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Review Article

Standardization of Licorice and TCM Formulations Using Eastern Blot Fingerprinting Analysis

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To prepare the antiglycyrrhizin (GC) monoclonal antibody (MAb), GC was treated with $NaIO_4$ resulting in aldehyde which can be combined with carrier protein. An antigen conjugate was performed by a matrix-assisted laser desorption/ionization TOF mass spectrometry to determine the hapten numbers in the conjugate. Anti-GC MAb was prepared from a hybridoma which was fixed from the spleen cells producing anti-GC MAb and the myeloma cells after immunization. The TCM and licorice extract were developed by TLC and blotted to a polyvinylidene difluoride (PVDF) membrane. The membrane was treated by $NaIO_4$ and protein, enzyme labeled secondary MAb, and finally substrate was added. Clear spot appeared on PVDF membrane identifying GC against a background containing large amount of impurities. In eastern blotting, the GC molecule was divided into two functions. The aglycone part is recognized as an epitope and the sugar moiety can be combined to membrane. The specific reactivity of sugar moiety in the GC molecule against anti-GC MAb might be modified by the $NaIO_4$ treatment on the membrane because glycyrrhetic acid 3-O-glucuronide can be stained although the cross-reactivity is only 4.3%. Eastern blotting for GC can not only apply for the standardization of licorice and TCM, but also it can open for the other bioactive products.

1. Introduction

Licorice is one of the most important herbal medicines used in traditional Chinese medicine (TCM) and is now prescribed in approximately 70% or more formulas with other herbal medicines in Japan as an antitussive, an expectorant, a taste-modifying agent, and for relieving pain [1]. This pharmacological evidence mainly depends on glycyrrhizin (GC) having various pharmacological activities like protein kinase inhibitory [2], antiulcer [3], and antiviral [4] activities, and it has been developed as a medicine for liver diseases [5] and as an antiallergenic phenomena [6] in Japan from approximately 60 years ago.

Since several *Glycyrrhiza* species are widely distributed mainly in China and through Europe to Siberia, the quality control is somehow complicated. Figure 1 indicates *G. uralensis* growing in Mongolia and its constituents. Therefore, we previously investigated the DNA analysis and components like glycyrrhizin (GC) and flavonoids of licorice in China for the standardization of licorice. The four kinds of DNA regions were internal transcribed spacer on nuclear ribosomal DNA, *rbcL* gene, *matK* gene, and trnH-*trnKI* intergenic region on chloroplast DNA. Among three species of licorice *G. glabra*, *G. inflata* and its hybrid have been distributed in the eastern area; on the other hand *G. uralensis* is distributed in the western zone in China [7]. These results are well correlated to those of the component analysis [8]. This evidence is also deeply related to the standardization of licorice.

Many methods have been employed in the determination of phytochemical constituents for the standardization of herbal medicines. They include spectral methods such as infrared, nuclear magnetic resonance, and circular dichroism and other chromatographic methods such as ion chromatography, capillary electrophoresis (CE), and high-speed counter current chromatography. Compared to TLC, GLC, and HPLC methods, the immunological method is more sensitive and selective.

Immunoassay systems using monoclonal antibody (MAb) against drugs and small molecular bioactive compounds have become an important tool for investigations like

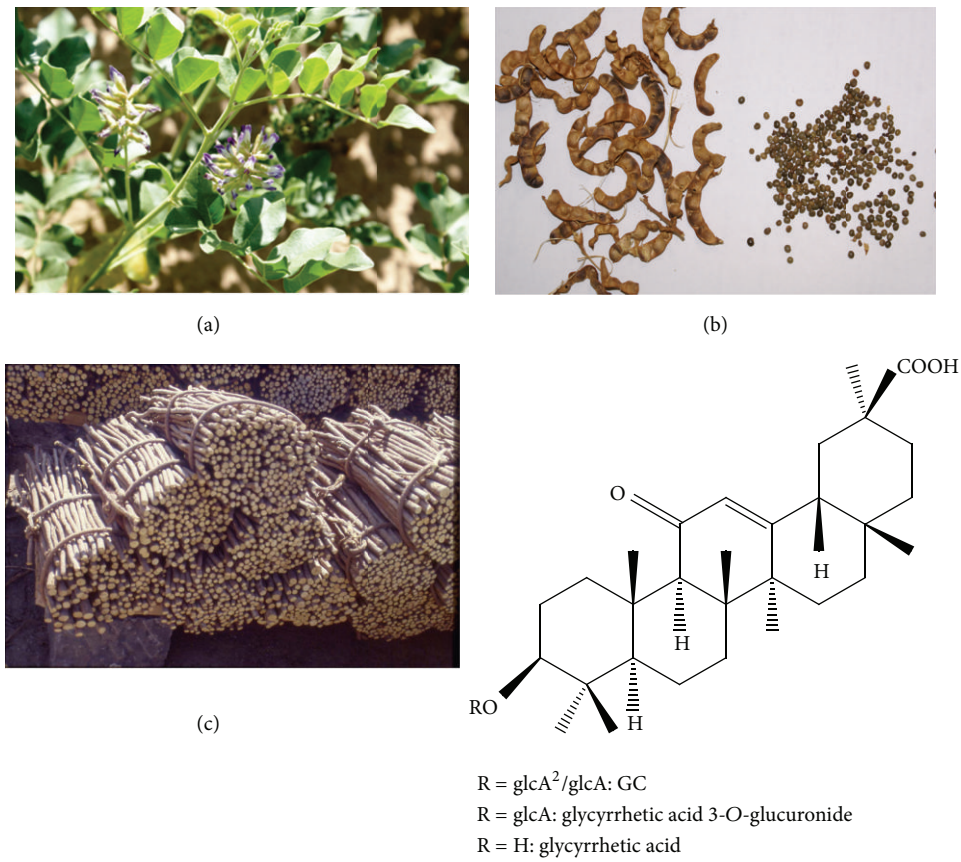

Figure 1: *Glycyrrhiza uralensis* Fischer and structures of glycyrrhizin and related compounds: (a) flowering, (b) fruits and seeds, and (c) licorice roots.

enzyme assay and quantitative and/or qualitative analytical techniques in herbal medicines owing to their specific affinity and high sensitivity. Previously we prepared various kinds of MAbs against natural products like forskolin [9], solamargine [10], crocin [11], marihuana compound [12], opium alkaloids [13], ginsenosides [14, 15], berberine [16], sennosides [17, 18], paeoniflorin [19], GC [20, 21], ginkgolic acid [22], aconitine alkaloid [23], baicalin [24], and so on and developed individual competitive enzyme-linked immunosorbent assay (ELISA) as a high sensitive, specific, and simple methodology. Furthermore, in our ongoing MAb investigation we succeeded to prepare knockout extract and its application using an immunoaffinity column conjugated with MAb [25, 26] and to create a transgenic plant constructed with compact MAb, single-chain Fv gene called missile-type molecular breeding resulting in higher concentration of target component, antigene compound [27, 28].

Since the confirmation of hapten number in a synthesized antigen is the most important in the first stage of MAb preparation, its determination method will be discussed first of all. An MAb against GC has been prepared resulting in that the ELISA is set up and the eastern blotting method is newly developed as an application of MAb and is named [21]. It will be introduced in this review.

2. Determination of Hapten Number in Hapten-Carrier Protein Conjugate

For production of MAb, the synthesis of hapten which is derived from immune antigen and linker bridge and carrier protein conjugates is necessary. There had been no direct and appropriate methods for the determination of hapten conjugated with carrier protein without differential UV analysis and radiochemical or chemical methods. Therefore, immunization by the injection of hapten-carrier protein conjugate was unreliable. We set up the direct analytical method of hapten and carrier protein conjugates by matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry using an internal standard.

A small amount of the antigen conjugate was mixed with an excess of sinapinic acid in an aqueous solution containing trifluoroacetic acid. The mixture was placed inside a MALDI-TOF mass monitor and irradiated with an N_2 laser. The ions formed by each pulse were accelerated and detected [29–31].

Figure 2 shows the co-MALDI-TOF mass spectrum of forskolin-BSA conjugate and BSA used as an internal standard. This shows only singly, doubly, and triply ionized molecule ions of the intact conjugate. The sharp peak at m/z 66928 (calculated m/z; 66267) is $[M + H]^+$ of BSA. A bigger

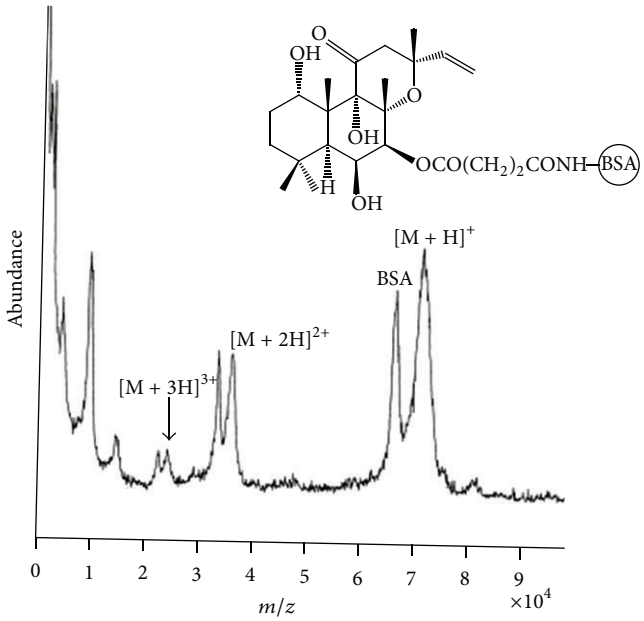

Figure 2: MALDI-TOF mass spectrum of forskolin-bovine serum albumin (BSA) conjugate and BSA as an internal standard.

$[M + H]^+$ peak of the forskolin-BSA conjugate is at m/z 72160 (calculated; 71447). The calculated molecule mass of the forskolin moiety is 5180. From this result, 11.5 molecules of forskolin combined with BSA [30]. This method is suitable for small molecule natural products including glycosides like GC [21]. From this result, all conjugates have been analyzed by MALDI-TOF mass to confirm the hapten number by this way before immunization.

3. Preparation of MAb against GC

GC having sugars in a molecule was treated by $NaIO_4$ solution to cleavage the sugar moiety and release aldehyde group which can be combined with a carrier protein. The synthesized antigen was injected to mice and antiglycyrrhizin MAb was prepared by the ordinal method in our laboratory [9] as follows.

A hybridoma producing MAb reactive to GC was obtained by the general procedure in our laboratory [9] and classified into IgG1 which had K light chains. The reactivity of IgG type MAb 5A8 was tested by varying antibody concentration and by performing a dilution curve. The antibody concentration was selected for competitive ELISA. The free MAb following competition is bound to polystyrene microtiter plates precoated with GC-human serum albumin (HAS). Under these conditions, the full measuring range of the assay extends from 20 to 200 ng/mL [20].

Cross-reactivity is the most important factor in determining the value of an antibody. Since the ELISA for GC was established for phytochemical investigations involving crude plant extract, the assay specificity was checked by determining the cross-reactivity of the MAb with various related compounds. The cross-reactivity data of MAb were examined by competitive ELISA and calculated using picomole of GC. The cross-reactivity of glycyrrhetic acid 3-O-glucuronide, glycyrrhetic acid, and 11-deoxy-18β-glycyrrhetic acid was weak compared to GC, 4.36%, 2.13%, and 2.32%, respectively. Other components in licorice showed no cross-reactivity (less than 0.02%). It is evident that the MAb reacted only and very weakly with a small number of structurally related GC molecules and did not react with other saponins like ginsenoside Rb1, saikosaponin A, and solamargine [20, 21].

The results of quantitative analysis of GC in licorice roots and TCM by ELISA were in good agreement with those from the HPLC analysis [32].

4. Fingerprinting by Eastern Blotting

Although the western blotting is a common assay methodology for substances of high molecular weights, this method has not been employed for small molecules, as direct immunostaining of such compounds on a TLC plate is yet unknown. Therefore, a new method for such small molecular compounds is required. Moreover, if small molecules can be blotted to a membrane, fixing it also needs a new methodology. Previously, we succeeded in separating the function of small molecule compounds such as solasodine glycosides into a part of epitope and fixing it on the membrane as follows [33].

Eastern blotting was started from the development of components by TLC plate. The developed TLC plate was covered by polyvinylidene difluoride (PVDF) membrane and a blotting solution was added, and then heated for a short period. The blotted PVDF membrane was treated by $NaIO_4$ solution following the addition of BSA [29]. The conjugated glycoside like GC on membrane was washed and treated with anti-GC MAb and then peroxidase-labeled goat anti-mouse IgG MAb. Finally, the PVDF membrane was exposed to substrate, 4-chloro-1-naphthol, and H_2O_2 solution. For staining by anti-GC MAb, the blotted PVDF membrane was treated in the same way as anti-GC MAb except that it was exposed to 3-amino-9-ethylcarbazole and H_2O_2 solution.

Figure 3 shows the H_2SO_4 staining and eastern blotting of GC standards, *Glycyrrhiza* species, and TCM using anti-GC MAb. It is impossible to determine the GC by TLC-stained by H_2SO_4 as indicated in Figure 3(a). Clear staining of GC occurred by eastern blotting (Figure 3(b)). Furthermore, it became evident that Da Chaihu Tang prescription (Figure 3(b) line 8) did not contain licorice indicating no band of GC. The eastern blotting method was considerably more sensitive than that of H_2SO_4 staining. The H_2SO_4 staining detected all compounds indicating complicated fingerprinting (Figure 3(a)). On the other hand, the eastern blotting indicated only limited staining of GC as shown in Figure 3(b). From these results, it is clear that this methodology can be applied for the chromatographic fingerprinting of GC in TCM formulas and/or in *Glycyrrhiza* species as already discussed above and reported previously [32].

We suggest that an aglycone, glycyrrhetic acid and part of the sugar may be of importance to immunization and may function as an epitope for the structure of GC since

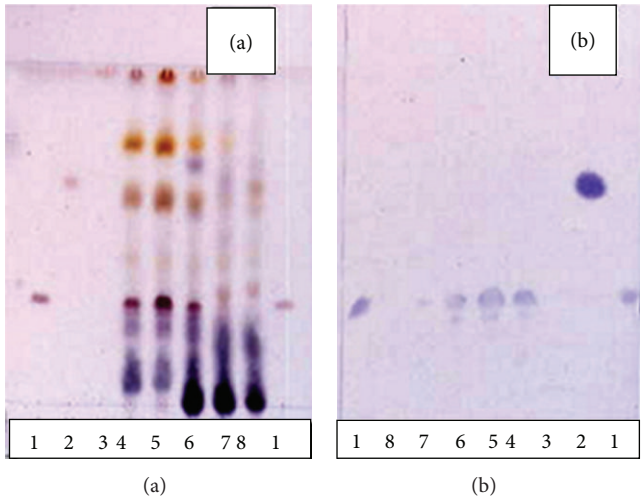

Figure 3: Chromatographic fingerprintings of TCM prescribed licorice, *Glycyrrhiza* species, standard of glycyrrhizin, and glycyrrhetic acid O-glucuronide by eastern blotting using antiglycyrrhizin MAb (b) and TLC stained by H_2SO_4 (a). 1: glycyrrhizin, 2: glycyrrhetic acid O-glucuronide, 3: glycyrrhetic acid, 4: *G. uralensis*, 5: *G. glabra*, 6: Shaoyao Gancao Tang, 7: Xiao Chaihu Tang, 8: Da Chaihu Tang.

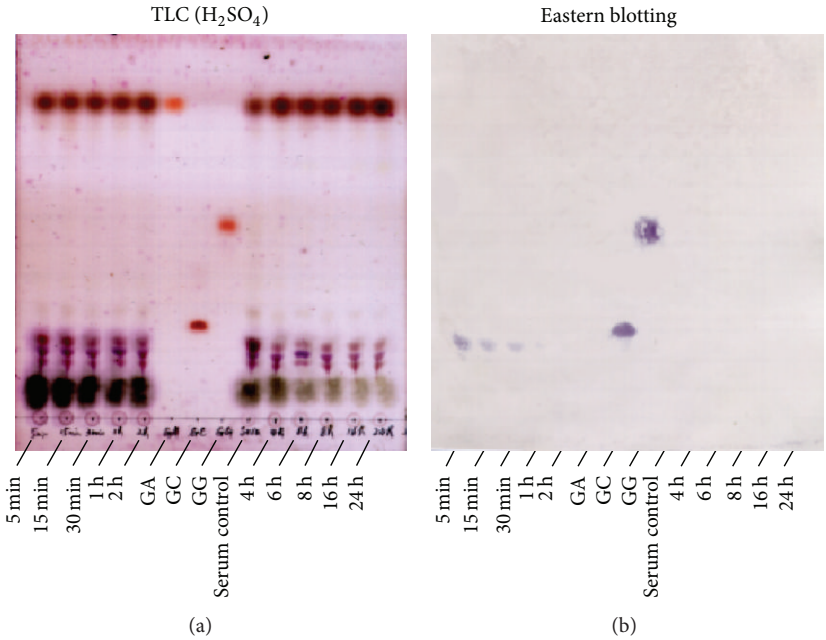

Figure 4: Chromatographic fingerprinting of glycyrrhizin after injection of glycyrrhizin in rat serum by TLC stained by H_2SO_4 and eastern blotting. (a) TLC chromatographic fingerprinting stained by H_2SO_4. (b) Chromatographic fingerprinting by eastern blotting. GA, GC, and GG were glycyrrhetic acid, glycyrrhizin, and glycyrrhetic acid O-glucuronide, respectively. GC was detected until 1 hour after injection.

glycyrrhetic acid 3-O-glucuronide (Figure 3(b) line 2) can be stained (see Figure 4 also) despite 4.3% of cross-reactivity (data not shown). From this evidence, it is suggested that the specific reactivity of sugar moiety in the GC molecule against anti-GC MAb may be modified by the $NaIO_4$ treatment of GC on the PVDF membrane.

Although not related to quality control of herbal medicines, the other chromatographic fingerprinting of GC can be indicated in Figure 4. Application of the eastern blotting method for the detection of GC in the serum samples was investigated. In general, it is difficult to detect GC in serum due to a large amount of impurities. The developed bands of impurities lapped over the band of GC, and we failed to find a proper developing solvent system that could separate GC and the impurities apart from each other clearly on the TLC plate. Figure 4 shows, however, the detection of GC by the eastern blotting technique in the rat serum samples. GC could not be identified on the TLC plate stained by H_2SO_4 though many bands were detected (Figure 4(a)). On the other hand, eastern blotting clearly shows the band of GC even after 1 h.

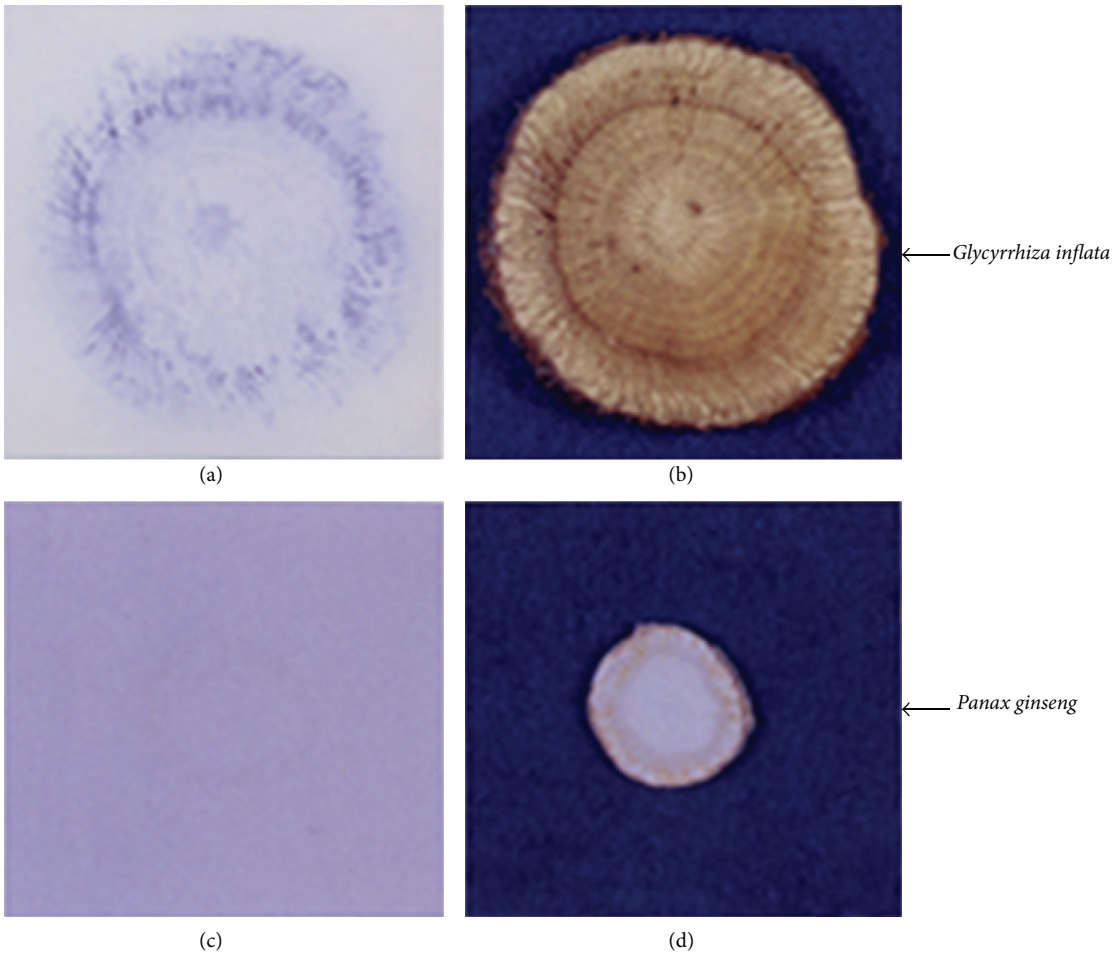

Figure 5: Direct eastern blotting of licorice slice using antiglycyrrhizin MAb. (a) Eastern blotting of licorice slice, (b) licorice slice, (c) eastern blotting of ginseng slice using antiglycyrrhizin MAb, and (d) slice of ginseng.

Although the sensitivity of the eastern blotting method was greatly affected by the impurities, the detection limit was still at the nanogram level. Interestingly glycyrrhetic acid was clearly detected by TLC stained by H_2SO_4 (Figure 4(a) line GA), but not in eastern blotting (Figure 4(b) line GA). This is the reason why the sugar moiety is essential for the eastern blotting of GA because the sugar part can be conjugated to a PVDF membrane through an aldehyde-type intermediate into GC-protein conjugate as discussed already. The results proved that the eastern blotting technique could be a unique method for identifying GC against a background of a large amount of impurities.

In the early stage, the blotted staining on PVDF membrane using MAb was successful in solasodine glycosides and was called western blotting [33]. From this evidence, we applied this new methodology to licorice glycoside, GC, and named it as eastern blotting [21] following ginsenosides [34], saikosaponin A [35], baicalin [36], sennoside A and B [37], and aristrochic acids [38].

Figure 5 indicates immunolocalization of GC in the licorice root slice using anti-GC MAb as another application of the eastern blotting method showing that the phloem contained a higher concentration of GC than the xylem [21]. When different *Glycyrrhiza* species like *G. uralensis*, *G. glabra,* and *G. inflata* were compared after stained by eastern blotting, the different distribution pattern was observed (data not shown). This direct eastern blotting also can help in the standardization of *Glycyrrhiza* species.

Figure 6 indicated the hypothetic scheme of eastern blotting for GC. In this staining system, GC molecule was divided into two functions. One is epitope, aglycone part of GC against MAb, and the other is fixing function, sugar moiety on membrane. However, as discussed previously the specific reactivity of sugar moiety in the GC molecule against anti-GC MAb might be modified by the $NaIO_4$ treatment of GC on the PVDF membrane because glycyrrhetic acid 3-O-glucuronide can be also stained although the cross-reactivity is only 4.3% [21].

On our ongoing studies, we developed a new assay system for GC called an immunochromatographic assay. This assay system is based on a competitive immunoassay using anti-GC MAb and a detector reagent that contains colloidal gold

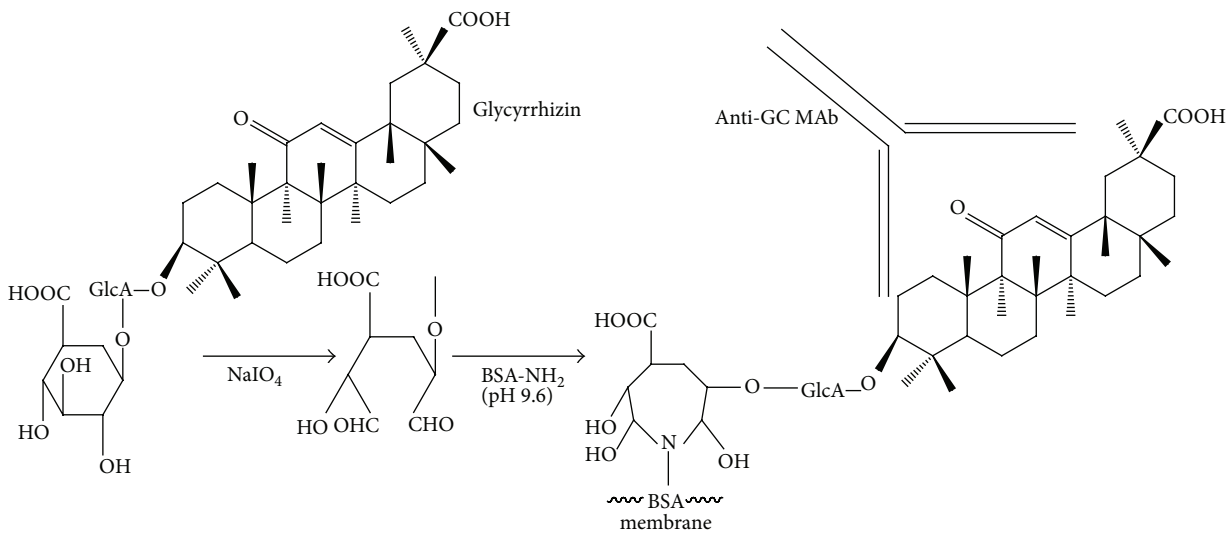

Figure 6: Scheme of eastern blotting for glycyrrhizin using antiglycyrrhizin MAb. Sugar moiety was cut by $NaIO_4$ to lease aldehyde group which conjugated to protein which can be fixed to membrane. An aglycone part was recognized by MAb resulting in staining.

particles coated with anti-GC MAb can be used [39]. This assay system also opened the standardization of TCM and/or *Glycyrrhiza* species by chromatographic fingerprinting. Furthermore, we prepared a surface plasmon resonance-based immunosensor using anti-GC MAb and succeeded the quite high sensitive detection of GC [40].

5. Conclusion

We developed a new staining method, eastern blotting. In this system, we found that GC molecule can be separated into two functional parts. The sugar moiety is oxidized to give dialdehydes, which react with amine groups of lysine and/or arginine of the protein that can bind strongly to the absorbent membrane, PVDF. The aglycone part of the GC molecule is bound by the anti-GC MAb for visualization of GC by the enzyme-labeled specific antibody. The method is shown diagrammatically in Figure 6. This hypothesis was confirmed from the evidences that an aglycone of GC, glycyrrhetic acid, could not be detected by eastern blotting but it could be detected on the TLC stained by H_2SO_4 as discussed already. The limit of detection (LOD) of eastern blotting against GC is 8 ng although the LOD is lower than that of ELISA indicating 1 ng of LOD against GC [21]. Therefore, this assay system can be used as a first screening of GC concentration in *Glycyrrhiza* species in the breeding program, for the analysis of TCM prescribed licorice and/or for the kinetic survey of GC in serum as a semiquantitative analysis because of good correlation to the ELISA using anti-GC MAb [32]. This methodology opened the chromatographic fingerprinting analysis of the other species like *Panax* [30], *Solanum* [29], *Bupleurum* [24], *Scutellaria* [32], *Rheum* [33], and *Aristolochia* [34] species. The eastern blotting for the late 2 species was successful by using the modification of carboxylic acid group in a molecule for conjugation with protein [33, 34].

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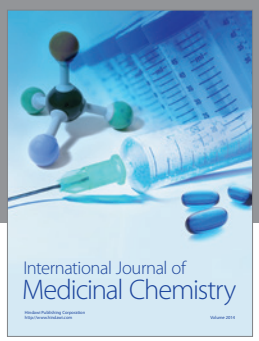

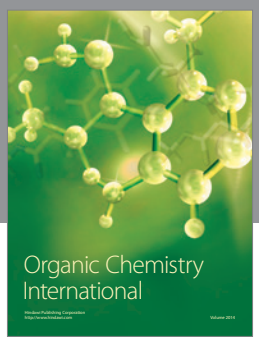

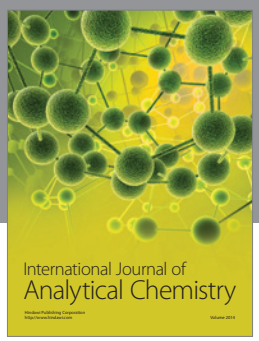

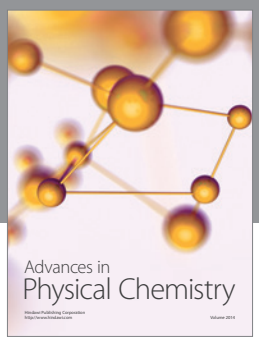

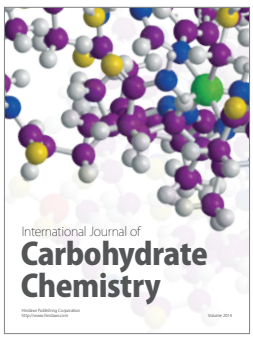

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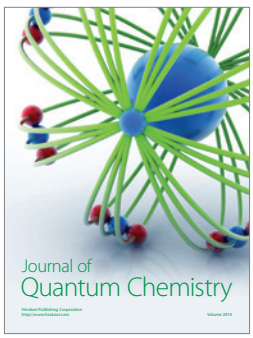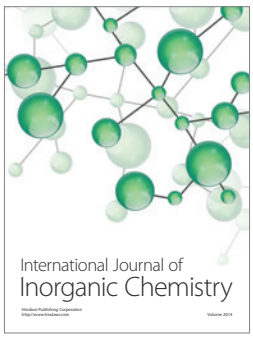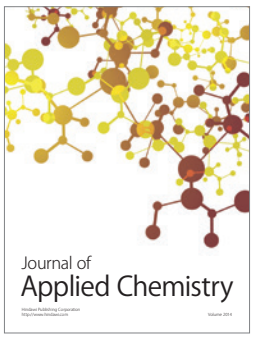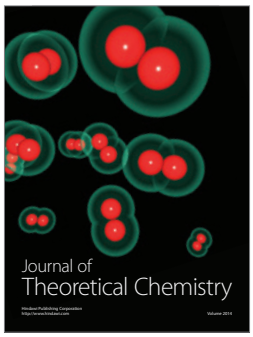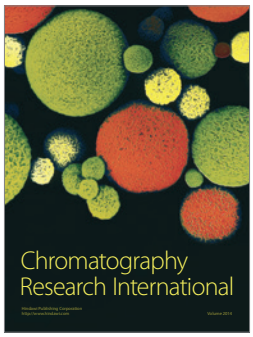
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