g/ml. The sample preparation and HPLC analysis were performed as described above. Calibration curves were constructed by plotting the measured peak area ratios of GA to internal standard versus concentrations of standard samples and the statistical analysis was performed. To establish the accuracy and precision of the method, three replicates of the standard plasma solutions at three different concentrations (0.1, 1 and 5 μ g/ml) were assayed on one day and three separate days. Within-day and between-days variations were calculated.

2.8. Preparation of licorice decoction

Twenty grams of licorice powder was soaked in 300 ml of water for 30 min. and then boiled for more than 2 h to about 100 ml. After filtering, sufficient water was added to 100 ml. This solution was stored at 4 °C until use. The concentration of glycyrrhizin in the decoction was determined by a modified HPLC method previously described [14].

2.9. Drug administration and blood sampling

The administered licorice aqueous extract was 2 ml via gastric gavage (the concentration of GL in the decoction was 5.28 mg/ml). Blood samples (about 5 ml) were collected in heparinized tubes at 2, 4, 6, 8, 10, 12 and 16 h after administration. Because large volumes

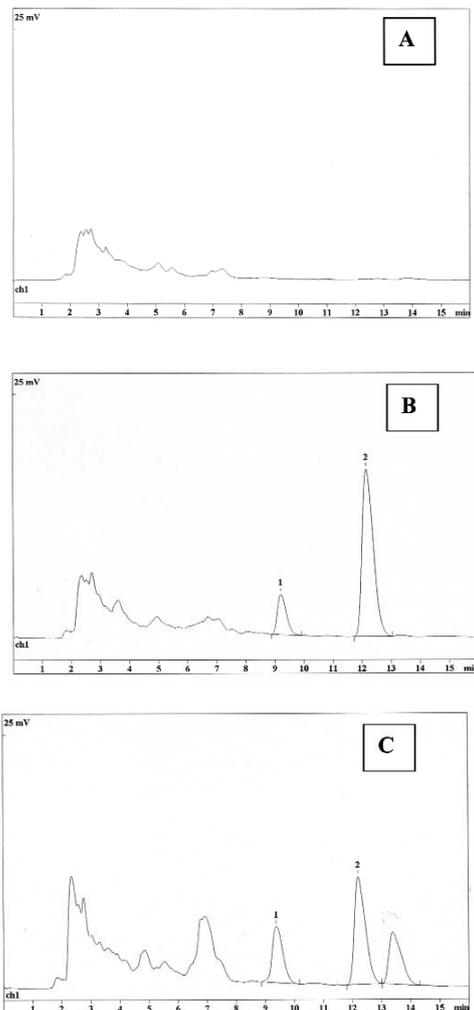


Figure 1. HPLC chromatogram of glycyrrhetic acid and internal standard (IS). (A) drug free plasma; (B) plasma sample spiked with glycyrrhetic acid and IS; (C) rat plasma sample, 8 h after oral administration of licorice decoction. 1: Glycyrrhetic acid, 2: IS.

of blood samples were required, seven rats were used for blood collecting at each time point. Samples were immediately centrifuged at 4500 rpm for 10 min. to separate the plasma.

3. Results and discussion

For the past 30 years, GL (the active ingredient of *G. glabra*) has found its way to the modern therapeutic applications. This is due to the fact that this compound is available in significant amounts in licorice (>3%) in addition to its various pharmacological effects [1-6]. Therefore, many researchers have developed various analytical methods for determination of GL in biological samples [9-14]. As *G. glabra* is naturally grown in Iran in different regions and exported in large amounts annually, it was decided to optimize an HPLC condition for analysis of GA in plasma after consumption of licorice aqueous extract. It is interesting to find that the C_{max} of GA is higher for Iranian licorice (2.12 ± 0.33 vs. 1.64 ± 0.77 mg/ml for pure GL), although GL was administered orally at 100 mg/kg to rats (Table 2) [13]. In addition, T_{max} is shorter for Iranian licorice than pure GL (8.0 vs. 11.2

h). These facts could be explained on the basis of the antimicrobial effects of GL [3, 6, 7] which might cause less available GA for absorption.

Under the chromatographic conditions described in the present procedure, GA and internal standard were well resolved from licorice extract or plasma samples with no interfering peaks (Figure 1). The calibration curve of GA was linear in the range of 0.1-5.0 $\mu\text{g/ml}$ plasma with LOD of 0.02 $\mu\text{g/ml}$ and typical equation of $Y = (0.732 \pm 0.002)X - 0.019 \pm 0.005$ and $r^2 > 0.999$. The linearity is validated by the high value of the correlation coefficient. The accuracy and precision of the method for analyzing GA in plasma were determined by assaying three samples at 0.1, 1.0, and 5.0 $\mu\text{g/ml}$ on three separate days. Concentrations were determined using calibration standard curve prepared in the range of 0.1-5.0 $\mu\text{g/ml}$. Good accuracy and precision were observed over the entire concentration range (Table 1). The within-day and between-day variability showed CV values less than 4.5% in all three selected concentrations.

The limit of quantification with $CV < 4.5\%$

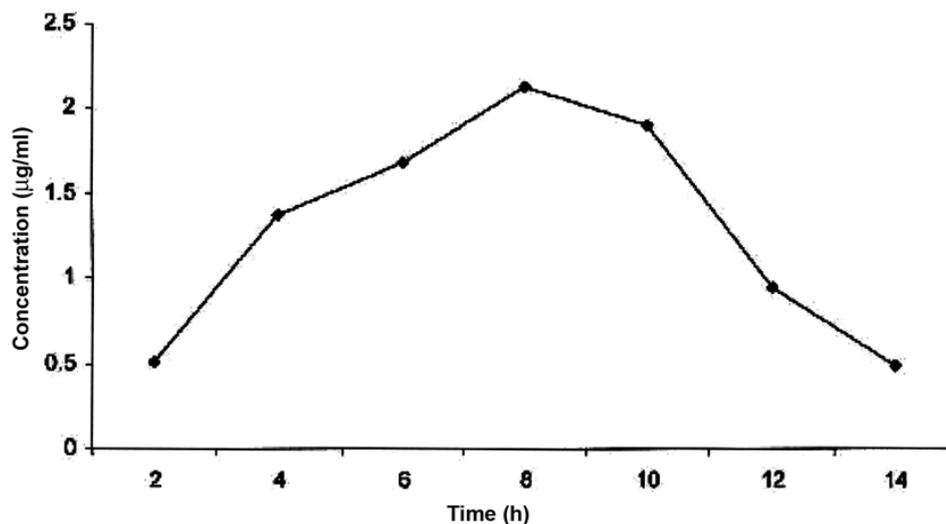


Figure 2. Mean plasma concentration-time curve of glycyrrhetic acid following oral administration of licorice decoction to 7 rats.

Table 2. Glycyrrhetic acid concentration in rat plasma at different periods of time after oral administration of licorice aqueous extract.

Rat	GA concentration ($\mu\text{g/ml}$) at time (h)						
	2	4	6	8	10	12	14
1	0.18	1.12	1.69	2.31	1.93	0.80	0.21
2	0.40	0.75	1.62	2.25	1.29	1.08	0.53
3	0.55	0.89	1.75	1.76	2.27	1.00	0.66
4	0.38	1.05	1.43	1.83	1.73	1.37	0.71
5	0.22	0.53	1.11	2.65	0.98	0.66	0.49
6	0.23	1.15	1.58	2.47	1.98	0.89	0.44
7	0.67	1.28	1.46	2.16	2.50	0.78	0.60
Mean	0.38	0.967	1.52	2.12	1.89	0.94	0.49
SD	0.23	0.48	0.72	0.33	0.56	0.41	0.24
CV%	60.50	ND	49.60	47.40	15.56	29.63	ND

ND: Not detected

was found to be 0.1 $\mu\text{g/ml}$ for GA. The limit of detection that can be reliably detected with S/N ratio of 3 was found to be 0.02 $\mu\text{g/ml}$. The method was successfully used for determination of GA in rat plasma. The plasma concentration of GA, the main metabolite of glycyrrhizin after oral administration of licorice extract to rats is presented in Table 2 and Figure 2. From the results of this study it might be concluded that this simple and reliable method could be used for determining GA in human plasma for pharmacokinetic and therapeutic drug monitoring studies.

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