Synthesis of ^{125}I Labeled Estradiol-17β-Hemisuccinate and Its Binding Study to Estrogen Receptors Using Scintillation Proximity Assay Method

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ARTICLE INFO

Article history:
Received 04 December 2012
Received in revised form 19 December 2012
Accepted 21 December 2012

Keywords:
Indirect labeling
Estrogen receptor
Estradiol
Scintillation proximity assay
Binding affinity
MCF7

ABSTRACT

Research was carried out to obtain a selective ligand which strongly bind to estrogen receptors through determination of binding affinity of estradiol-17β-hemisuccinate. Selectivity of these compounds for estrogen receptor was studied using Scintillation Proximity Assay (SPA) method. Primary reagents required in the SPA method including radioligand and receptor, the former was obtained by labeling of estradiol-17β-hemisuccinate with ^{125}I, while MCF7 was used as the receptor. The labeling process was performed by indirect method via two-stage reaction. In this procedure, first step was activation of estradiol-17β-hemisuccinate using isobutylchloroformate and tributylamine as a catalist, while labeling of histamine with ^{125}I was carried out using chloramin-T method to produce ^{125}I-histamine. The second stage was conjugation of activated estradiol-17β-hemisuccinate with ^{125}I-histamine. The product of estradiol-17β-hemisuccinate labeled ^{125}I was extracted using toluene. Furtherly, the organic layer was purified by TLC system. Characterization of estradiol-17β-hemisuccinate labeled ^{125}I from this solvent extraction was carried out by determining its radiochemical purity and the result was obtained using paper electrophoresis and TLC were 79.8% and 84.4% respectively. Radiochemical purity could be increased when purification step was repeated using TLC system, the result showed up to 97.8%. Determination of binding affinity by the SPA method was carried out using MCF7 cell lines which express estrogen receptors showed the value of Kd at 7.192 x 10^{-3} nM and maximum binding at 336.1 nM. This low value of Kd indicated that binding affinity of estradiol-17β-hemisuccinate was high or strongly binds to estrogen receptor.

INTRODUCTION

Breast cancer is a malignant condition of the cells contained in the breast. Breast cancer is one of the leading causes of cancer death among women and more than one million cases a year are found around the world [1]. Molecular mechanisms of breast cancer begin with a mutation in the normal gene. Mutation occur are usually point mutations, among others, could caused to rearrangement or deletion [1]. Two-thirds of breast cancers express estrogen receptor (ER). The ER status is important as a prognostic indicator in breast cancer. Women with estrogen receptor positive breast tumors have a better prognosis than women with estrogen receptor negative tumors in the treatment with the antiestrogen [2].

Estrogen receptor is one of the intracellular receptor (nuclear receptor) that mediates the action of 17β-estradiol (estrogen) hormone in the body. The presence of estrogen is able to initiate growth, proliferation and metastasis of various types of cancers (breast, ovarian, colorectal, prostate, and endometrial) [3].

If an estrogen receptor (ER) binds to the ligand, there will be a change in receptor conformation that allows binding with coactivator. Estrogen receptor complex then binds with estrogen receptor element (ERE). After binding with ERE,

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the complex binds to a protein coactivator and activates transcription factors. Activation of gene transcription was going to produce mRNA that directs the synthesis of specific proteins, which then affects cell function, depending on their target cells [4].

Ligand that would bind to estrogen receptors was then compete with estrogen for binding to its receptor and was called Selective Estrogen Receptor Modulators (SERMs). This selectivity may be achieved due to the estrogen receptor in different tissues vary in their chemical structure that allows a group of SERMs drugs to selectively interact on estrogen receptors in certain tissues [4].

To obtain a selective ligand which strongly bind to estrogen receptor, study is needed to determine the selectivity of compound to be used as a ligand. In this study, analog of the ligand that will bind to estrogen receptors of the steroid compound will be used, which is estradiol-17β-hemisuccinate.

The presence of hydroxyl groups on the C3 aromatic ring of estradiol-17β-hemisuccinate indicates an ability to bind to estrogen receptors. This is reasonable because one of pharmacophore was required to bind to the estrogen receptor is the presence of an aromatic ring substituted with a hydroxyl group [5]. The main frame of estradiol-17β-hemisuccinate contributed an estimated affinity for estrogen receptor α through hydrophobic interactions with binding of the ligand and estrogen receptor α [6].

The binding affinity of estradiol-17β-hemisuccinate to the estrogen receptor in MCF7 cell lines was studied using scintillation proximity assay (SPA) method. The SPA is a technique which determine binding of antibodies or receptor molecules to a bead which emit light from the energy of labeled radioactive ligand. This occurrence triggers the release of light from the beads and therefore can be detected by detector [7]. The SPA method does not require any separation or washing step because of its homogeneous form [8], thus the method is easy to be applied for drug screening [9]. The factors affecting the determination by the SPA technique are the use of solvent (assay buffer), the type of ligand or compound to be determined and the type of bead [10].

In this study determination of binding affinity of estradiol-17β-hemisuccinate to the estrogen receptor was performed by in vitro test with SPA method using estradiol-17β-hemisuccinate labeled ^{125}I. The aim of this research is to find an optimum condition for preparation and purification of ^{125}I labeled estradiol-17β-hemisuccinate using an indirect method, especially to obtain the value of dissociation constant (Kd) and maximum binding (Bmax) of binding affinity of estradiol-17β-hemisuccinate labeled ^{125}I. The previous study related to this research (reference no 15) using radioligand binding assay method. In this study using SPA method, is expected to obtain a lower value of Kd.

EXPERIMENTAL METHOD

Indirect Labeling of Estradiol-17β-hemisuccinate

The labeling process of estradiol-17β-hemisuccinate was performed in similar manner to procedure reported in Refs [11-14]. This process involved three stages of reactions. It was began by activating the estradiol-17β-hemisuccinate with isobutilchloroformate using tributylamine as catalyst to form an active compound of estradiol-17β-hemisuccinate-isobutylchloroformate (Fig. 1). The second stage was preparation of histamine labeled with ^{125}I using chloramin-T method (Fig. 2). The final stage was the conjugation of histamine labeled ^{125}I to the activated estradiol-17β-hemisuccinate-isobutylchloroformate (Fig. 3). The obtained ^{125}I-estradiol-17β-hemisuccinate-iodohistamine was then purified and tested using labelling efficiency and radiochemical purity parameters.

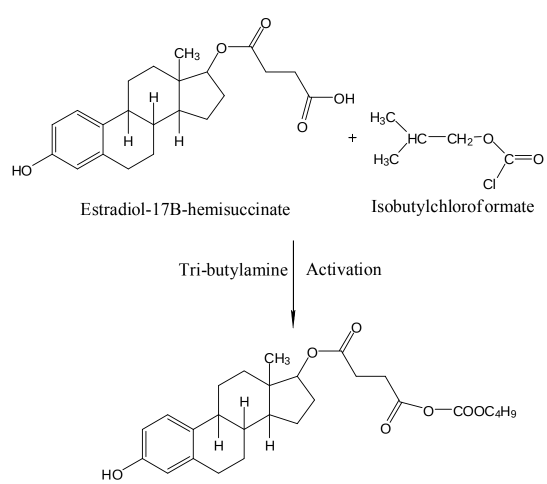


Fig. 1. Activation of estradiol-17β-hemisuccinate with isobutilchloroformate using tributylamine as catalyst.

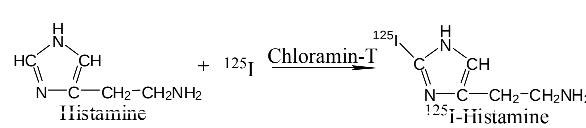


Fig. 2. Labeling histamine with ^{125}I using chloramin-T method.

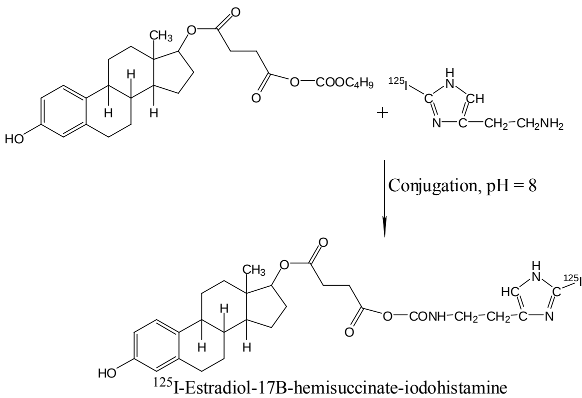


Fig. 3. Conjugation of [125]I-histamine to the activated estradiol-17β-hemisuccinate

Scintillation Proximity Assay (SPA)

Assay protocol with varying the amount and type of SPA bead

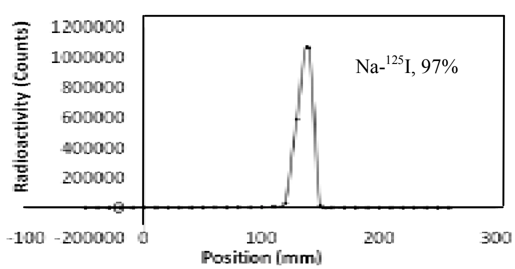
In a Laminar Air Flow, a 100 μL of sterile phosphate buffer saline containing MCF7 cells was added to a plate of 96 well. For well of non spesific binding, a 25 μL of unlabeled estradiol-17β-hemisuccinate was placed in the well, while for total binding the well containing a 25 μL phosphate buffer saline. Then a 100 μL of ^{125}I-estradiol-17β-hemisuccinate-iodohistamine in assay buffer and 50 μL of SPA beads suspension were added to each well. The plate was sealed and incubated for 1 h at 25ºC, with shaking. Final assay consentrations were : MCF7 cell lines of 500,000/well, 0.08 nM unlabeled estradiol-17β-hemisuccinate, 400 nM ^{125}I-estradiol-17β-hemisuccinate-iodohistamine, SPA beads (12.5 to 250 μg/μL).Varying types of beads were used including YSi-WGA SPA, YSi-antirabbit SPA dan PVT-antirabbit SPA beads. The plate was counted using Liquid Scintillation Counter (LSC) at 1 min per well. Results from this experiment can identify the proper type of SPA bead to be used in future experiments.

Assay protocol for binding study of ^{125}I-estradiol-17β-hemisuccinate-iodohistamine [15]

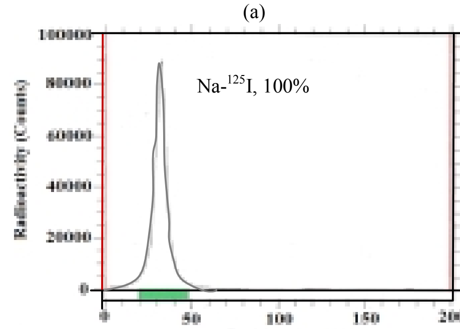
To each well, a 100 μL of sterile phosphate buffer saline containing MCF7 cells was added. A 25 μL of unlabeled estradiol-17β-hemisuccinate was placed in the well for non spesific binding. For total binding the well, a 25 μL phosphate buffer saline was added. Varying consentration of ^{125}I-estradiol-17β-hemisuccinate-iodohistamine as a tracer was diluted in assay buffer, then a 100 μL of tracer and 50 μL of SPA beads suspension were added to each well. The plate was sealed and incubated for 1 h at 25ºC, with shaking. Final assay consentrations : MCF7 cell lines of 500,000/well, 0.08 nM unlabeled estradiol-17β-hemisuccinate, concentration of ^{125}I-estradiol-17β-hemisuccinate-iodohistamine (10 nM to 400 nM), SPA beads (25 μg/μL). The plate was counted using LSC at 1 min per well. Results from this experiment were used to calculate the value of Kd and maximum binding (Bmax).

RESULTS AND DISCUSSION

The labeling of estradiol-17β-hemisuccinate with ^{125}I was carried out using indirect method [11-14]. Evaluations of labeling and radiochemical purity were monitored using paper electrophoresis and TLC. Prior to labeling process, a radiochromatogram of Na-^{125}I was prepared and showed in Fig. 4 for paper electrophoresis and TLC. Whatman paper No. 1 was used as stationary phase and 0,025 M phosphate buffer pH 7,4 was used as mobile phase in the electrophoresis method. While for TLC, silica 60F_{254} was used as the stationary phase and the mixture of chloroform : ethanol (9 : 1) as mobile phase. The radiochromatogram was used as a refference in identifying the result of labeling process (^{125}I-estradiol-17β-hemisuccinate-iodohistamine). The radiochromatogram of Na-^{125}I (Fig. 4) showed the radiochemical purity at more than 97%, which was good enough for this research [13].



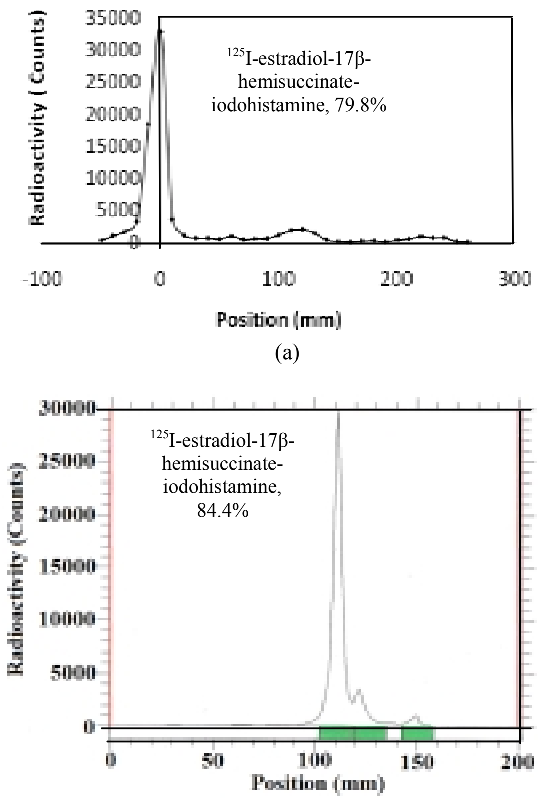
(a)



(b)

Fig. 4. Radiochromatogram of Na-^{125}I using paper electrophoresis (a) and using TLC (b).

The labeling process was performed by indirect method via two-stage reaction. First activation of estradiol-17β-hemisuccinate using isobutylchloroformate and tributylamine as a catalist, and labeling of histamine was carried out by ^{125}I using chloramin-T method. The second stage was conjugation of activated estradiol-17β-hemisuccinate with ^{125}I-histamine. The estradiol-17β-hemisuccinate labeled ^{125}I was extracted using toluene and organic phase was purified by TLC system. The radiochemical purity of ^{125}I-estradiol-17β-hemisuccinate-iodohistamine was obtained at 79.8% using paper electrophoresis while using TLC was 84.4% as shown in Fig. 5.



(a)

(b)

Fig. 5. Radiochromatogram of ^{125}I-estradiol-17β-hemisuccinate-iodohistamine using paper electrophoresis (a). and using TLC (b).

It can be seen from Fig. 5, that radiochemical purity of the purified ^{125}I-estradiol-17β-hemisuccinate-iodohistamine was still low. Therefore further purification was a necessary. Further purification was carried out by a semi-preparative TLC glass plate of silica gel 60 F_{254} as the stationary phase and a mixture of benzene : ethanol : acetic acid (75 : 24 : 1) as the mobile phase. Radiochromatogam of ^{125}I-estradiol-17β-hemisuccinate-iodohistamine purified with TLC system can be seen in Fig. 6.

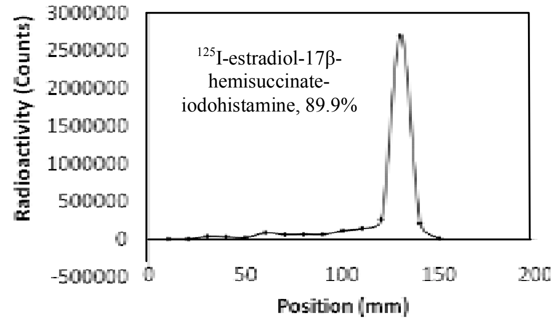


Fig. 6. Radiochromatogram of ^{125}I-estradiol-17β-hemisuccinate-iodohistamine using TLC glass plate.

Figure 6 showed an increasing purity of ^{125}I-estradiol-17β-hemisuccinate-iodohistamine. Increased purification yield of ^{125}I-estradiol-17β-hemisuccinate-iodohistamine reached 89.9%. Radiochemical purity of purified ^{125}I-estradiol-17β-hemisuccinate-iodohistamine was then monitored by TLC, using silica 60 F_{254} as stationary phase and a mixture of chloroform : ethanol (9 : 1) as a mobile phase. Radiochromatogram for radiochemical purity of purified ^{125}I-estradiol-17β-hemisuccinate-iodohistamine was obtained at 97.8% as showed in Fig. 7.

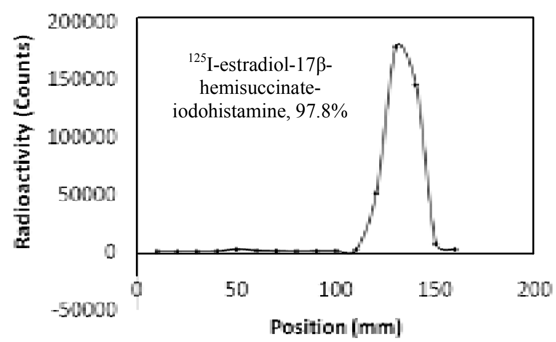
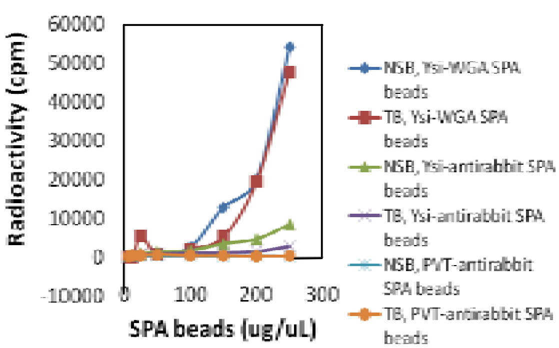


Fig. 7. Radiochromatogram of ^{125}I-estradiol-17β-hemisuccinate-iodohistamine using TLC.

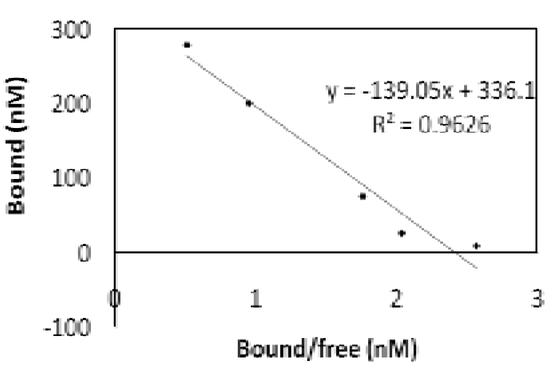
Purified ^{125}I-estradiol-17β-hemisuccinate-iodohistamine with high radiochemical purity was then used to observe its binding to estrogen receptors. The estrogen receptors used in this study was breast cancer cells (MCF7 cell lines) that express estrogen receptor α. The binding study performed after optimization on the amount and type of SPA beads. Various types of beads were used including YSI-WGA SPA, YSI-antirabbit SPA and PVT-antirabbit SPA. At varying amount of SPA beads (12.5 μg/mL to 250 μg/mL). Figure 8a. showed optimum beads was YSI-WGA SPA beads. The results showed that the YSI-WGA SPA beads with concentration of 25 ug/mL gave total binding (TB) higher than the value of non specific binding

(NSB). For other types of beads (YSI-antirabbit SPA and PVT-antirabbit SPA) gave low values of NSB and TB.

After obtaining the type and amount of the optimum of beads, assay protocol was performed to obtained value of the dissociation constant (Kd) and maximum binding values (Bmax). The data from assay protocol was radioactivity of NSB and TB. The data were then calculated to obtain the concentration of NSB and TB, then rosenthal plot curve was made to get linear regression equation. As shown in Fig. 8b, linear regression equation following formula y = -ax + b, y = -139.05x + 336.1. The regression equation gave the value of Kd = - (1/slope) = - (1/-139,05) = 7.192 x 10^{-3} nM and maximum binding = Bmax = intercept = 336.1 nM. The values of Kd was requirements for Kd values of estradiol are based on literature at < 0.003 – 0.87 nM [16]. Binding affinity of estradiol-17β-hemisuccinate represented by the value of Kd at 7.192 x 10^{-3} nM. This low value of Kd indicated that binding affinity of estradiol-17β-hemisuccinate was high or strongly bind to estrogen receptor. When compared with literature written by Neto et al, values of Kd was obtained at 6.14 nM. The difference in affinity is probably related to the ligand lipophilicity [17].



(a)



(b)

Fig. 8. Optimization of the type and amount of SPA bead (a), and Rosenthal Plot curve (b)

CONCLUSION

Preparations of ^{125}I-estradiol-17β-hemisuccinate-iodohistamine with indirect labelling method has been carried out. Two stages of purification, solvent extraction and TLC system, were managed to obtain radiochemical purity of ^{125}I-estradiol-17β-hemisuccinate-iodohistamine at more than 90%. Purification stage yielded at 89,9% and the radiochemical purity at 97,8% was obtained. Determination of binding affinity by the SPA method using MCF7 cell lines which express estrogen receptors, represented by the value of Kd at 7.192 x 10^{-3} nM and maximum binding at 336.1 nM. This low value of Kd indicated that binding affinity of estradiol-17β-hemisuccinate was high or strongly bind to estrogen receptor.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Abdul Mutalib, Ex-Head, Centre for Radioisotopes and Radiopharmaceuticals, Radioisotope Division counterparts for their keen interest and valuable support during the work. The authors are thankful to Gadjah Mada University for supplying us with MCF7 cell lines.

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