Enzyme-Linked Immunosorbent Assay for Determination of Miroestrol in *Pueraria candollei*

วิธีวิเคราะห์เชิงปริมาณสารไมโรเอสทรอลในกวาวเครือขาวโดยเทคนิคทางภูมิคุ้มกันวิทยา

Gorawit Yusakul (กรวิทย์ อยู่สกุล) *,*** Dr.Waraporn Putalun (ดร.วราภรณ์ ภูตะลุน) **,***
Orapin Udomsin (อรพินทร์ อุดมศิลป์)****,*** Dr.Thaweesak Juengwatanatrakul (ดร.ทวีศักดิ์ จึงวัฒนตระกูล)*****
Dr.Hiroyuki Tanaka (ดร.ฮิโรยูกิ ทานากะ)****** Dr.Chaiyo Chaichantipyuth (ดร.ชัยโย ชัยชาญทิพยุทธ)*******

ABSTRACT

Miroestrol, a potent and stable phytoestrogen from *Pueraria candollei*, exhibited potential to be biomarker for quality control of *P. candollei* samples in research or industrial development. Competitive enzyme-linked immunosorbent assay (ELISA) for miroestrol determination was developed and validated by using polyclonal antibody from rabbit immunization. The polyclonal antibody recognized specifically to miroestrol, which exhibited cross-reactivity to deoxymiroestrol and isomiroestrol with 6.68% and 1.05% respectively. The linearity rage of ELISA was at 0.73-3000 ng/ml, which relative standard deviation (RSD) of both intra- and interpolate determination was less than 5%. The percentage of recovery was 98.80-104.37% with maximum RSD of 7%. Validated ELISA was comparable with published HPLC method (R^2= 0.9996) in samples with various miroestrol contents.

บทคัดย่อ

ไมโรเอสทรอล ออกฤทธิ์เป็นฮอร์โมนเอสโตรเจนสูง และมีความคงตัวทางเคมี ซึ่งมีศักยภาพเป็นตัวบ่งชี้ทางชีวภาพเพื่อควบคุมคุณภาพกวาวเครือขาวในระดับการวิจัยและอุตสาหกรรม วิธี*เอนไซม์*-ลิงค์ อิมมูโนแอสเซย์แบบแข่งขันถูกพัฒนาและตรวจสอบเพื่อวัดปริมาณสารไมโรเอสทรอลโดยใช้โพลีโคลนอลแอนติบอดีจากกระต่าย โดยพบว่าแอนติบอดีมีความจำเพาะสูงต่อสารไมโรเอสทรอล แต่มีปฏิกิริยาข้ามกับสารดีออกซีไมโรเอสทรอล และสารไอโซไมโรเอสทรอลเพียง 6.68 % และ 1.05 % ตามลำดับ วิธี*วิเคราะห์*มีช่วงการวัดที่ 1.46-1500 นาโนกรัมต่อมิลลิลิตร โดยค่าการเบี่ยงเบนมาตรฐานสัมพัทธ์ไม่เกิน 5% วิธีวิเคราะห์มีค่าความเที่ยงในช่วง 98.80-104.37% และค่าเบี่ยงเบนมาตรฐานสัมพัทธ์สูงสุดที่ 7% เมื่อเปรียบเทียบระหว่างวิธีอีไลซ่าและเอชพีแอลซีเพื่อการวิเคราะห์สารไมโรเอสทรอลในตัวอย่างต่างๆพบว่ามีค่า*สัมประสิทธิ์*ของการตัดสินใจ (Coefficient of determination, r^2) เท่ากับ 0.9996

Key Words: ELISA, Miroestrol, *Pueraria candollei*

คำสำคัญ: อีไลซา ไมโรเอสทรอล กวาวเครือขาว

* *Student, Master of Pharmacy in Pharmaceuticals, Faculty of Pharmaceutical Sciences, Khon Kaen University*

** *Associate Professor, Department of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen University*

*** *Research Group for Pharmaceutical Activities of Natural Products using Pharmaceutical Biotechnology (PANPB), National Research University-Khon Kaen University*

**** *Student, Doctor of Philosophy in Pharmaceuticals, Faculty of Pharmaceutical Sciences, Khon Kaen University*

***** *Assistant Professor, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Ubon Rajathanee University*

****** *Associate Professor, Graduate School of Pharmaceutical Sciences, Kyushu University, Japan*

******* *Associate Professor, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University*

Introduction

Pueraria candollei var. *mirifica* belonging to Leguminosae family, is phytoestrogen enrich plant which its tuberous root was used widely in traditional Thai medicine system for smoothening skin, enhancing memory and rejuvenating in aged man and woman (Anusarnsoondhorn, 1931). The randomized clinical trials to evaluate efficacy and safety of *P. candollei* var. *mirifica* showed that the climacteric score of menopause woman were declined after treatment, whereas no significant side effects were observed with different dose from 25-100 mg daily of *P. candollei* var. *mirifica* (Chandeying & Lamlertkittikul, 2007; Lamlertkittikul & Chandeying, 2004; Virojchaiwong et al., 2011). In comparison with conjugated equine estrogen, efficacy of 50 mg daily dose of *P. candollei* var. *mirifica* on alleviation of climacteric symptom was not significantly different with daily dose of 0.625 mg conjugated equine estrogen with/without medroxyprogesterone acetate (Chandeying & Sangthawan, 2007). Interestingly, the randomized, double-blind, placebo control trials informed that *P. candollei* var. *mirifica* (20, 30 and 50 mg) decreased bone turnover significantly in postmenopausal woman, suggesting the anti-osteoporosis effect like estradiol (Manonai et al., 2008), moreover vaginal health in postmenopausal woman was improved also after treatment with *P. candollei* var. *mirifica* (Manonai et al., 2007).

Potent phytoestrogen, miroestrol (ME) was found only from *P. candollei* (Chansakaow et al., 2000). ME significantly increased uterus weight and volume being similar to the same dose of estradiol benzoate, moreover the compounds also decreased lipid peroxidation in mice brain significantly (Udomsuk et al., 2012) correlated to Thai traditionally usage (Anusarnsoondhorn, 1931).

It is very necessity to standardize ME contents in *P. mirifica* samples before its using in pharmacological research and related product development because of these reasons: firstly there are different ME content between two *P. candollei* varities (Yusakul et al., 2011), secondly different culturing place, and season of harvest effect on secondary metabolite variance in *P. candollei* var. *mirifica* (Cherdshewasart & Sriwatcharakul, 2007; Cherdshewasart et al., 2007) and finally ME is product of deoxymiroestrol degradation occurring with molecular oxygen in air under various conditions (Chansakaow et al., 2000). Standardized material of strong phytoestrogen marker ME is essential to compare efficacy of product or results of different trials associated *P. candollei*. Analytical method of ME using HPLC-UV was developed for its determination in *P. candollei* (Yusakul et al., 2011). With disadvantage of HPLC method for minor compounds, immunoassay based on antigen-antibody reaction is optional analysis method with high selectivity, sensitivity, rapidity, thus it more appropriate than HPLC for low concentration analyte in various samples with different interferences to chromatographic method, especially with large number of sample required to analyze.

Objectives of the study

We aim to develop enzyme-linked immunosorbant assay using polyclonal antibody (PAb) against ME for its determination in plant samples

Methodology

With periodate oxidation, ME was coupled with bovine serum albumin (BSA) as immunogen, whereas the compound was coupled with ovalbumin (OVA) as non-relevant coating agent for ELISA test. To synthesize ME-BSA conjugate, ten milligrams of ME dissolved in DMSO was added dropwise to aqueous solution of 5 mg $NaIO_4$, and then stirred at room temperature for 1 hour. After that, the reaction mixture was added to BSA solution (10 mg in 50 mM carbonate buffer pH 9.6), and stirred for 5 hours. All of above reaction were performed at room temperature. The reaction mixture was dialyzed against five changes of water at 4°C, and then lyophilized to obtain ME-BSA. The same method was applied to synthesize ME-OVA conjugate.

To ensure immunogenicity of ME-BSA conjugate, the hapten number of the conjugate was determined by using MALDI-TOF-MS. Briefly a small amount (1-10 pmol) of an antigen conjugate was mixed with a 10^3-fold molar excess of sinapinic acid in an aqueous acetonitrile solution containing 0.10% trifluoroacetic acid. The mixture was subjected to a high performance MALDI-TOF-MS system, autoflex III (Brucker Daltonics, Bremen, Germany).

A male New Zealand White rabbit (National Animal Centre, Mahidol University, Nakhon Pathom, Thailand) was immunized subcutaneously with a 1:1 emulsion mixture of Freund's complete adjuvant and ME-BSA conjugate (1.5 mg). Three week later, the intramuscular booster of the same conjugate (1.0 mg) emulsified with Freund's incomplete adjuvant was administered for three times with two weeks interval. When desired antibody was obtained, the final immunization was carried out intravenously with 500 µg of ME-BSA conjugates without any adjuvant. Seven days after the last immunization, the rabbit was bled to collect serum as source of polyclonal antibody, which total IgG was purified using a Protein G FF column.

Indirect non-competitive ELISA was used to evaluate whether the antibody reacts to fixed ME-OVA, whereas quantitative ELISA was developed using principal of competition between fixed ME-OVA and free ME, and validated for specificity, sensitivity, precision and accuracy. Published High Performance Liquid Chromatography or HPLC method for ME determination (Yusakul et al., 2011) was set as comparator to developed ELISA.

The plant materials of *P. candollei* were dried under hot air oven at 50 °C and then ground to a fine powder. The plant samples (1 g) were washed a time with 5 ml hexane, and then were extracted five times with 5 ml chloroform-ethyl acetate (1:3, v/v) with sonication for 1 hour of each extraction. All extracts of each sample were pooled and evaporated at room 60 °C. Solid residues of extract were dissolved with 1 ml absolute ethanol for analysis by ELISA and HPLC.

Results

Mass spectrum showed the peak of ME-BSA at approximately 71785.95 m/z, therefore at least 14 molecules of ME were coupled to a molecule of BSA.

Indirect non-competitive ELISA suggested that PAb reacted fixed ME-OVA with concentration dependent manner, which PAb concentration of 12.5 µg/ml was set as appropriate concentration for development of competitive ELISA for ME determination.

With indirect competitive ELISA, the result suggested that the PAb reacted to free ME with concentration dependent. The limit of detection which was calculated at 10% inhibition was 0.73 ng/ml. The linearity rage of ELISA was at 0.73-3000 ng/ml (figure 1).

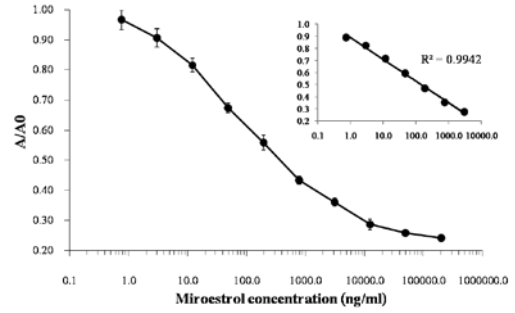

Figure 1 The inhibitory curve of miroestrol (where A0 is the absorbance with no miroestrol present and A is the absorbance with miroestrol present)

Although ME structure is very similar to deoxymiroestrol and isomiroestrol, PAb exhibited low cross-reactivity of 6.68% and 1.05% respectively. With known isoflavonoids found in *P. candollei* and other chemicals, their cross-reactivates are less than 0.01%, which can be considered as negligible. The inhibitory variation of each concentrations, which were in rang of measurement, were tested within a plate (n=6) and between plate (n=12). The relative standard deviation of both intra- and inter-plate were less than 5%, which the developed ELISA showed low variation for reliable ME analysis. After the *P. candollei* extract was spiked with ME solution (0.5, 1, 5 and 10 µg/ml), the ME concentration was determined by the developed ELISA method. The percent of recovery extended from 98.80% to 104.37% with a maximum RSD of 7 %. To compare performance of our validated ELISA with published HPLC method for ME determination, *P. candollei* samples with different content of ME were determined using both assays. ME contents in each sample was correlated with value from HPLC method with high confident of determination (R^2=0.9996).

Discussion and Conclusions

The reaction applied to link ME to BSA was successful to produce antibody with high affinity and selectivity to free ME. Because zero-length crosslinking was used for ME-BSA conjugate synthesis, PAb responding to the conjugate was specific to the attached hapten without cross-reactivity to linker (Hermanson, 2008). The overall results of cross-reactivity experiment indicated that the antibody were specific for ME determination in *P. candollei* samples. The indirect competitive ELISA exhibited much higher sensitivity than published HPLC method (Yusakul et al., 2011), which the assay could be applied to various ME content of samples. With precision experiment, the developed ELISA showed low variation for ME determination Moreover the ELISA is reliable for ME determination in *P. candollei* samples because of its high recovery in spiked samples. ME contents in each sample was correlated with value from HPLC method with high confident of determination. The high correlation indicated that developed ELISA was comparable with HPLC method in high ME containing samples. In additionally, some samples undetectable by HPLC method could be quantified by ELISA due to its higher sensitivity**.** With the higher performance of ELISA over HPLC method, it could be applied with the more convenient, which require less sample amount, easier sample preparation, smaller time of analysis than HPLC with UV detector.

Acknowledgements

This work was supported in part by the Faculty of Pharmaceutical Science, Khon Kaen University, Thailand and Graduate School, Khon Kaen University, Thailand. The authors thank Suranaree University of Technology, Nakhon Ratchasima province, and Ubon Rajathanee University, Ubon

Ratchathani province, Thailand for providing of *P. candollei* plant samples.

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