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Journal of Chromatography B, 850 (2007) 53–58
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

One-step immunochromatographic separation and ELISA quantification of glycyrrhizin from traditional Chinese medicines

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Received 20 February 2006; accepted 31 October 2006
Available online 16 November 2006

Abstract

The bioactive constituent, glycyrrhizin or glycyrrhizic acid (GA), was purified from two traditional Chinese medicines (TCM), *Shaoyao gancao tang* and *Dahuang gancao tang*, and from crude extracts from licorice roots by means of immunoaffinity chromatography using anti-GA monoclonal antibody (MAb) and was quantified with an enzyme-linked immunosorbent assay (ELISA). Laboratory preparations included the synthesis of conjugate GA-human serum albumin (GA-HSA), the production of anti-GA-MAb, the optimization of the immunoaffinity column packed with the anti-GA-MAb coupled to hydrazide gel and the determination of the GA content in TCM and crude drugs from five different sources by ELISA and high performance liquid chromatography (HPLC). The experimental results reveal that the anti-GA-MAb coupled to Affi-Gel Hz gel results in a coupling efficiency of 95.2%, and the immunoaffinity chromatography gives a mean recovery of 97.6% of GA with a capacity of 33.5 ± 2.40 μg/mL of immunoaffinity gel under the given conditions. The GA content of the crude extracts (ranging 74.8–114.6 μg/mg) from different sources by the ELISA method is much greater than that of the TCM (16.4–25.1 μg/mg) which is, in good agreement with the results of the HPLC method. Our report provides a rapid, reliable and sensitive approach for one-step separation and quantification of GA.

Keywords: One-step separation; Glycyrrhizin; Immunoaffinity chromatography; Monoclonal antibody; ELISA

1. Introduction

Licorice (*Glycyrrhiza* spp.) is one of the most important remedies used in traditional Chinese medicine (TCM). It mainly consists of dried roots and stolons of the perennial plants, *Glycyrrhizae uralensis*, *G. inflate* and *G. glabra*. Licorice is prescribed with other herbal medicines as a demulcent in the treatment of sore throats, an expectorant for coughs and bronchial catarrh, an antitussive, a taste-modifying agent for relieving pain, an anti-inflammatory agent for anti-allergic reactions, rheumatism and arthritis, a prophylactic for liver disease and tuberculosis and adrenocorticoid insufficiency [1–5].

The phamacological properties of licorice depend upon glycyrrhizin or glycyrrhizin acid (GA), which is considered to be its main active constituent. Fig. 1 shows the chemical structure of GA; it is a triterpenoid saponin that can be converted into glycyrrhetic acid by GA β-D-glucuronidase [6].

Quality standardization of licorice is usually based on its GA content. GA is a protein-kinase inhibitor with anti-ulcer and anti-viral activities that is now used in the treatment of hepatitis in China and Japan [7–9]. Interferon-inducing activity and inhibition of HIV-1 replication by GA have also been reported [10–12]. Moreover, GA is used in food additives and cosmetics as a well-known natural sweetener [13].

Recently, the demand for licorice has been increasing, whilst the availability of wild licorice has declined [14,15]. The promotion of cultivation for licorice as an additional and stable source of the medicinal plant requires a determination of the GA content in different sources of the herb. Therefore, it has become important to develop a rapid and sensitive method with high reproducibility and repeatability for monitoring the GA concentration in drug production and pharmacological research.

Various methods for separation or quantification of GA from licorice have been reported, such as gas chromatography, high performance liquid chromatography (HPLC) and micellar electrokinetic chromatography [16–18]. Commercial purification of GA typically includes several isolation steps, such as crystallization, column chromatography and liquid partitioning. However,

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doi:10.1016/j.jchromb.2006.10.062

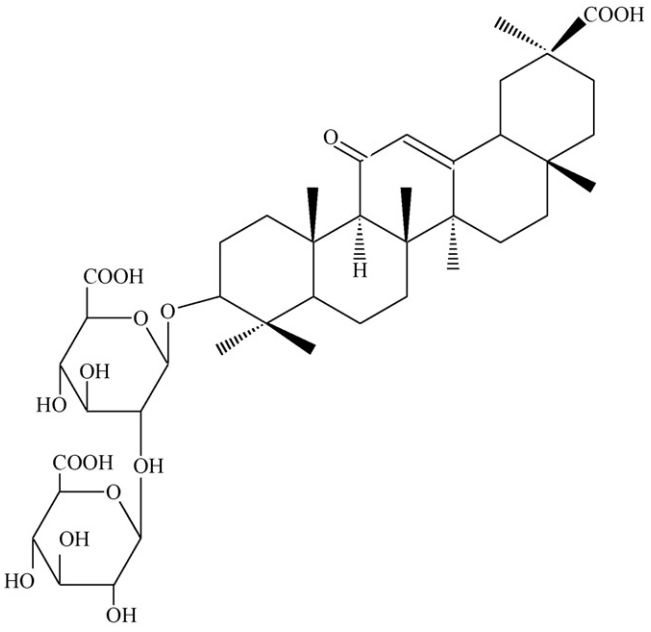

Fig. 1. Chemical structure of glycyrrhizic acid (GA) showing the carbohydrate moiety positions.

these methods are far from satisfactory for analytical purpose in terms of high sensitivity, reproducibility, large amounts of extraction solvents and time-consuming factors.

Previously, we established a fast and sensitive assay system for the screening of GA concentration in large numbers of small samples with low concentration and for the quality control of drug production, pharmacological research and other applications [19–21]. As a second step, the purpose of our present work is to purify and quantify GA from two traditional Chinese medicines and crude drug extracts of licorice roots by means of one-step immunoaffinity chromatography using anti-GA monoclonal antibody (anti-GA-MAb) with detection by means of an enzyme-linked immunosorbent assay (ELISA). We demonstrate that this is a rapid and sensitive technique for one-step separation and quantification of the GA content of TCM that is suitable for high-throughput laboratory analysis.

2. Experimental

2.1. Immunochemicals and chemicals

Pure GA and 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Wako Pure Industrial Ltd., Osaka, Japan. Human serum albumin (HSA) was provided by Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse immunoglobulin G (POD-IgG) was from Organon Teknika Cappel Products (West Chester, PA, USA). Affi-Gel Hz gel was from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals of analytical grade were commercially available.

2.2. Extraction of GA from different sources

Crude GA extracts were prepared from either powdered TCM, known as *Shakuyaku kanzo to* and *Daio kanzo to* in Japanese (*Shaoyao gancao tang* and *Dahuang gancao tang* in Chinese), or from the powdered crude *G. uralensis* and *G. glabra* in the laboratory. The compositions and ratios were adjusted according to the prescriptions in the Chinese Pharmacopoeia [22] and the Japanese Pharmacopoeia [23] as shown in Table 1.

Table 1
Extraction of GC from different sources

No.	Compositions and ratio	Notes
A	*Paeonia lactiflora*:*Glycurrhiza glabra* (1:1)	*Shaoyao* and *gancao*, lab preparation
B	*P. lactiflora*:*G. uralensis* (1:1)	*Shaoyao* and *gancao*, lab preparation
C	*Rheum palmatum*:*G. glabra* (4:1)	*Dahuang* and *gancao*, lab preparation
D	*Tsumura Shakuyaku kanzo to*	No. 68, commercial Kampo medicine
E	*Tsumura Daio kanzo to*	No. 84, commercial Kampo medicine

The powdered *P. lactiflora* and *G. glabra* (each 0.8 g) were first extracted thoroughly with methanol (8 mL) and repeatedly extracted 5 times (totally 40 mL of solvent used) in an ultrasonic bath at 30 °C for 15 min. The pooled extract was centrifuged at 4000 rpm for 5 min; the supernatant was collected and evaporated at 40 °C under a nitrogen stream to remove the residual methanol. The residue was dissolved in distilled water and frozen at −18 °C for 1 h before lyophilization, which was conducted in a freeze dryer (Eyela FD-5N, Tokyo, Japan). The dried extracts were kept in a refrigerator (4 °C) for subsequent use.

2.3. Preparation of anti-GA-MAb

The preparation of the anti-GA-MAb has been reported in our previous work [24]. In brief, immunization of BALB/c female mice with GA-HSA conjugate emulsified in Freund's complete adjuvant was performed by an intraperitoneal injection to stimulate antibody production. Then the antibody-forming splenocytes were fused by polyethylene glycol method to form hybridomas with a hypoxanthine-aminopterin-thymidine-sensitive myeloma cell line (P3-X63-Ag8-653). After hybridomas screening (ELISA method), the antibody-producing hybridomas were cloned and expanded. At last, the anti-GA-MAbs were harvested and purified from the cultivation using a protein G FF column (0.46 cm × 11 cm, Pharmacia Biotech, Uppsala, Sweden) [20].

2.4. Preparation and optimization of the immunoaffinity column

The purified anti-GA-MAb (50 mg) was coupled to an Affi-Gel Hz gel (25 mL) and used to prepare the immunoaffinity column.

Prior to coupling to Affi-Gel Hz gel, the purified anti-GA-MAb was dialyzed in a 10^3-fold excess coupling buffer (Bio-Rad Affi-gel Hz coupling buffer, commercially available), pH 5.5, overnight at 4 °C and then oxidized with $NaIO_4$ by mixing gently for 1 h in a container covered with foil at ambient temperature. Glycerol was then added at a final concentration of 20 mM

Table 2
Different buffer systems and the GC recovery percentage

Name	Compositions	Recovery (%)[a]
Loading buffer	5 mM PB–5% MeOH–50 mM NaCl, pH 7.0	
Washing buffer	5 mM PB–50 mM NaCl, pH 7.0	
Elution buffer	20 mM PB–30% MeOH–500 mM NaCl, pH 7.0	100.00
	50 mM PB–40% EG–500 NaCl, pH 7.0[b]	91.22
	10 mM PB–500 mM KSCN, pH 7.0	74.10
	20 mM PB–30% MeOH, pH 7.0	78.67

[a] The recovery of 20 mM PB–30% MeOH–500 mM NaCl (pH 7.0) was taken as 100%, and the others were calculated as relative percentage to the former.
[b] EG stands for ethylene glycol.

immediately after the 1 h oxidation, and mixed for 10 min. The oxidized anti-GA-MAb was dialyzed in the above-mentioned conditions.

The oxidized and desalted anti-GA-MAb was then coupled to the washed Affi-Gel Hz hydrazide gel for 24 h with gentle stirring at ambient temperature. After completing the coupling reaction, the gel/MAb slurry was poured into a plastic column (300 mm × 28 mm i.d.) and washed with one bed volume of 20 mM phosphate buffer (PB, 0.1167% monosodium phosphate monohydrate and 0.3093% disodium phosphate, heptahydrate) containing 0.5 M NaCl, pH 7.0. The eluates were collected and saved for coupling efficiency determination. The prepared column was washed with phosphate buffered saline (PBS, 0.15 M NaCl in 10 mM potassium phosphate, pH 7.4) containing 0.02% sodium azide and stored at 4 °C until ready for use.

The coupling efficiency of the anti-GA-MAb coupled to Affi-Gel Hz gel was determined by a sandwich ELISA and calculation of the ratio.

To determine the best elution conditions, pure GA (40 μg) dissolved in 1 mL of loading buffer (5 mM PB-5% MeOH–50 mM NaCl, pH 7.0) was loaded onto the immunoaffinity column packed with 2 mL of anti-GA-MAb hydrazide gel. The column was incubated for 2 h at 4 °C, washed with 20 mL of 5 mM PB–50 mM NaCl, pH 7.0 and finally eluted sequentially with different elution buffers (Table 2). The GA concentration of each fraction was determined by using competitive ELISA, and the optimal elution buffer was selected on the basis of the GA recovery.

2.5. Immunoaffinity chromatography of GA from the extracts

Before use, the immunoaffinity column was washed with PBS. The crude extract (containing GA 1.0–1.5 mg) dissolved in 5 mL of loading buffer was applied consecutively on the prepared immunoaffinity column with 25 mL anti-GA-MAb hydrazide gel. The column was eluted with one bed volume of washing buffer to remove the unbounding GA and then eluted with three bed volumes of elution buffer. The column was eluted at a flow-rate of 1 mL/min. After use, the column was washed with PBS, and finally equilibrated with PBS buffer containing 0.02% of sodium azide and stored at 4 °C until ready for reuse.

2.6. Determination of GA concentrations by ELISA and HPLC

A direct ELISA method was used for determining the affinity of the anti-GA-MAb for the GA-HSA conjugate and a competitive ELISA was used for the quantification of GA in fractions eluted from the immunoaffinity column.

For direct ELISA, 100 μL of the GA-HSA solution (1 μg/mL in 50 mM sodium carbonate buffer, pH 9.6) was immobilized on to the wells of a 96-well immunoplate (Nunc Roskilde, Denmark) for 1 h. The plate was washed 3 times with PBS containing 0.05% Tween 20 (PBST), and then blocked for 1 h with a 300 μL of PBS containing 5% skimmed milk to reduce nonspecific absorption. The plate was washed again 3 times with PBST and reacted with 100 μL of anti-GA-MAb solution for 1 h. After washing the Plate 3 times with PBST, the MAbs were combined with 10^3-fold diluted POD-IgG for 1 h. The plate was washed 3 times with PBST and then 100 μL of substrate solution containing 200 mM citrate buffer, pH4.0, and 6 mg/mL of ABTS were added and incubated for 15 min to develop the color. The absorbance at 405 nm was monitored.

The competitive ELISA was basically similar to the direct ELISA, except 50 μL of a serial of different concentrations of standard GA and/or the GA extracts dissolved in 10% of MeOH was incubated with 50 μL of the anti-GA-MAb for 1 h to competitively react with the GA-HSA, whereas 100 μL of the anti-GA-MAb was used as described in the direct ELISA.

To determine the immunoassay specificity, cross-reactivity (CR) of the anti-GA-MAb against GA and some chemically structure-related compounds were evaluated by competitive ELISA using Weiler and Zenk's method [24] as follows:

$$\mathrm{CR}\,(\%) = \frac{\text{GA concentration at } A/A_0 = 50\%}{\text{Test compound concentration at } A/A_0 = 50\%} \times 100\%$$

Here A stands for the absorbance in the presence of the test compound and A_0 for the absorbance in the absence of the test compound.

An analytical HPLC system (pump model LC-10ADVP and equipped with a UV-vis detector, Shimadzu, Tokyo, Japan) was also used to confirm the GA concentrations in the fractions of immunoaffinity chromatography under the following operating conditions: Cosmosil column 5C18-AR-3 (150 × 4.6 i.d. mm, code no. 34246-21, Nacalai Tesque, Kyoto, Japan), 30 °C; UV detector: 254 nm; mobile phase solution: acetonitrile:water:acetic acid (32:45:3, v/v); flow-rate: 0.5 mL/min; linear gradient of acetonitrile varied from 0% to 60% in 40 min.

2.7. Statistical analysis

Present data are the mean values of triplicates. ANOVA analysis was conducted to test if the differences between data were statistically significant. Correlations were performed with software CurveExpert version 1.38 (copyright by Daniel Hyams, MS, USA).

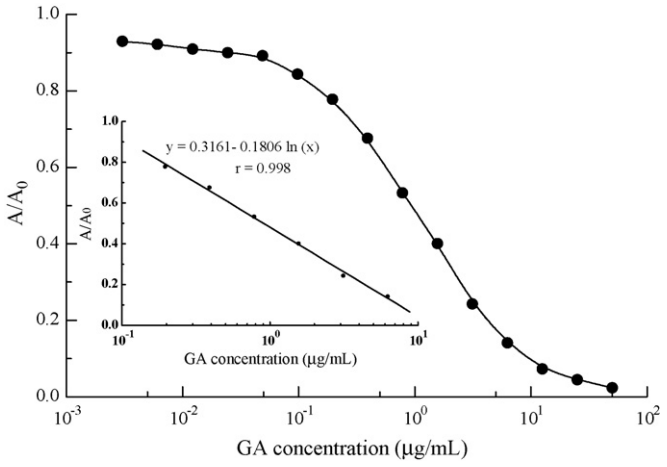

Fig. 2. The curve of absorbance at 405 nm against the GA concentration and the linear calibration curve used for the determination of the GA concentration by the competitive ELISA method.

3. Results and discussion

3.1. GA concentration from different extracts

It is well known that the GA quantity of licorice may vary with both the analytical methods and the sources of crude materials in prescriptions (*e.g.* cultivars, habits and harvest time, *etc.*).

Fig. 2 demonstrates the standard curve of the GA concentration against the abosorbance at 405 nm (A/A_0) by the competitive ELISA method. The full linear calibration ranged from 0.1 to 6.25 μg/mL, which was used in the determination of the GA concentrations from different sources. The GA concentrations from the TCM and the crude extracts of laboratory preparations are shown in Table 3. It indicates that the GA concentrations from the laboratory preparations (74.8–114.6 μg/mL powder) are larger than those (16.4–25.1 μg/mL powder) from the TCM of commercial source (Kampo). At the same time, the results obtained by ELISA and HPLC show a good agreement between the two methods in terms of precision and reproducibility. Quantification of the GA concentrations by anti-GA-MAb ELISA is highly sensitive and reproducible and offers advantages of speed and reduced sample preparation over alternative techniques using HPLC.

3.2. Validation of the prepared immunoaffinity column and optimal buffer system

Since IgG contains approximately 3% carbohydrate localized in the Fc region (heavy chain) of the MAbs, periodate oxidation of vicinal hydroxyl groups in the carbohydrates moiety is carried out to prepare aldehydes that can be conjugated with Affi-Gel Hz gel to form stable, covalent hydrazones. The coupling efficiency of the anti-GA-MAb to Affi-Gel Hz was determined to be 95.21% using a sandwich ELISA to measure uncoupled MAb. The capacity of immunoaffinity column against GA was determined to be about 33 μg/mL gel (Table 5).

Table 3
The GC contents (μg/mg powder) from different extracts determined by ELISA and HPLC

No.	ELISA	HPLC
A	85.20 ± 0.05	85.06 ± 0.06
B	74.80 ± 0.10	75.11 ± 0.21
C	114.58 ± 1.31	115.09 ± 2.10
D	25.11 ± 0.46	25.97 ± 0.13
E	16.36 ± 0.33	15.98 ± 0.15

Table 4
Cross-reactivities (CR) of the anti-GC-MAb against the GC and some of its analogues

Analogues	CR (%)
Glycyrrhizin	100.00
Glycyrrhetic acid-3-*O*-glucuronide	0.585
Glycyrrhetic acid	1.865
Deoxycholic acid	<0.005
Ursolic acid	<0.005
Oleanolic acid	<0.005

Cross-reactivity is one of the important parameters in optimizing competitive ELISA conditions. Actually, lower cross-reactivity for chemically similar analogues of GA means higher selectivity for the ELISA determination of GA. The cross-reactivities of the anti-GA-MAb against GA and some other chemically structure-related compounds are shown in Table 4. The cross-reactivities of the anti-GA-MAb against glycyrrhetic acid-3-*O*-glucuronide and glycyrrhetic acid were 0.585% and 1.865%. However, the other three analogues were all less than 0.005%, respectively. This implies that the anti-GA-MAb had a weak cross-reaction with those related compounds, but specifically reacted with GA. The result also suggests that the aglycone and a part of the glucuronic acid function as epitopes, and the configuration of the carboxyl group at C-20 of the GA molecules plays an important role in the cross-reaction.

From these results, the newly prepared immunoaffinity column is available for the rapid separation of GA. Experimentally, a typical column could be regenerated in excess of 20 times without an obvious loss of capacity (from about 33 μg/mL gel to about 29 μg/mL gel, data in detail not shown).

3.3. Optimal buffer systems for separation of GA

As shown in Table 2, the elution buffer of 20 mM PB–30% MeOH–500 mM NaCl (pH 7.0) gave the best recovery of GA; this was selected to be an elution buffer and applied in the subsequent immunoaffinity chromatography.

3.4. Chromatography of GA with anti-GA-MAb Affi-Gel Hz gel column

The elution profile of GA eluted with the selected buffer system (20 mM PB–30% MeOH–500 mM NaCl, pH 7.0) is shown in Fig. 3. Forty micrograms of GA was loaded to the column packed with 2 mL of prepared anti-GA-MAb Affi-Gel Hz gel. The GA concentration in the collected fractions was determined by the competitive ELISA. A peak of GA is observed in the elution profile indicating the successful separation of GA from different extracts.

Table 5
Separation result of the five different GA extracts

No.	Loaded sample (mg)	Fractions (μg)		Total (μg)	Recovery (%)	Capacity (μg/mL gel)
A	1.2	Loading	50.19			
		Washing	281.48			
		Elution	829.85	1161.52	96.79	33.19
B	1.2	Loading	52.21			
		Washing	304.45			
		Elution	853.53	1210.18	100.85	34.14
C	1.2	Loading	59.66			
		Washing	268.56			
		Elution	923.52	1251.74	104.25	36.94
D	1.2	Loading	40.04			
		Washing	288.08			
		Elution	756.42	1084.53	90.38	30.26
E	1.2	Loading	46.29			
		Washing	272.47			
		Elution	829.61	1148.37	95.70	33.18
Average					97.59	33.54

Table 5 shows the practical immunoaffinity recovery of GA from different extracts. When 1.0–1.2 mg of GA was applied on the immunoaffinity column, 90–100% (average 97.6%) recovery was obtained. Meanwhile, when compared with the previous reports on the capacities of an immunoaffinity column for forskolin (9.41 μg/mL gel) [25,26], the capacity (averaged 33.5 μg/mL gel) of the prepared immunoaffinity column revealed rather to be effective to capture the immunogen as described above. This result may also infer that when the concentration of GA is very low and cannot be analyzed by ELISA method, the newly established immunoaffinity column can possibly function to concentrate for the ELISA analysis. In fact, the capacity of the gel is not strictly related to the operating concentration range. The capacity reflects the number of active sites, but this can show concentration dependence. The uptake and release of the analytes are at equilibrium, and therefore, concentration-dependent. Consequently, the selection of the correct monoclonal antibody includes the selection of an antibody that has near ideal adsorbing characteristics.

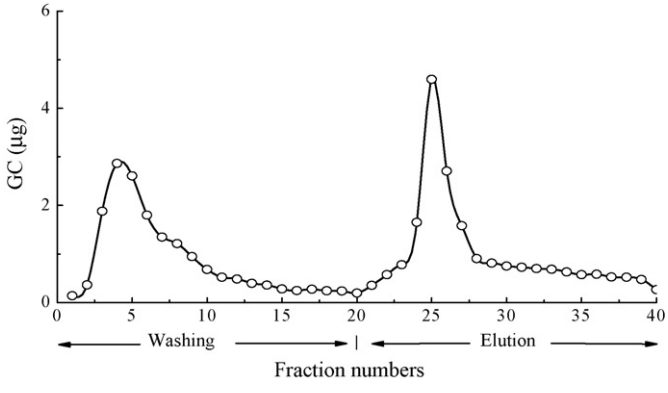

Fig. 3. Elution profile of GA from the crude extracts of *Shakuyaku kanzo to* (*Shaoyao gancao tang*) with an immunoaffinity column coupled with the anti-GA-MAb. For buffer systems and eluting conditions, see the text.

The rapid separation and quantification of bioactive constituents from the TCM or crude extracts using MAb techniques are a new approach in compound TCM (as *Shaoyao gancao tang* in this case) studies. Moreover, compared to HPLC method, the combination of the established immunoaffinity chromatography using anti-GA-MAb and competitive ELISA method provided a reliable and very high sensitive analysis for GA from different extracts of various medicinal herbs or other drugs. Therefore, it might be especially applicable to the screening of a large number of small samples with low concentration. It also might be a novel means for quality control in monitoring the GA concentration variation in TCM production, and in biochemical and pharmaceutical sciences.

Acknowledgements

The authors thank Dr. O. Morinaga, Dr. S.J. Shan, Ms M. Yoshida and Ms A. Fujino for their co-operative technical assistance in the experiment. This work is also in part supported by NSFC (Grant No. 30572316) and key laboratory of the Ministry of Education for cell biology and tumor cell engineering, Xiamen University (Grant No. 2005110).

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