DEVELOPMENT OF BIOASSAYS FOR THE DETECTION OF BENZYLISOQUINOLINE ALKALOIDS,HIGENAMINE AND BERBERINE

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Development of bioassays for the detection of benzylisoquinoline alkaloids, higenamine and berberine

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【Introduction】

Benzylisoquinoline alkaloids (BIA) are the one of the valuable plant secondary metabolites in the term of pharmacological activities. There are over 2,500 BIA originated from plants. To humankind, the BIA has been long utilized in the form of crude drugs and plant extracts for various diseases treatments. The content of BIA in the plant could affect the desired pharmacological effects of that plants. Thus, the quality of the plants is generally controlled by means of standardization of biologically active compounds. By these circumstances, the reliable analytical methods for determination of BIA content in plant matrix are needed.

Because of BIA pharmacological effects, some of them was prohibited in the sport games as it was the doping agent. Higenamine (HM; Fig. 1A) is the BIA which widely known as cardiac stimulating alkaloids isolated from various medicinal plants. HM was included in the β2 agonist prohibited list of World Anti-Doping Agency (WADA)[1]. Therefore, the presence of HM in the intakes is the critical issue for athletes. Food and medicines should be carefully screened prior served to prevent the unintentional use of HM. To facilitate this process, the high throughput and reliable analytical method so-called indirect competitive enzyme-linked immunosorbent assay (icELISA) was successfully established. The specific antibody against HM (MAb E8) was produced in-house and served as the main detection element in icELISA.

To make the detection procedure possible to more detection scenarios, the MAb E8 was used for development of lateral flow immunoassay (LFA). The result interpretation could be easily done promptly without any requirement of signal interpreter using paper-based device.

Berberine (BBR; Fig. 1B) is the quaternary ammonium BIA isolated from several crude drugs included in traditional medicines. Because of its proved pharmacological effects, BBR was believed to be one of the lead compounds in traditional medicine preparations[2]. Thus, the content of BBR in the medicine preparations was a point of concern. The aptamer-based fluorometric assay for BBR determination in Kampo medicine sample was established.

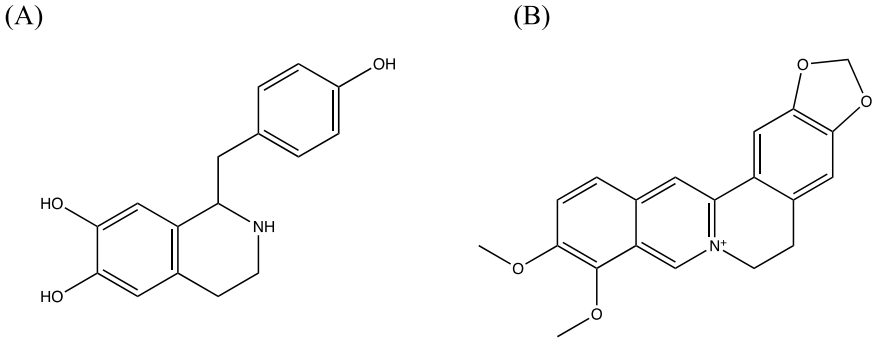

Figure 1 Structures of BIA of interest, (A) HM and (B) BBR

【Method】

To produce the MAb E8, the HM-BSA conjugates was intraperitoneally administered to the BALB/c mice with appropriate interval and doses. The anti-(*S*)-HM antibody in mice serum was

confirmed in each immunization via ELISA. After obtained the appropriate antibody titer and activity against free (*S*)-HM, the splenocytes of the immunized mice were isolated and immortalized through polyethylene glycol-mediated somatic fusion to a myeloma cell line partner (SP2/0) yielding hybridomas. The hybridomas were screened and homogenized to obtain the clone which produce the best characteristic antibody (colony E8). The supernatant was purified to obtain the MAb E8. The MAb E8 was systemically characterized prior icELISA development. Reactivity of MAb E8 against immobilized antigen, cross-reactivity, and reactivity of MAb E8 against free (*S*)-HM were evaluated. To develop the icELISA for (*S*)-HM determination in plant samples, various validation methods including intra-,and inter-plate assay, spike-recovery assay were conducted to confirm the precision and accuracy of the developed icELISA. Moreover, the comparative analysis between icELISA and HPLC-UV was performed.

For an alternative way of detection, the LFA was developed. The colloidal gold nanoparticle conjugated with MAb E8 was used as the signal indicator. The LFA comprised of 2 main components. First component is the paper detection strip which contained control zone (rabbit anti-mouse IgG antibodies) and test zone (HM-γ-globulin conjugates). The other component is detection solution where detection sensor located in. The (*S*)-HM in the samples were competitively reacted with the detection sensor. The concentration of the (*S*)-HM in the sample was reported by pink spot on the test zone which the intensity was inversely relate to the content of free (*S*)-HM in the sample. The sensitivity, selectivity, repeatability, and accuracy of the LFA was conducted to ensure the reliability of the assay.

To produce the fluorogenic aptamer against BBR, colloidal gold nanoparticle-based systematic evolution of ligands by exponential enrichment (GOLD-SELEX) was performed. The aptamer candidates which can enhance the fluorescence of BBR were selected by fluorescence measurement[3]. The best aptamer candidate was used as the template for structure modification i.e., truncation, mutation, and polymerization. Then, the aptamer-based fluorometric assay for BBR determination in Kampo medicines was developed. The assay was validated by performing the intra-, and inter- plate assay and spike-recovery assay to ensure the repeatability and accuracy. Moreover, the content of BBR in 128 Kampo medicine samples was analyzed and compared with those obtained from HPLC-UV.

【Results】

After immunization, the titer of anti-(*S*)-HM was raising dose by dose as well as the reactivity against (*S*)-HM. The splenocyte obtained from the best titer mouse was successfully fused with SP2/0 cells. After screening and homogenization of the hybridomas, the best characteristic clone (colony E8) supernatant (800 mL) was purified yield sufficient amount of MAb E8 (59.3 mg). The cross-reactivity test revealed that the MAb E8 showed less cross-reaction than 5% to the compound candidates. However, MAb E8 showed high cross-reactivity against norlaudanosoline (223%) which never been reported to be isolated in plant so far. The MAb E8-based icELISA was successfully developed with the determination range of 7.81–125 ng/mL (Fig. 2) with

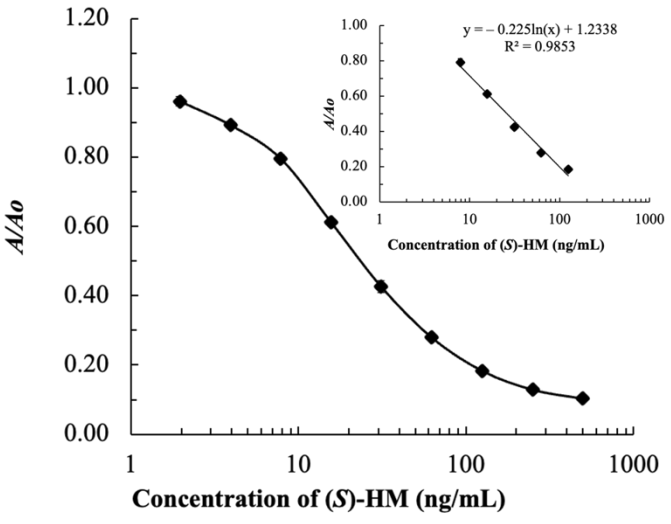

Figure 2 Standard curve for determination of (*S*)-HM using icELISA.

the limit of detection (LOD) of 4.41 ng/mL. The validation process revealed that the developed icELISA was robust enough as the intra- and inter- plate assay showed the coefficient of variation (CV) of less than 15%. Moreover, the accuracy of the assay was confirmed by the recovery rate obtained from spike-recovery assay in the range of 100–117%. Moreover, the correlation of the (*S*)-HM amount in plant sample determined by icELISA and HPLC-UV was in positive agreement. This indicated that the developed icELISA was suitable for (*S*)-HM determination in plant samples.

For LFA developed using MAb E8, the sensitivity of the LFA was demonstrated as the limit of detection of 156 ng/mL (Fig. 2A; lane 5) visualized by naked eyes. The concentration dependent range was between 19.0–156 ng/mL when using photoanalysis software (Fig. 2B). The cross-reaction profile of the LFA was similar to that of icELISA. The CV of the intensity of test zone from various concentration of (*S*)-HM was lower than 15% both intra- and inter- day assay. This indicated that the LFA was precise enough. Moreover, the LFA results obtained from actual plant samples were corelated with the results obtained from icELISA and HPLC-UV. This can be the alternative way for a quick detection of (*S*)-HM in plant sample.

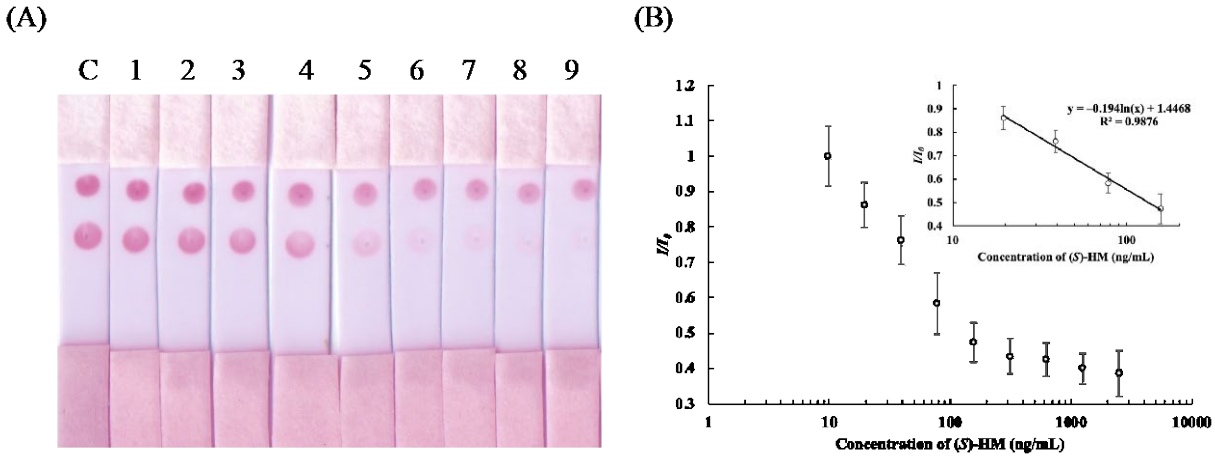

Figure 2 Challenging various concentrations of (S)-HM to the LFA. (A) The actual strip of LFA using a set of (S)-HM concentration that was 2-fold serially diluted from 9.77 ng/mL (Lane 1) to 2.50 µg/mL (Lane 9) and the negative control (5% MeOH; lane C). (B) The standard curve was obtained from the analysis of the strip photos.

The best fluorogenic aptamer against BBR (BBR38) was successfully selected with 7th round of GOLD-SELEX. The truncation of BBR38 showed that the fluorescent was enhanced when the base of the BBR38 was systemically cut. Then, the minimal structure of 22-mer aptamer was obtained. The 22-mer aptamer was used as the mutation template. With systemically mutation, the fluorogenicity of the 22-mer aptamer was enhanced (Fig. 3). The finalized aptamer was trimerized using poly-T linker to obtain the suitable aptamer (TBBR38s) for aptamer-based fluorometric assay. The aptamer-based fluorometric assay was successfully developed with the linearity range of 0.780–25.0 µg/mL (Fig. 4). The CV of the intra- and inter assay was less than 5%. The recovery (%) obtained from spike and recovery assay was ranging from 91.2% to 108%. This indicated that the assay was precise and accurate for BBR determination in Kampo medicine samples. Moreover, the comparative results analyzing the content of BBR in Kampo medicine samples obtained from aptamer-based fluorometric assay and HPLC-UV was correlated. This affirmed that the developed assay was suitable for BBR determination in Kampo medicine samples.

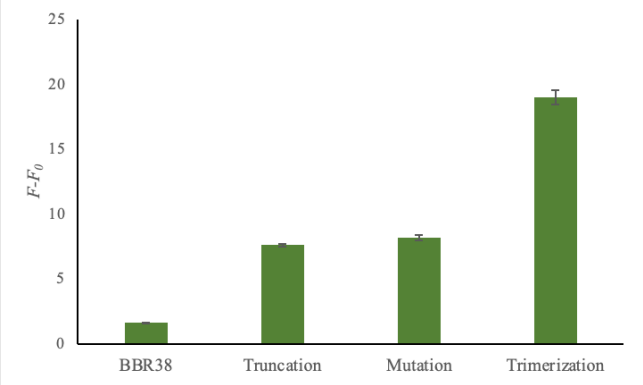

Figure 3 Measurement of the normalized fluorescence intensity of each post-SELEX modification

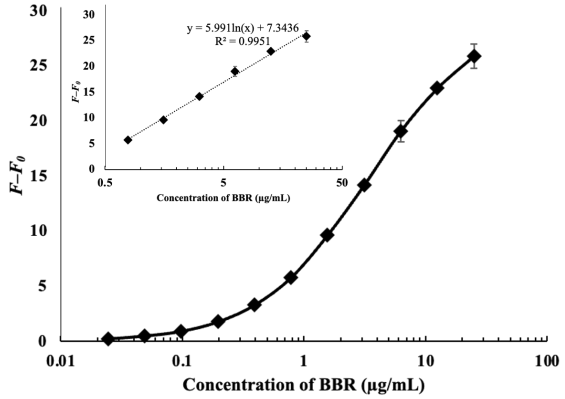

Figure 4 Standard curve for determination of BBR using fluorometric-based assay

【Discussion】

The BIA detection methods based on binding affinity of the macromolecule (antibody or aptamer) and ligand were shown to be effective in such a complex matrix as plant extracts and Kampo medicines. The developed detection method in this study was not the best in the term of sensitivity compared to the established methods. However, the benefit of these methods were the cost-performance, selectivity, and rapidity especially when the multiple samples have to be handled. These methods can be used as an alternative way of detection or use alongside with the conventional methods.

【References】

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【Publication list】

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