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Synthesis of Sulochrin-^{125}I and Its Binding Affinity as α-Glucosidase Inhibitor using Radioligand Binding Assay (RBA) Method

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ABSTRACT

Most of diabetics patients have type 2 diabetes mellitus or non insulin dependent diabetes mellitus. Treatment type 2 diabetes mellitus can be done by inhibiting α-glucosidase enzyme which converts carbohydrates into glucose. Sulochrin is one of the potential compounds which can inhibit the function of α-glucosidase enzyme. This study was carried out to obtain data of sulochrin binding with α-glucosidase enzyme as α-glucosidase inhibitor using Radioligand Binding Assay (RBA) method. Primary reagent required in RBA method is labeled radioactive ligand (radioligand). In this study, the radioligand was sulochrin-^{125}I and prior to sulochrin-^{125}I synthesis, the sulochrin-I was synthesized. Sulochrin-I and sulochrin-^{125}I were synthesized and their bindings were studied using Radioligand Binding Assay method. Sulochrin-I was synthesized with molecular formula $C_{17}H_{15}O_7I$ and molecular weight 457.9940. Sulochrin-^{125}I was synthesized from sulochrin-I by isotope exchange method. From the RBA method, dissociation constant (Kd) and maximum binding (Bmax) were obtained 26.316 nM and Bmax 9.302 nM respectively. This low Kd indicated that sulochrin was can bind to α-glucosidase.

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INTRODUCTION

Diabetes mellitus is a chronic disease related to insulin resistance. In 2010, people with diabetes reached 220 million and in 2025, its estimated reaching to 300 million people [1]. Based on IDF (International Diabetes Federation) data, Indonesia is the fourth largest country for prevalence of diabetes [2]. About 90% of diabetics are from type 2 diabetes mellitus (non insulin-dependent diabetes mellitus). Type 2 diabetes mellitus is characterized by the occurrence of postprandial hyperglycemia [3]. The control of postprandial hyperglycemia is an important step for the treatment of diabetes. This can be done by inhibiting the absorption of glucose through the inhibition of the carbohydrates hydrolyzed enzyme such as α-glucosidase and α-amylase [4]. The α - glucosidase is an enzyme that converts carbohydrates into glucose [5,6]. The mechanism of action α-glucosidase inhibitors is a reversible inhibition, competitive with α-amylase and pancreatic digestive enzymes in the small intestine such as isomaltase, sucrase and maltase. These enzymes play a role in the hydrolyzing of carbohydrates into glucose and other monosaccharide. Inhibition of these enzymes can inhibit the absorption of glucose thereby reducing the state hyperglycemia after meal [6,7].

Recently, α-glucosidase inhibitors have been studied, *in silico*,as well as both *in vivo* and *in vitro*. Isolation α-glucosidase inhibitor has widely been done, such as, isolation acarbose from *Actinoplanes uthanesis* [3,4], isolation vasicine and vasicinol from *Adhatoda vasica* [8]. Voglibose, miglitol were also known as α-glucosidase inhibitor and they were used in type 2 diabetes therapy [9]. Sulochrin (methyl 2-(2,6-dihydroxy-4-methylbenzoyl)-5-hydroxy-3-methoxybenzoate) isolated from *Aspegillus terreus*, showed its potency as an α-glucosidase inhibitor with IC_{50} value 8.5 μg/mL

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[10]. Sulochrin derivates, the methyl-4-hydroxy-5,7-diiodo-6′-methoxy-6-methyl-3,4′-dioxospiro [benzofuran-2(3H), 1′-[2,5] cyclohexadiene]-2′-carboxylate and methyl 5,7 dibromo-4-hydroxy-6-methoxy-6-methyl-3,4′dioxospiro[benzofuran-2(3H), 1′-[2,5] cyclohexdiene-2′-carboxylate showed their glucose uptake activity in rat adiposite [11]. *In silico* field, α-glucosidase inhibitor interacted with α-glucosidase residues through formation of hydrogen bond. Derivates of sulfonamide interacted with Asp349, His348, Phe157, Tyr71, Phe177, Arg212 in α-glucosidase residues from *Saccharomyces cerevisiae* [5]. Derivatives of salacinol were also known as α-glucosidase inhibitors through interaction with Asp327, His600, Asp542, Asp203, Arg526 in NtMGAM residues [12].

In this study, a derivate of sulochrin was synthesized and its binding affinity was studied using Radioligand Binding Assay (RBA) method. The RBA is a method which can be used to determine binding affinity of antibodies or receptors with ligand using a radioactive as tracer [13]. Radioligand (L*) is reacted with receptor (R) and reach equilibrium to get radioligand receptor complex (L*R).

Purification of radioligand receptor complex (L*R) from unreacted ligand (L*) can be done by filtration, centrifugation, dialysis, size exclusion chromatography [14]. This purified complex can be counted their radioactivity, and from this data, Kd and Bmax values will be known. Kd is dissociation constant and Bmax is maximum binding that can be occurred between ligand and receptor. The RBA method needs a ligand compoud labeled with radioactive element such as iodine-125 (I-125). The radioisotope I-125 is widely used due to its physical half life (59.4 days, long enough for *in vitro* treatment) and its low energy gamma radiation (about 25 keV, small enough for being save for user) [15].

EXPERIMENTAL METHODS

The materials used were sulochrin (was received from Research Center for Chemistry LIPI), N,N-dimethylformamide, potassium iodide, iodine, sodium acetate, sodium hydrogen sulfate, sodium sulfate anhydrate, methanol, chloroform (Merck), Iodine-125 as $Na^{125}I$ (PRR-BATAN), α-glucosidase enzyme from *Shaccharomyces sp* (Wako Pure Chemistry Industry), bovine serum albumin, polyethylene glycol (Sigma-Aldrich). The equipments used were gamma counter (Nucleus), micropipette (Eppendorf), magnetic stirrer, analytical balance (Sartorius).

Synthesis of sulochrin-I [11]

Procedure of synthesis sulochrin-I was performed in similar manner to procedure reported previously [11]. Sulochrin (4 mg, 12 μmol) was dissolved in 1 mL N,N-dimethylformamide (DMF). Potassium iodide (2.2 mg, 13.2 μmol), iodine (3.2 mg, 12.6 μmol) and sodium acetate (1 mg, 12.2 μmol) were added to the solution of sulochrin. The reaction mixture was stirred for 17 hours (over-night) at room temperature. Then ethyl acetate was added into the reaction mixture, and the resulting mixture was washed with 5% sodium hydrogen sulphite. The organic layer was dried with Na_2SO_4. The resulting product was purified by silica gel TLC developed with $CHCl_3$/MeOH (96 : 4). The final product was analyzed using LC-MS (Liquid Chromatography-Mass Spectrometry).

Synthesis of sulochrin-^{125}I

$Na^{125}I$ (2 μL, 1.5 mCi) was added into sulochrin-I solution (150 μL, 50μgram). The reaction mixture was stirred for 17 hours (over night) at room temperature and then purified by silica TLC developed with $CHCl_3$/MeOH (95 : 5).

Assay protocol with RBA method

A 50 μL of phosphate buffer saline containing 5% of bovine serum albumin and 2.5 μg α-glucosidase was added into Total Binding (TB) and Non Spesific Binding (NSB) tubes. A 50 μL unlabeled sulochrin-I was added in the NSB tube and 50 μL of phosphate buffer saline was added into TB tube. Varying concentration of sulochrin-^{125}I was diluted in phosphate buffer saline, then a 50 μL of this solution was added to each tube. The tubes were incubated for 2 hour at 37 ^oC (1st incubation). Separation of the Receptor-Ligand Complex (L*R) from unbound ligand was done by adding solution of 10% polyethylene glycol in phosphate buffer saline to the tubes. The tubes were incubated for 30 minute at 37 ^oC (2nd incubation). After incubation, the tubes were decanted and counted using gamma counter. Results from this experiment were used to calculate value of Kd and maximum binding (Bmax).

RESULTS AND DISCUSSION

Synthesis of sulochrin-I

Synthesis of sulochrin labeled by non radioactive iodine (sulochrin-I) was carried out prior to synthesis of sulochrin radioactive iodine (sulochrin-^{125}I) by reacting sulochrin with iodine and potassium iodide at room temperature (Fig. 1). Extraction method was used to get sulochrin-I with addition of ethyl acetate and sodium hydrogen sulphite solution for binding the iodide excess. The sulochrin-I as the product will be in ethyl acetate phase. Purification of sulochrin-I was done by thin layer chromatography (TLC) method whereas using silica 60F_{254} as stationary phase and mixture of chloroform:methanol (96:4) as mobile phase. The Rf of sulochrin and sulochrin-I were 0.5 and 0.7 respectively. This result showed that TLC method can be used as purification of sulochrin-I.

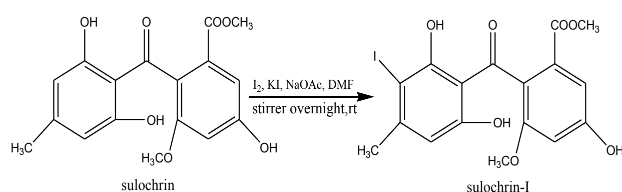

Fig. 1. Reaction of sulochrin-I.

Purified sulochrin-I was characterized by LC-MS. The results showed that the synthesized product had molecular weight 458.9940 $(M+H)^+$ (Fig. 2). We predict that this compound has molecular formula $C_{17}H_{15}O_7I$. This result showed that iodine was bound to the sulochrin molecule. This compound will be used to synthesis the sulochrin labeled radioactive iodine (sulochrin-^{125}I).

Synthesis of sulochrin-^{125}I

Prior to synthesis of sulochrin labeled radioactive iodine (sulochrin-^{125}I), a radiochromatogram of Na^{125}I was prepared using TLC method and showed in Fig. 3. Whatman paper No. 1 was used as stationary phase and 75% methanol solution as mobile phase. The radiochromatogram of Na^{125}I showed the radiochemical purity at more than 99% which qualify for labeling process [16].

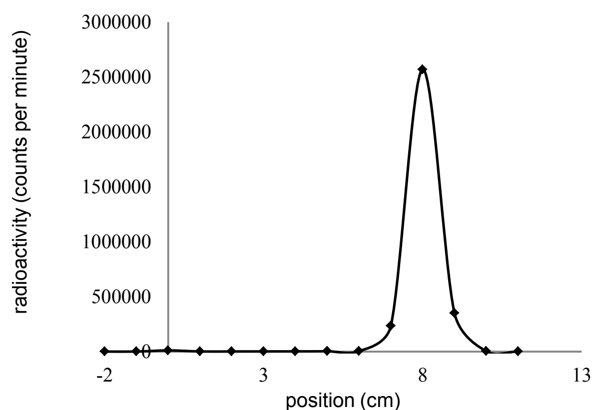

Fig. 3. Radiochromatogram of Na^{125}I using TLC method, whatman paper No.1 as stationary phase and 75% methanol solution as mobile phase.

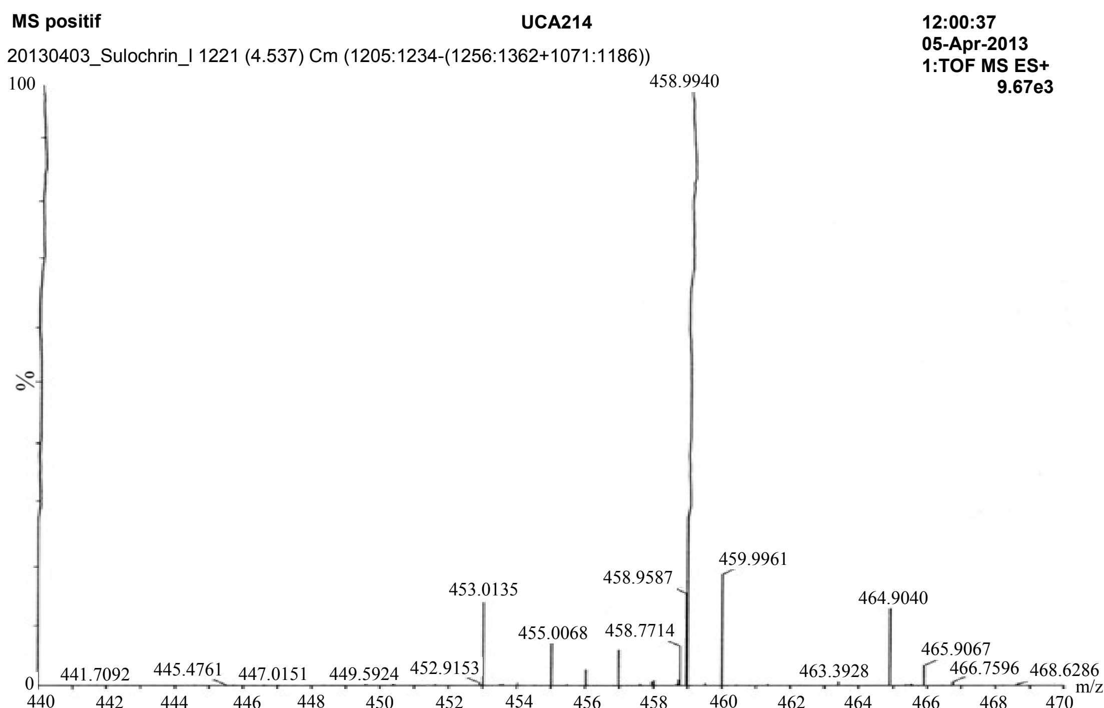

Fig. 2. The Mass Spectrum of sulochrin-I from synthesized product had molecular weight 458.9940 $(M+H)^+$.

In this study, sulochrin-^{125}I was used as tracer to study binding affinity of sulochrin as α-glucosidase inhibitor using Radioligand Binding Assay (RBA) method. The method which was used for synthesis of sulochrin-^{125}I was isotope exchange reaction, in which the non radioactive iodide was interchanged with radioactive iodide-^{125}I. Sulochrin-I was reacted with Na^{125}I and stirred overnight at room temperature. TLC was used as purification method, using silica as stationary phase and mixture of chloroform : methanol (95:5) as mobile phase. Radiochromatogram of sulochrin-^{125}I synthesis in Fig. 4 showed that retention time of sulochrin-^{125}I and Na^{125}I was different. Retention time of sulochrin-^{125}I and ^{125}I unreacted were 0.6 and 0 respectively. This result showed that sulochrin-^{125}I separated from unreacted Na^{125}I.

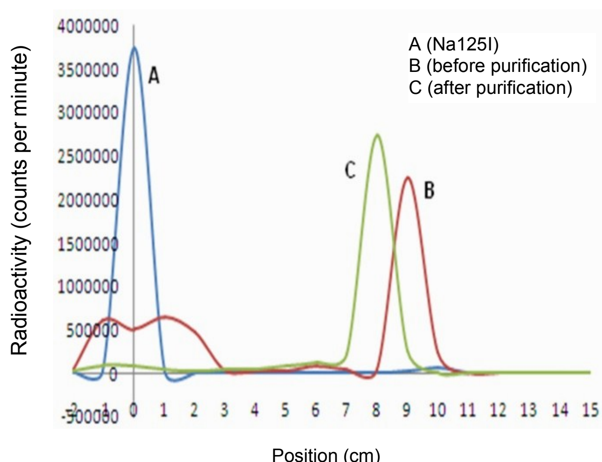

Fig. 4. Radiochromatogram of (A) Na^{125}I, (B) sulochrin-^{125}I before purification and (C) after purification using TLC method, silica 60F$_{254}$ as stationary phase and mixture of chloroform:methanol (95:5) as mobile phase.

Purified sulochrin-^{125}I was then used to observe its binding affinity with α-glucosidase receptors using Radioligand Binding Assay (RBA) method. The RBA method was done to obtained value of dissociation constant (Kd) and maximun binding (Bmax). The data from assay protocol was radioactivity of NSB (Non Spesific Binding), TB (Total Binding) and TRA (Total Added). The data was converted to concentration of NSB, TB and TRA, then Rosenthal curve was made to get the value of Kd and Bmax. As shown in Fig. 5, the curve gave equation y = -0.038x + 9.302 with linearity (r) 0.871. The low value of this linearity of curve (r) is might be due to less optimal separation of bound radioligand from free radioligand. The separation is crucial step in RBA method, it is important to prevent dissociation of the receptor-radioligand complex which this parameter is measured [17]. The value of Kd and Bmax was obtained from this equation i.e Kd = -(1/slope) = -(1/-0.038) = 26.316 nM and Bmax = intercept = 9.302 nM. The Kd is equal to the concentration of ligand that will occupy 50% receptors [18], and it is mean that the concentration of sulochrin-^{125}I needed to occupy 1.25 μg receptors is 26.316 nM.

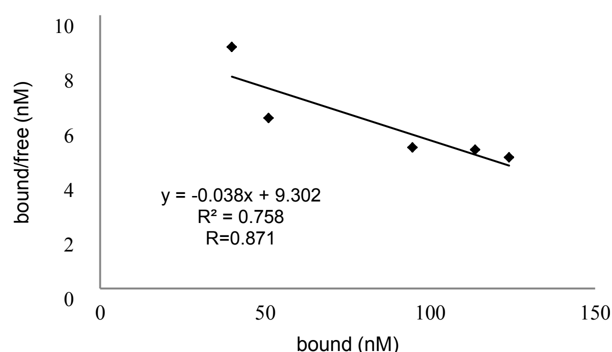

Fig. 5. Rosenthal curve for obtaining Kd and Bmax value.

CONCLUSION

Sulochrin showed its potency as an α-glucosidase inhibitor. Sulochrin binding with α-glucosidase enzyme as α-glucosidase inhibitor was studied using Radioligand Binding Assay (RBA) method. Assay sulochrin with RBA method needed sulochrin-^{125}I as a tracer. Prior synthesis of sulochrin-^{125}I, the procedure was firstly synthesizing the sulochrin-I. Synthesis of sulochrin-I and sulochrin-^{125}I has been carried out. Sulochrin-I had molecular weight 457.9940 with molecular formula $C_{17}H_{15}O_7I$. Sulochrin-^{125}I was synthesized from sulochrin-I using isotope exchange method. Kd and Bmax value of sulochrin were 26.316 nM and 9.302 nM respectively. This low Kd indicated that sulochrin was can bind to α-glucosidase.

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