

Quality Control of *Bupleurum* Species by Newly Established Eastern Blotting

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1. Introduction

In the investigation of staining technique for several compounds, a polyvinylidene difluoride (PVDF) membrane was found as transfer membrane (Towbin et al., 1979), and applied to western blotting technique that utilizes antigen-antibody binding property for the specific and sensitive detection of peptides and proteins by immunostaining (Granger, 1988; Reig & Klein, 1988). In the case of low molecule compounds, thin-layer chromatography (TLC) immunoblotting of glycosphingolipids was reported using a nitrocellulose membrane (Towbin et al., 1984), but the transfer efficiency from TLC plate to the membrane was poor and not constant. Recently, the direct TLC immunostaining of glycolipids and glycosphingolipids was demonstrated without the blotting step from TLC plate to the membrane (Meisen, 2004; Suetake & Yu, 2003). Furthermore, TLC immunostaining of gangliosides (Miyamoto et al., 2006) and dot blot analysis of gangliosides on PVDF membrane (Chabraoui et al., 1993) were reported as the direct immunostaining. These immunostaining techniques were limitedly applied for glycolipids, glycosphingolipids and gangliosides because the other low molecule compounds were easily washed out into the buffer solution without fixing on the TLC plate or PVDF membrane.

Since small molecular compound such as saponin cannot fix on the PVDF membrane or TLC plate, no success of immunostaining for saponin has been reported. We reached to a new idea that a saponin was divided into two functional parts, sugar and aglycone moieties. Sugar parts in saponin could be oxidized to give aldehyde groups which were conjugated with carrier protein like bovine serum albumin (BSA) and then fixed onto the PVDF membrane as saponin-BSA conjugate because BSA was strongly binded on the membrane. In contrast, the aglycone part was recognized as epitope by monoclonal antibody (MAb) against saponin and then we succeeded to immunostaining of saponin on the PVDF membrane. The first success was reported on the immunostaining of solasodine glycosides by our group (Tanaka et al., 1997) followed ginsenosides (Fukuda, 1999, 2000, 2001). In 2001 we suggested a new name, [eastern blotting] for glycyrrhizin as a new immunostaining technique (Shan et al., 2001).

In eastern blotting the developed TLC plate was covered with PVDF membrane and all contents were blotted from TLC plate to the membrane by press and heating. After the

blotting, the membrane was treated with sodium periodide and then immersed in BSA solution at alkaline condition for the preparation of hapten-BSA conjugate on the membrane. After blocking with skimmed-milk, the membrane was treated by MAb which recognize a hapten and then a second antibody labeled with peroxidase directed against first antibody. Finally a substrate is added resulting in staining (Fig. 1). Fig. 2 shows the mechanism of binding and immunostaining of saikosaponin a (SSa) onto the PVDF membrane as previously reported (Zhu et al., 2007). Eastern blotting could stain only hapten molecule on the PVDF membrane. However, since the transfer efficiency at blotting step was difficult to control, this method could not be applied for the quantitative analysis system. Therefore, we improved staining system into the direct staining without transfer to membrane from TLC plate. In this chapter we will discuss about a newly developed eastern blotting.

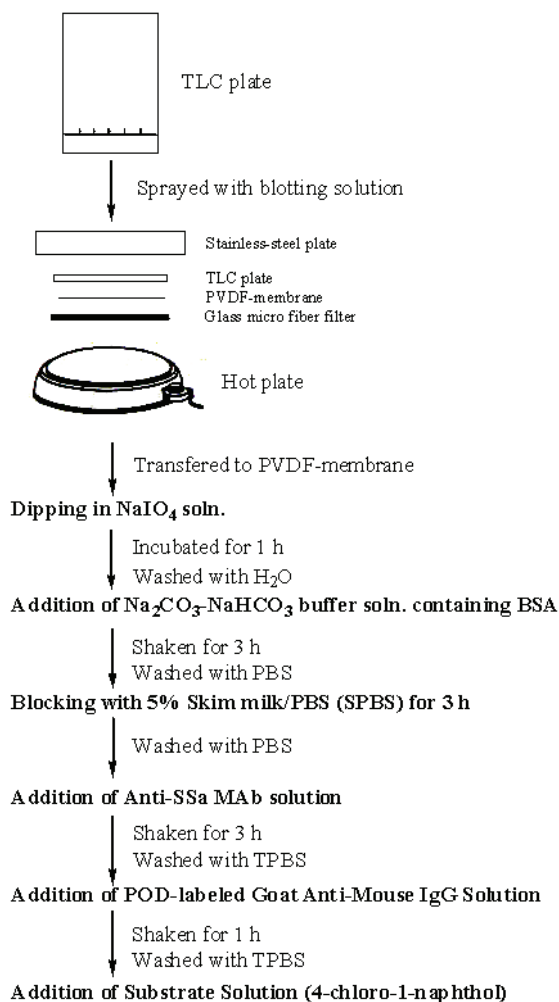


Fig. 1. Eastern blotting protocol

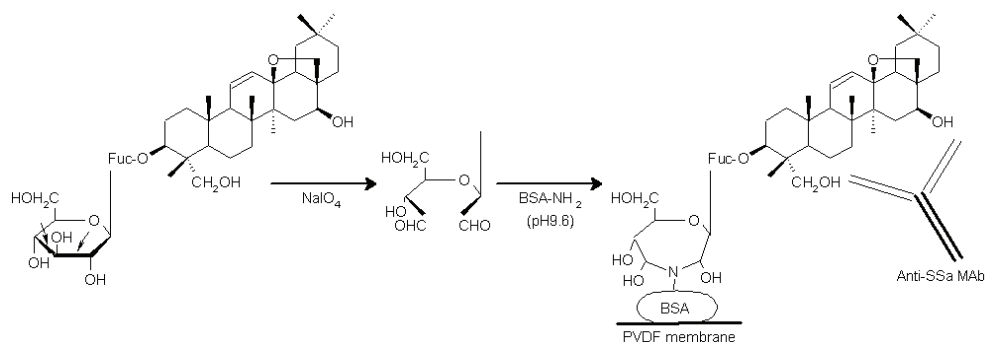


Fig. 2. Schematic diagram illustrating the immunodetection of SSa onto a PVDF membrane

Three major oleanane-saponins named saikosaponins such as SSa, SSc, and SSd (Fig. 3) and many minor saponins are isolated from the roots of *Bupleurum* species. SSa has anti-cancer, anti-inflammatory, corticosterone secreting and plasma-cholesterol decreasing activities. SSb₂ performs phagocytosis-increasing activity on macrophages. SSd shows liver protective effect, anti-inflammatory, immunoregulatory, virus inactivating and anti-cancer activities. Furthermore, in a prescription of Kampo medicine (Shosaikoto) containing *Bupleuri radix*, SSa, SSb₂, SSc, and SSd showed anti-tumor effects. Phytochemical and pharmacological analyses of Kampo medicine is extremely difficult due to combinatorial use of multiple herbs. In addition, because Kampo medicines are manufactured as highly standardized ethical drugs in Japan, a method of comprehensive analysis of the compounds for scientific quality control is urgently needed.

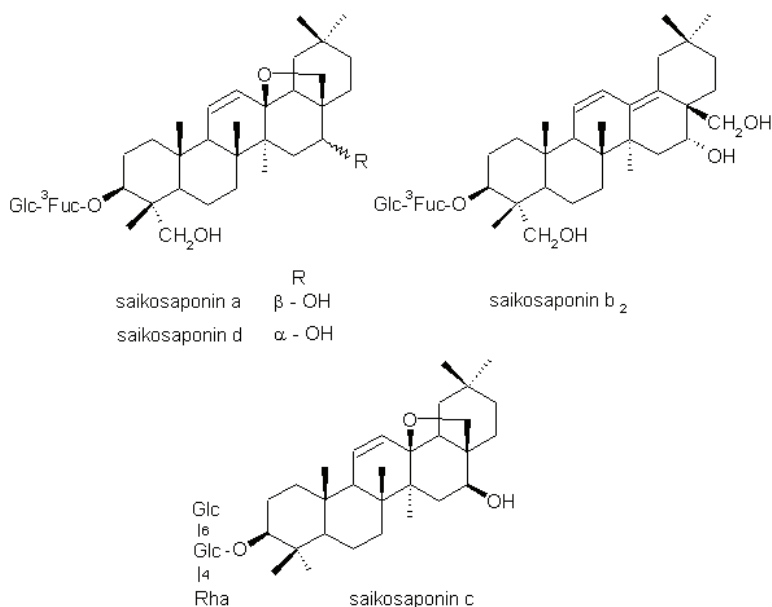


Fig. 3. Chemical structures of saikosaponins

Recently we prepared a specific MAb against SSa (Zhu et al., 2004) and a MAb having wide cross-reactivity to saikosaponins (Zhu et al., 2006), and developed an enzyme-linked immunosorbent assay (ELISA) system for quantitative measurement of SSa and/or total saikosaponins. However, it is difficult to gain the each content of SSa, SSb₂, SS_c and SS_d by these competitive ELISA methods. In pharmacological investigation of Kampo medicine, it is very important to grasp each bio-active compound concentration in each crude drugs prescribed individually, because individual saikosaponins have significant pharmacological effects as indicated above.

Therefore, we described here an unique approach, immunodetection of SSa, SS_c, and SS_d by new eastern blotting using anti-SSa MAb and its application to quantitative immunoassay using NIH Image for the estimation of saikosaponins in crude drugs and Kampo medicines containing Bupleuri radix for quality control as previously reported (Morinaga et al., 2006).

2. MAbs against SSa and saikosaponins and their developments for ELISA system

Immunizations, hybridizations and purifications of MAbs against SSa (1G6) and saikosaponins (3G10) and their developments of ELISA system for determination of SSa and total saikosaponins have been established (Zhu, 2004, 2006). Cross-reactivities are the most important factor in phytochemical investigations in which there are many structurally related compounds (see Table 1).

Compound	MAb-1G6	MAb-3G10
Saikosaponin a	100	100
Saikosaponin b ₂	<0.25	63.77
Saikosaponin c	2.65	28.52
Saikosaponin d	3.76	15.62
Digitonin	<0.25	1.42
Solasonine	<0.25	<0.25
Solamargine	<0.25	<0.25
Glycyrrhizin	<0.25	<0.25
Ginsenoside Rb1	<0.25	<0.25
Ergosterol	<0.25	<0.25
Cholesterol	<0.25	<0.25
Sitosterol	<0.25	<0.25
Deoxycholic acid	0.45	<0.25

Table 1. Cross-reactivities (%) of anti-SSa MAbs (1G6) and (3G10)

Assay specificities were examined by competitive ELISA with various related compounds, then calculated following by Weiler and Zenk's method (Weiler & Zenk, 1976). The cross-reactivities (CR) of saikosaponins and related compounds were calculated by following equation.

$$CR\% = \frac{\mu\text{g/ml of SSa yielding } A/A_0 = 50\%}{\mu\text{g/ml of compound under investigation yielding } A/A_0 = 50\%} \times 100 \quad (1)$$

A: absorbance in the presence of the test compound

A₀: absorbance in the absence of the test compound (10% MeOH)

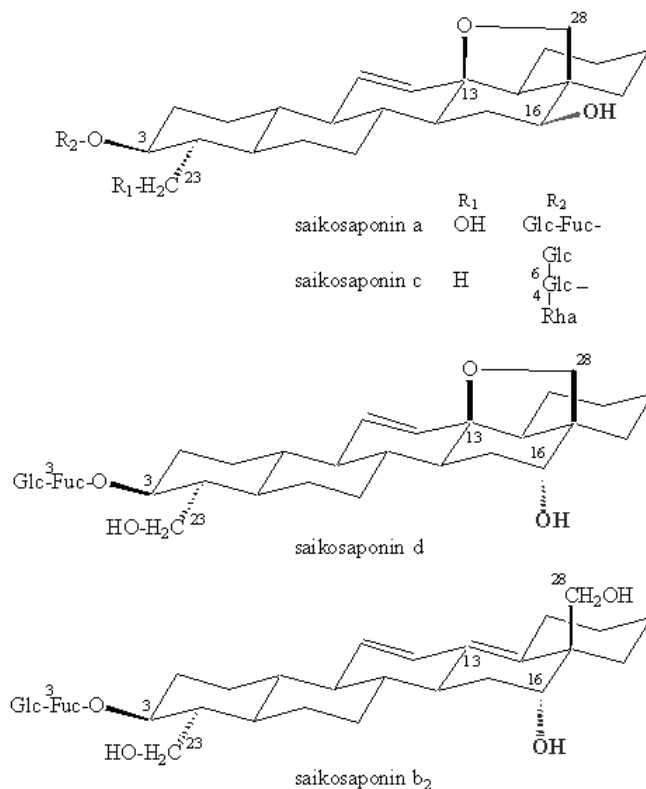


Fig. 4. Stereochemistry of saikosaponins

MAB-1G6 reacted only with structurally related SSc and SSd, weakly resulting in the high specificity of MAB. An undetectable cross-reaction with other steroidal compounds was shown. When compared the cross-reactivities between SSa having a β -hydroxyl group at the C-16 position and SSd (α -hydroxyl group), MAB-1G6 had cross-reactivities against SSd (see Fig. 4). From this, the hydroxyl group at the C-16 position is distinguished stereochemically by MAB-1G6.

MAB-3G10 cross-reacted with SSb₂, SSc and SSd at a rate of 63.8, 28.5 and 15.6%, respectively, easily suggesting that the ether ring between the C-13 and C-28 position in SSa might be opened in a mouse body during the immunization period, and then immunized. This wide cross-reaction is the major advantage of antibody reagent used in this ELISA, because when total saikosaponin concentrations in body fluid and/or in plant sample are needed, this MAB can be widely available, similarly to the results of anti-solamargine MAB (Ishiyama et al., 1996).

From these results, we decided that the concentration of the major saikosaponin, SSa, in the crude drug of *Bupleuri radix* can be analyzed by ELISA using MAB-1G6. On the other hand, when it is necessary to determine the concentration of total saikosaponins in the extracts of *Kampo* medicines, MAB-3G10 functions well for quantitative analysis because the ether ring at the C-13 and C-28 positions opens during the extraction process, as previously reported (Akahori, 1980).

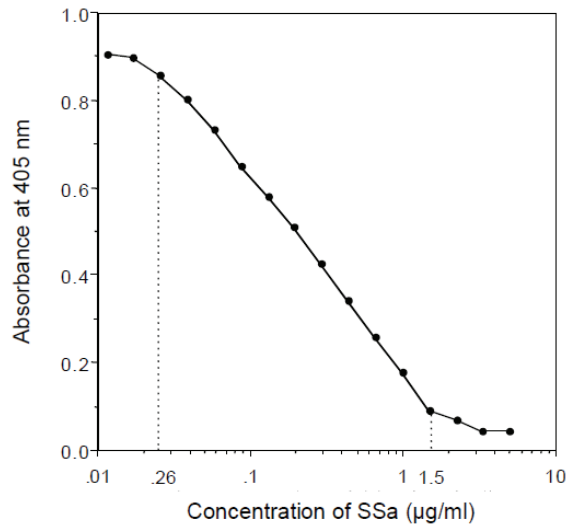


Fig. 5. Calibration curve of SSa by ELISA with MAb-1G6. Various concentrations of SSa were incubated with MAb-1G6 in the microtiter plate adsorbed with SSa-HSA (1 µg/ml). After washing with TPBS, the wells were again incubated with peroxidase-labeled anti-mouse IgG. Absorbance was measured at 405 nm

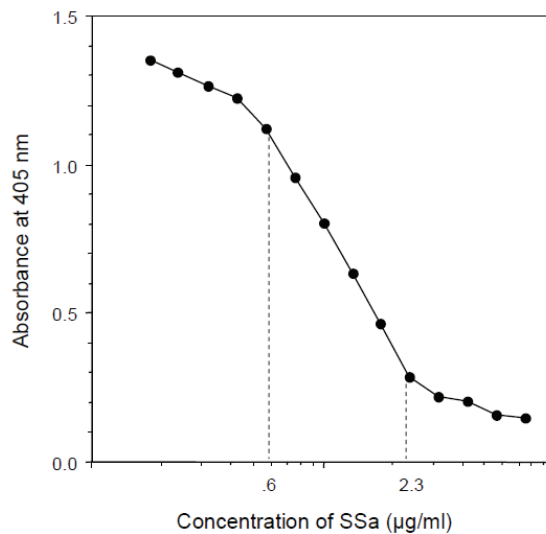


Fig. 6. Calibration curve of SSa by ELISA with MAb-3G10. Various concentrations of SSa were incubated with MAb-3G10 in the microtiter plate adsorbed with SSa-HSA (1 µg/ml). After washing with TPBS, the wells were again incubated with peroxidase-labeled anti-mouse IgG. Absorbance was measured at 405 nm

Fig. 5 indicates the calibration curve of SSa using MAb-1G6 by ELISA, showing that the full measuring range of the assay extended from 26 ng /ml to 1.5 $\mu\text{g}/\text{ml}$. Since the available measuring range of the HPLC method performed in our laboratory was 25 $\mu\text{g}/\text{ml}$ to 400 $\mu\text{g}/\text{ml}$, it becomes evident that the ELISA is about 1000 times more sensitive than that of the HPLC method.

Fig. 6 indicates the calibration curve of SSa using MAb-3G10 by ELISA, showing that the full measuring range of the assay covers from 0.6 $\mu\text{g}/\text{ml}$ to 2.3 $\mu\text{g}/\text{ml}$. Since the available measuring range of the HPLC method performed in our laboratory was 25 $\mu\text{g}/\text{ml}$ to 400 $\mu\text{g}/\text{ml}$, it becomes evident that the ELISA is about 50 times more sensitive than that of the HPLC method.

3. New eastern blotting for saikosaponins

3.1 Extraction of crude drugs and Kampo medicines

Dried powders of various *Bupleuri radix* (20 mg) and Kampo medicine (50 mg) were extracted five times with 0.5 ml methanol with sonication and filtered using a Cosmonice filter W (0.45 μm filter unit, Nacalai Tesque, Kyoto, Japan). The combined extracts were diluted with methanol for ELISA and new eastern blotting analyses.

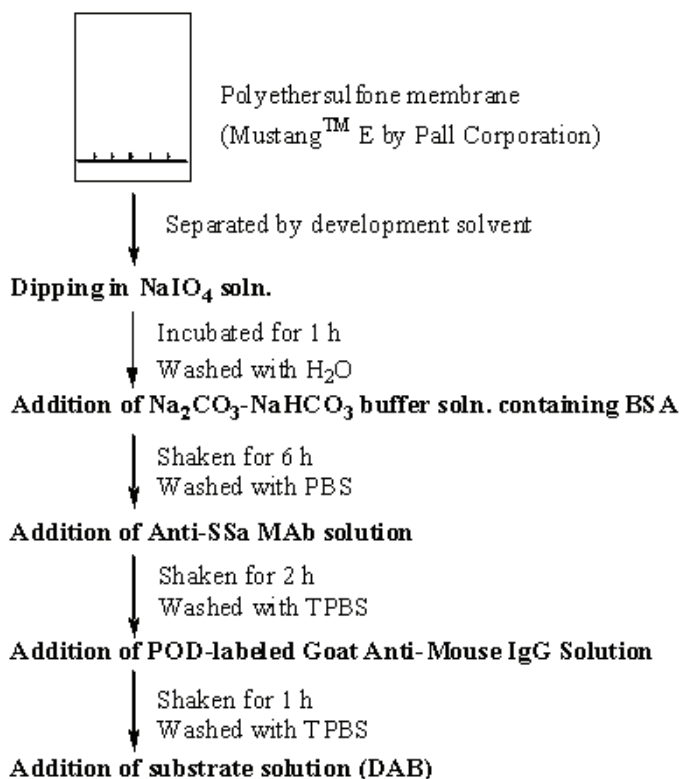


Fig. 7. New eastern blotting protocol

3.2 Visual detection of SSa, SSb, and SSd by new eastern blotting

New eastern blotting was performed except for separation by TLC plate and transfer to a PVDF membrane as reported previously (Tanaka et al., 1997) as following the protocol indicated in Fig. 7.

Saikosaponins and the extracts of *Bupleuri radix* and *Kampo* medicine were applied onto a positive-charged polyethersulfone (PES) membrane (Mustang™ E, Pall Corporation, East Hills, NY, USA). After drying, this membrane was hung in the tank and immersed and developed by acetonitrile-water (1:4, by volume). The developed PES membrane was dried and dipped into water containing NaIO_4 (10 mg/ml) for 1 h. After washing with water, 50 mM carbonate buffer solution (pH 9.6) containing BSA (1%) was added, and was stirred at room temperature for 3 h. After washing PES membrane with phosphate-buffered solution (PBS), the membrane was treated with PBS containing 5% skimmed milk (S-PBS) for 2 h to reduce nonspecific adsorption. The PES membrane was immersed in anti-SSa MAb (1G6) and stirred at room temperature for 2 h. After washing the PES membrane twice with PBS containing 0.05% Tween 20 (T-PBS) and water, a 1:1000 dilution of peroxidase-labeled goat anti-mouse IgG in PBS containing 0.02% gelatin was added, and the mixture was stirred at room temperature for 1 h. Then the PES membrane was washed twice with T-PBS and water and then exposed in Tris-HCl buffer (pH 7.5) containing 1 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.02% H_2O_2 for 30 min at room temperature. The reaction was stopped by washing with water, and the immunostained PES membrane was allowed to dry.

3.3 Image analysis system and image acquisition

A graphic analysis system, which consisted of a Macintosh computer, a public domain program NIH Imaging 1.63 (developed at the U.S. National Institutes of Health and available on the internet at <http://rsb.info.nih.gov/nih-image/>), a desktop scanner and a scanning software Photoshop CS2 (Adobe Systems Inc., San Jose, CA, USA) was used. Images were captured as 256 levels grayscale. The file size was 300 k bytes (200 dots per inch resolution). Digital output was transferred from the scanner to the computer and stored as a PICT file.

3.4 Image analysis using NIH Image

The immunostained PES membrane grayscale image was loaded and thresholded to make a binary image for the separation of objects and background. Then, we chose the analyze menu options and selected the area, ellipse major axis and ellipse minor axis commands to calculate the objects area as previously described (Masters et al., 1992; Root & Wang, 1993).

4. Establishment of new eastern blotting for immunodetection of saikosaponins

In a new approach, we demonstrated the immunodetection of saikosaponins on the positive-charged PES membrane. PES membrane is widely used for the ultrafiltration system (Duarte et al., 2003) and enzyme immobilization unit (Gomes et al., 2004). However, no success with immunodetection of small molecular compounds like saikosaponins using PES membrane has been reported yet. Fortunately, we found a new fact that the positive-charged PES membrane was suitable for the immunodetection of saikosaponins by dot blot analysis using anti-SSa MAb (1G6) (data not shown).

We also noticed its intrinsic hydrophilicity and strong physical property against organic solvents and believed it makes possible to separate saikosaponins chromatographically on this membrane. Although we tested various ratios of methanol-water as the solvent system, acceptable separation data was not obtained. However, fortunately we found its reverse phase property and tested various ratios of acetonitrile-water just following HPLC mobile phase for saikosaponins. All saikosaponin standards and *Bupleuri radix* (Mishima) extract were separated well with acetonitrile-water (1:4, by volume) as shown in Fig. 8.

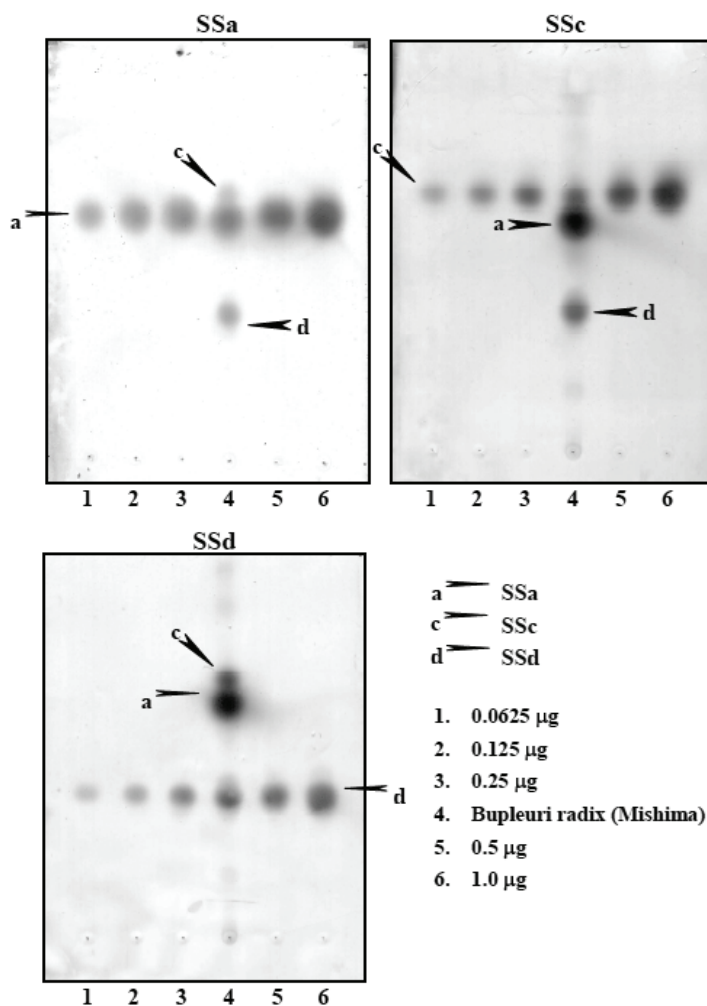


Fig. 8. Separation and immunodetection of saikosaponins by new eastern blotting using anti-SSa MAb (1G6)

The other important merit is the same as PVDF membrane depending on the increase of cross-reactivity as reported in the previous paper (Fukuda et al., 1999). The eastern blotting

of SSa on a PES membrane using anti-SSa MAb (1G6) resulting in staining together with SSc and SSd. In this methodology, we separated the SSa molecule into two functional parts, the epitope part (mainly aglycone) and the sugar parts as commented above (See Fig. 2). The sugar parts in saikosaponins were oxidatively cleaved to release aldehyde groups which were conjugated with protein to fix on a PES membrane. Because it was evident that a part of sugar moiety in SSa was immunized, the cleavage of sugar moiety by NaIO_4 expanded its cross-reactivity against other saikosaponins resulting in possibility of staining for SSc and SSd, though anti-SSa MAb (1G6) had weak cross-reactivity with SSc and SSd (2.65% and 3.76%, respectively).

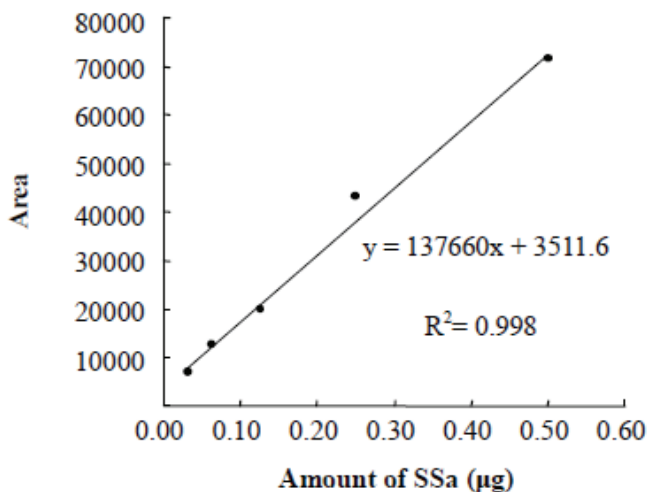


Fig. 9. Calibration curve of SSa obtained by new eastern blotting using anti-SSa MAb (1G6)

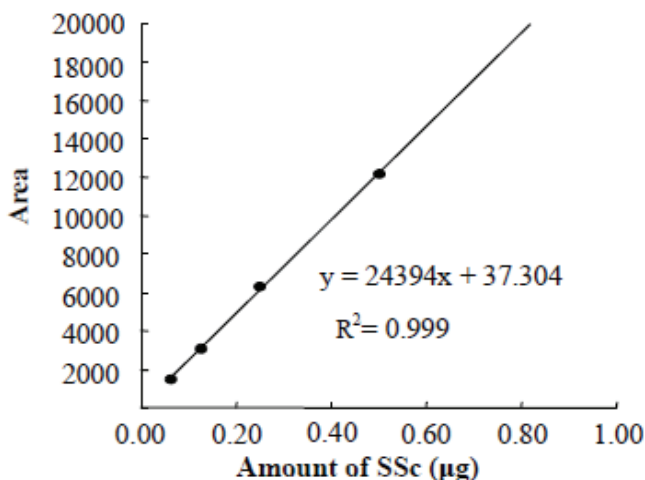


Fig. 10. Calibration curve of SSc obtained by new eastern blotting using anti-SSa MAb (1G6)

5. Application of new eastern blotting to quantitative immunoassay for saikosaponins

We applied the new eastern blotting to the quantitative immunoassay for saikosaponins using NIH Image because newly established method reflected direct sample amounts without transfer efficiency. For the calibration curves, each saikosaponin standard was spotted in five kinds concentration. By this method, the contents of saikosaponins can be determined individually. Calibration curve of each saikosaponin is indicated in Fig. 9 - 11.

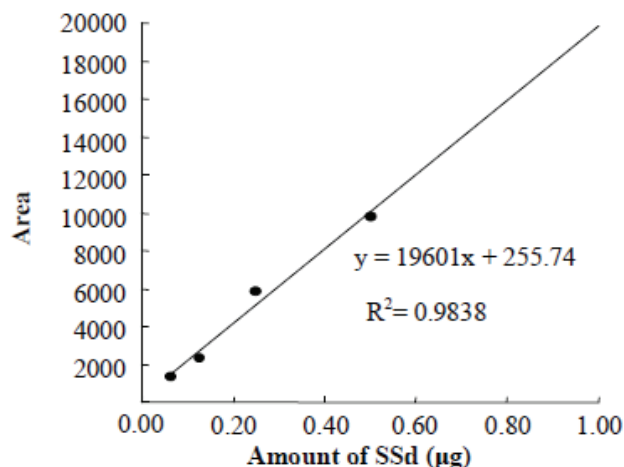


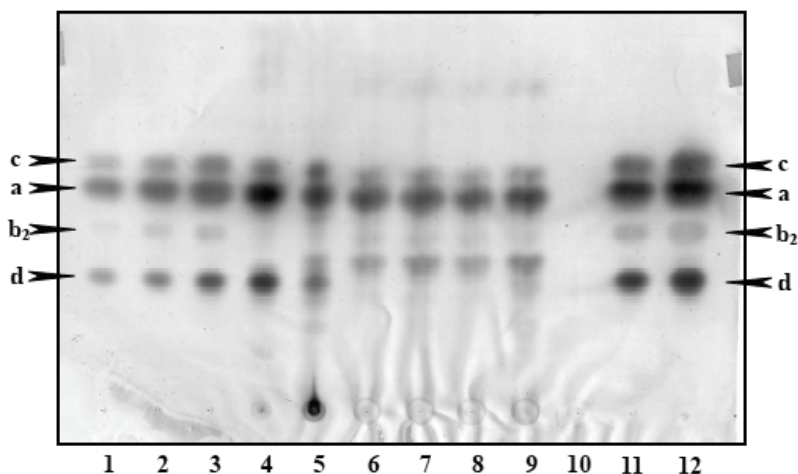
Fig. 11. Calibration curve of SSd obtained by new eastern blotting using anti-SSa MAb (1G6)

6. New eastern blotting as a tool for the quality control of Kampo medicines containing *Bupleuri radix*

New eastern blotting was also applied to the estimation of various Kampo medicines. Fig. 12 shows the new eastern blotting of *Bupleuri radix* and Kampo medicines prescribed with and without *Bupleuri radix*. Lane 10 shows Shoseiryuto prescription extract, which contains no *Bupleuri radix* resulting that no band was appeared by new eastern blotting. Compared to the result of *Bupleuri radix*, there was no SSd detected from Kampo medicines, because SSd will be converted into SSb₂ during the extraction processing.

On the other hand, the bands of SSa and SSc in Daisaikoto, Shosaikoto, Saikokyoyoto and Saikokaryukotsuboreito extracts (Fig. 12, lanes 6, 7, 8 and 9), which contain *Bupleuri radix* clearly appeared. These areas of the coloring spots on this membrane were calculated using graphic analysis by the NIH Image and the saikosaponins concentrations were determined as indicated in Table 2. These results were in a good agreement with those from the ELISA analysis (see Table 2).

In the case of SSb₂, only weak color development was observed in this method and no determination data was shown by using NIH Image analysis. It is suggested to be due to the quantitative determination limits of this NIH Image analysis system. However, this problem can be solved by using more sensitive anti-saikosaponin MABs and/or color developing substrate and chemiluminescence system.



- | | |
|---|---|
| 1. Saikosaponins standard (0.0625 μg) | 7. Shosaikoto |
| 2. Saikosaponins standard (0.125 μg) | 8. Saikokyoyoto |
| 3. Saikosaponins standard (0.25 μg) | 9. Saikokaryukotsuboreito |
| 4. Bupleuri radix (Mishima, Japanese species) | 10. Shoseiryuto (negative control) |
| 5. Bupleuri radix (Austria, Europe species) | 11. Saikosaponins standard (0.5 μg) |
| 6. Daisaikoto | 12. Saikosaponins standard (1.0 μg) |

Fig. 12. Immunodetection of saikosaponins in Bupleuri radix and various Kampo medicines by new eastern blotting using anti-SSa MAb (1G6)

Sample	Concentration ($\mu\text{g}/\text{mg}$ dry weight powder)						
	New Eastern Blotting					ELISA	
	SSa	SSc	SSb ₂	SSd	Total	SSa	Total
Bupleuri radix (Mishima)	8.38	1.39	ND	3.76	13.53	9.31	11.46
Bupleuri radix (Austria)	7.65	1.35	ND	1.55	10.55	7.90	8.94
Daisaikoto	0.63	0.19	(+)	ND	0.82	0.62	1.06
Shosaikoto	0.80	0.22	(+)	ND	1.02	0.76	1.19
Saikokyoyoto	0.69	0.13	(+)	ND	0.82	0.68	1.13
Saikokaryukotsuboreito	0.83	0.23	(+)	0.19	1.25	0.89	1.43
Shoseiryuto	ND	ND	ND	ND	ND	ND	ND

Table 2. Saikosaponins concentrations in Bupleuri radix and Kampo medicines determined by new eastern blotting using NIH Image. Data are the means of triplicate assays. ND= not detectable

7. Conclusion

Many analytical approaches have been used to identify saikosaponins in crude drugs and/or Kampo medicines. Among these methods, the use of HPLC in conjunction with mass spectrometry (MS) or photodiode array (PDA) is most frequently and widely used

(Ohtake et al., 2004; Li et al., 2005). The advantages of the new eastern blotting over the HPLC method are mainly its saving cost-performance (e.g., organic solvents and analytical equipments), speed, ease of use and more environmentally-soft determination method. Moreover, the new eastern blotting can analyze quantitatively and/or qualitatively at least three saikosaponins, SSa, SSc, and SSd by using a single MAb, anti-SSa MAb (1G6).

This is the first report of the separation and immunodetection of saikosaponins on positive-charged PES membrane and its application for quantitative immunoassay for saikosaponins. Although it has been believed difficult to determine the concentrations of small molecular compounds by western blotting technique, the methodology described here may open a wide field of comparable studies with other families of carbohydrates containing compounds of low molecular weight, such as glycosides, glucuronides, aminosugar conjugates and/or glycolipids and glycosphingolipids. It also should be conveniently used to survey for low concentrations of saikosaponins in plants and/or in experimental animals and humans.

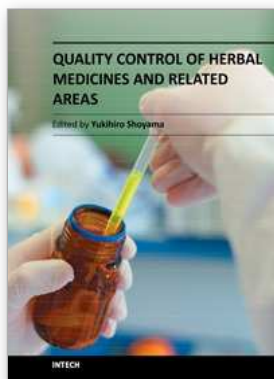
8. Acknowledgement

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The authors of this thematic issue provide a comprehensive summary of most recent knowledge and references on quality control in wide fields. Quality control is essential for natural products like natural medicine and related food products. In this issue fifteen chapters have been included, discussing in detail various aspects of quality control. It will certainly prove useful not only for phytochemical researchers, but also many scientists working in numerous fields. Much effort has been invested by the contributors to share current information. Without their efforts and input 'Quality Control of Herbal Medicine and Related Areas' could not exist.

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