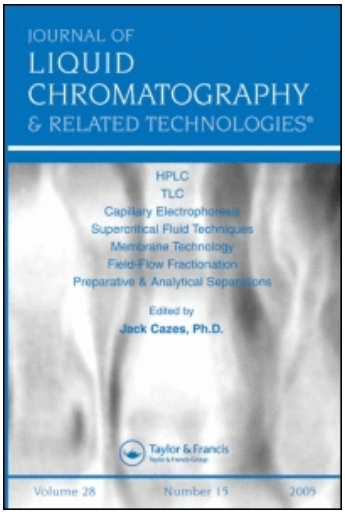

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S. J. Shan[a]; H. Tanaka[a]; J. Hayashi[b]; Y. Shoyama[a]
[a] Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan [b] Department of Medicine, Graduate School of Medicine, Kyushu University, Fukuoka, Japan

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WESTERN BLOTTING OF GLYCYRRHETINIC ACID GLUCURONIDES USING ANTI-GLYCYRRHIZIN MONOCLONAL ANTIBODY

S. J. Shan,[1] H. Tanaka,[1] J. Hayashi,[2] and Y. Shoyama[1,]*

[1] Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, and
[2] Department of Medicine, Graduate School of Medicine, Kyushu University, 3-1-1 Maidashi, Higahshi-ku, Fukuoka 812, Japan

ABSTRACT

A western blotting method for determining glycyrrhizin by using anti-glycyrrhizin monoclonal antibody was investigated. In this method, we found a new way to separate the glycyrrhizin molecule into two functional parts by using a simple and well-known chemical reaction. The sugar parts are oxidized to give dialdehydes, which react with amino groups on the protein that ties to the adsorbent membrane. The monoclonal antibody binds to the aglycone part of glycyrrhizin molecule. The immunocytolocalization of glycyrrhizin in *Glycyrrhiza* organs was investigated to indicate that the phloem contains a higher concentration of glycyrrhizin than that of the xylem. This result was confirmed by quantifying glycyrrhizin in the corresponding organs by an enzyme-linked

*Corresponding author.

immunosorbent assay using anti-glycyrrhizin monoclonal antibody.

INTRODUCTION

Licorice (*Glycyrrhiza* spp.) is one of the most important medicines used in traditional Chinese medicine and is prescribed with other herbal medicines as an antitussive, expectorant, and for improving taste.[1] These pharmaceutical properties are due to glycyrrhizin (GC) which has protein kinase inhibitory activity,[2] anti-ulcer,[3] and anti-viral[4] activities, and is now used in Japan as an antiallergic[5] and liver protection drug[6]. Although immunoassay systems using monoclonal antibodies (MAbs) are now indispensable in various investigations, not as many are available for naturally occurring bioactive compounds with smaller molecular weights, except against drugs like morphine.

No formation of MAb against GC has been reported yet, although immunological approaches for assaying quantities of glycyrrhetic acid using the anti-glycyrrhetic acid MAb have been investigated by Mizugaki et al.[7] In our studies of the formation of MAbs against naturally occurring bioactive compounds, we have established MAbs against forskolin,[8] solamargine,[9] opium alkaloid,[10] marihuana compounds,[11] crocin,[12] ginsenoside Rb1,[13] and developed their applications.[14,15]

Recently, we reported on the formation of MAb against GC.[16] Enzyme-linked immunosorbent assay (ELISA) was approximately 500 times more sensitive than the HPLC methods.[17-20] Moreover, this methodology may make possible the facile in vitro assay of biomedical samples, suitable for large numbers of samples, or of small samples in clinical uses of GC. Quality and quantity control of GC in traditional Chinese medicines, which are necessary for clinical use, are difficult because the concentration of GC in licorice depends on the species of *Glycyrrhiza* used and other factors. It may be possible to analyze the crude drugs by the combination of ELISA and western blotting. We now report on analysis of GC using ELISA and western blotting for the crude drugs of licorice and the traditional Chinese medicines containing licorice.

EXPERIMENTAL

Chemicals and Immunochemicals

GC, glycyrrhetic acid and its monoglucuronide were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Bovine serum albumin (BSA) and human serum albumin (HSA) were provided by Pierce (Rockford, IL, USA).

Peroxidase-labeled anti-mouse IgG was provided from Organon Teknika Cappel Products (West Chester, PA, USA). Polyvinylidene difluoride (PVDF) membranes (Immobilon™-N) were purchased from Millipore Corporation (Bedford, MA, USA). Glass microfiber filter sheets (GF/A) were purchased from Whatman International Ltd. (Maidstone, England). All other chemicals were standard commercial products of analytical grade.

ELISA for Glycyrrhetinic Acid Glucuronides

ELISA procedure was performed as described in a previous paper.[16] GC-HSA conjugate (5 molecules of GC per molecule of HSA) (100 μL, 1 μg/mL) was adsorbed to the wells of a 96-well immunoplate and then treated with 300 μL phosphate buffered saline (PBS) containing 5% of skim milk (SPBS) for 1 hr to reduce non-specific adsorption. Fifty μL of various concentrations of GC dissolved in 10% of methanol solution was incubated with 50 μL of IgG solution for 1 hr. The plate was washed three times with PBS containing 0.05% between 20 (TPBS), and then the MAb was combined with 100 μL of a 1:1000 dilution of peroxidase-labeled anti-mouse IgG for 1 hr. After washing the plate three times with TPBS, 100 μL of substrate solution was added to each well and incubated for 15 min. The absorbance was measured by a micro plate reader at 405 nm.

The cross-reactivities of anti-GC MAbs and related compounds were determined according to Weiler and Zenk's equation.[21]

Western Blotting for GC and Its Related Compound Using Anti-GC Mab, 5A8

Glycyrrhetic acid, its monoglucuronide, GC (1 μg each) and extracts of the traditional Chinese medicines including licorice or no licorice (named as Shaoyao Gancao Tang, Xiao Chaihu Tang, and Da Chaihu Tang, Tsumura Pharmaceutical Company, Tokyo, Japan), were applied to two silica gel TLC plates and developed with n-butanol-water-acetic acid (7 : 2 : 1). The developed TLC plate was dried and then sprayed with a blotting solution mixture of isopropanol-methanol-water (1 : 4 : 20, by volume). It was placed on a stainless steel plate, then covered with a piece of PVDF membrane sheet. After covering with a glass microfiber filter sheet, the preparation was pressed evenly for 50 sec at 120? on a hot plate as previously described.[22] The PVDF membrane was separated from the TLC plate and dried. The blotted PVDF membrane was dipped in water containing $NaIO_4$ (10 mg/mL) and stirred at room temperature for 1 hr.

After washing with water, 50 mM carbonate buffer solution (pH 9.6) containing BSA (1%) was added, and the preparation was stirred at room tempera-

ture for 3 hr. After washing the PVDF membrane with PBS, the membrane was treated with SPBS for 3 hr to reduce non-specific adsorption. The PVDF membrane was immersed in anti-GC MAb and stirred at room temperature for 1 hr. After washing the PVDF membrane twice with TPBS and water, 1000 times dilution of peroxidase-labeled goat anti-mouse IgG in PBS containing 0.2% of gelatin (GPBS) was added and stirred at room temperature for 1 hr. The PVDF membrane was washed twice with TPBS and water, then exposed to 1 mg/mL 4-chloro-1-naphthol-0.03% H_2O_2 in PBS solution, which was freshly prepared before use for 10 min at room temperature, and the reaction was stopped by washing with water. The immunostained PVDF membrane was allowed to dry.

Immunocytolocalization of GC

A fresh sliced *Glycyrrhiza* root was placed on the PVDF membrane, and pressed evenly for 6 h. The blotted PVDF membrane was dipped in water containing $NaIO_4$ (10 mg/mL) while stirring at room temperature for 1 hr, and then treated by the same procedure as described above.

RESULTS AND DISCUSSION

Direct detection of small molecular compounds on a TLC plate by immunostaining is impossible. GC and its related compounds on PVDF membrane are washed out by buffer solution or water without fixing. Therefore, we found a new method that separates the GC molecule into two functional parts by using a simple and well-known chemical reaction. The sugar parts are oxidized by $NaIO_4$ to give dialdehydes, which react with amino groups in protein that ties to the adsorbent membrane. The monoclonal antibody binds to the aglycone part of molecule. This reaction enhanced the fixing of GC-BSA conjugates on the PVDF membrane.

The PVDF membrane incubated in the absence of $NaIO_4$ was essentially free of staining of GC (data not shown). Therefore, the conjugation step between sugar moiety and protein is necessary for this western blotting. Fig. 1 indicates the schematic process of western blotting of GC. Fig. 2 shows the H_2SO_4 staining (A) and western blotting (B) of GC and various samples. Lanes 1 to 3 show the spots of GC, glycyrrhetic acid monoglucuronide and glycyrrhetic acid, respectively.

The western blotting method was considerably more sensitive than the H_2SO_4 staining. A GC aglycone, glycyrrhetic acid (1ane 3) was not detected by western blotting, indicating that the newly established western blotting requires sugar moieties. It is suggested that the specific reactivity of sugar moiety in GC

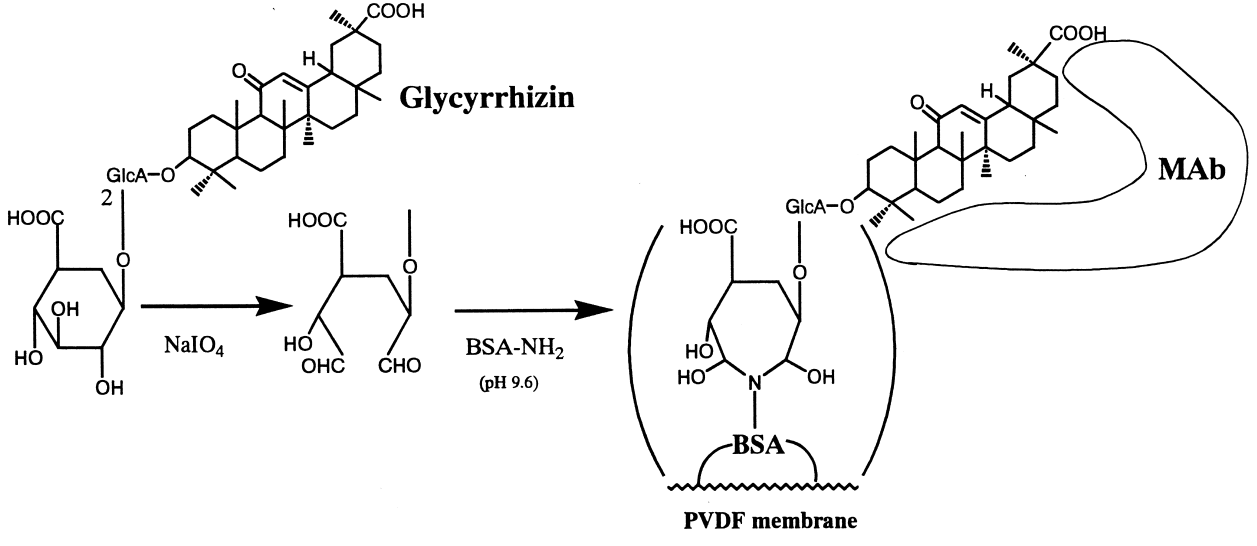

Figure 1. Schematic process for western blotting of GC in PVDF membrane using Anti-GC MAb. GC developed on TLC was transferred to PVDF membrane, and the membrane was treated by $NaIO_4$ to cleave the glucuronic acid moiety. Addition of BSA gave GC-BSA conjugate to fix to PVDF membrane. GC moiety was detected and stained by MAb.

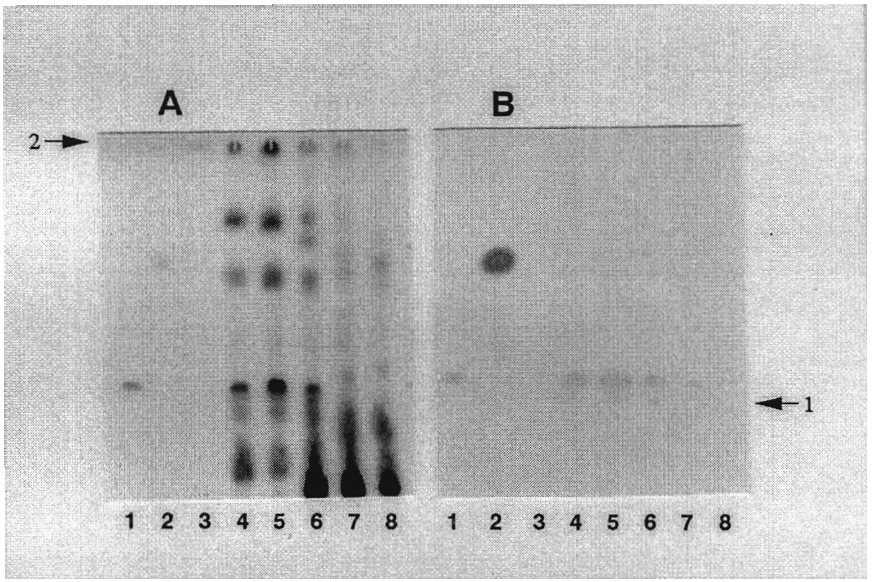

Figure 2. Western blotting of GC and related compounds. A was stained by H_2SO_4. GC (lane 1), glycyrrhetic acid 3-O-glucuronide (lane 2), glycyrrhetic acid (lane 3), *Glycyrrhiza uralensis* (lane 4), *Glycyrrhiza glabra* (lane 5), Shaoyao Gancao Tang (lane 6), and Xiao Chaihu Tang (lane 7) containing licorice, Da Chaihu Tang without licorice (lane 8).

against MAb may be modified by the $NaIO_4$ treatment of GC on the membrane, causing glycyrrhetic acid monoglucuronide which has a small cross-reactivity (4.36%) as reported previously,[16] to become detectable by the western blotting as indicated in Fig. 1-B, lane 2. The phenomenon that glycyrrhetic acid monoglucuronide is more sensitive than that of GC may depend on their blotting efficiencies, because GC is strongly bonded to the silica gel TLC plate due to two carboxylic acid groups in a molecule.

Although the H_2SO_4 staining (Fig. 2-A) detected more spots including sugars and different types of saponins (Fig. 2-A lanes 4 and 5) in the traditional Chinese medicine samples containing licorice, the western blotting (Fig. 2-B) detected only GC, together with a small amount of unknown compound (arrow-1) which may be structurally related to GC. One prescription without licorice, Da Chaihu Tang, is probably free of staining by the western blotting, as indicated in Fig. 2-B, lane 8.

We analyzed the GC concentrations contained in licorice and the traditional Chinese medicine samples prescribed with and without licorice by ELISA. Table 1 indicates that the crude drugs of *G. uralensis* and *G. glabra* contained higher

Table 1. Glycyrrhizin Contents in Different Licorice Roots and Traditional Chinese Prescriptions

Sample	Content (mg/g dry drug)
Licorice root	
G. glabra	34.87 ± 3.39
G. uralensis	27.48 ± 1.25
Prescription	
Shaoyao Gancao Tang	15.77 ± 2.89
Xiao Chaihu Tang	1.42 ± 0.34
Da Chaihu Tang	0

Data were mean ± SD from three replicate wells for each concentration.

concentration of GC, 27.48 and 34.7 mg/g dry weight, respectively. Although Shaoyao Gancao Tang and Xiao Chaihu Tang prescribed with licorice also contained GC, 15.77 and 1.42 mg/g dry weight, respectively, Da Chaihu Tang prescribed without licorice did not. These results showed good agreement with the strength of staining as indicated in Fig. 2-B.

One other application of this method, the immunocytolocalization of GC in *Glycyrrhiza* root, was investigated. Fig. 3 indicates the immunocytolocalization

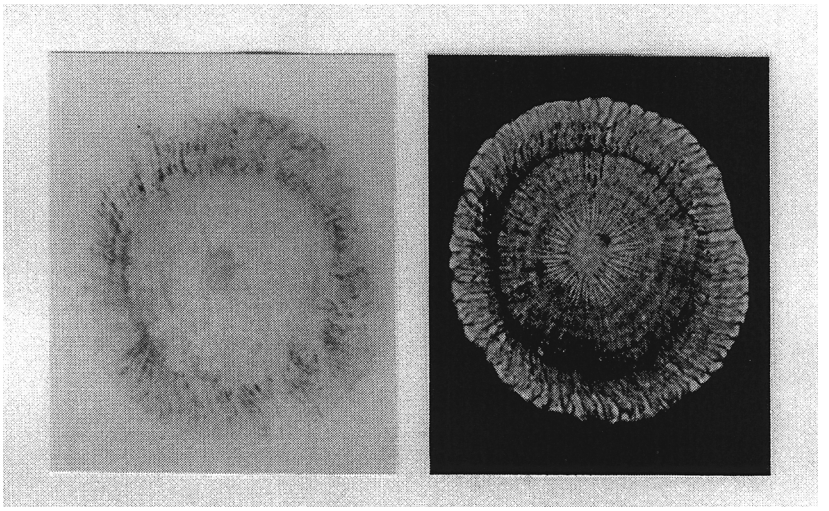

Figure 3. Immunocytolocalization of GC using anti-GC MAb in *Glycyrrhiza inflata* root. Left: western blotting, right: *G. inflata* root.

Table 2. Glycyrrhizin Contents in Different Parts of *G. inflata* Root Sample Used for Western Blotting

Part	Content (mg/g dry drug)
Cork layer	2.05 ± 0.25
Phloem	54.92 ± 1.48
Xylem	32.97 ± 2.34

Data were mean ± SD from three replicate wells for each concentration.

of GC in fresh *Glycyrrhiza inflata* root. The phloem contains a higher concentration of GC than that of the xylem. Since it became evident that the distribution of GC was different in organs, individual organs were assayed by ELISA. The concentrations of GC in cork, phloem, and xylem are 2.05, 54.92, and 32.97 mg/g dry weight, respectively (Table.2), indicating good agreement with a previous report.[23] Therefore, a newly established western blotting method can be routinely used to survey concentrations of GC in drugs and/or in animal plasma samples as a simple and rapid analysis. In future studies on this topic, this method will be described for the histochemical analysis of the GC distribution in animal organs or tissues. These results will be communicated elsewhere.

ABBREVIATIONS

MAb, monoclonal antibody; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HSA, human serum albumin; PBS, phosphate buffered saline; GPBS, PBS containing 0.2% of gelatin; TPBS, PBS containing 0.05% of Tween 20; SPBS, PBS containing 5% of skim milk; ABTS, 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt.

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