Synthesis of DOTA-TOC Conjugate as a Precursor of ^{177}Lu-DOTA-TOC Radiopharmaceutical for Therapy and Diagnosis of Somatostatin Receptor Positive Cancer

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Received: 15-Jan-2016 Revised: 25-Mar-2016 Accepted: 17-Mei-2016

ABSTRACT

Synthesis of DOTA-TOC Conjugate as a Precursor of ^{177}Lu-DOTA-TOC Radiopharmaceutical for Therapy and Diagnosis of Somatostatin Receptor Positive Cancer. Somatostatin receptor is overexpressed in some cancers such as neuroendocrine, breast cancers, etc. Somatostatin analog, octreotide, which is labelled as lutetium-177 (^{177}Lu), can act as a ligand that serves as both diagnostic and therapeutic agent of cancer. The chosen of ^{177}Lu is mainly due to its favourable characteristic. It emits gamma and beta particles that are useful for the above mentioned purposes. This research aims to prepare a conjugate of N,N',N'',N'''-tetraazacyclododecane tetra acetic acid (DOTA)-tyr^3-octreotide (DOTA-TOC) a precursor of ^{177}Lu-DOTA-Tyr^3-Octreotide, a candidate of radiopharmaceutical for diagnosis and therapy of somatostatin receptors positive cancers. The DOTA-TOC conjugate was synthesised using two-step reactions: conjugation of ester NHS-DOTA to BOC-TOC and hydrolisis to form DOTA-TOC. The chromatogram of the DOTA-TOC conjugate gave peaks with retention time (tR) of 11.2 mins. ESI-MS analysis revealed +2 charge of the DOTA-TOC conjugate gave a peak at 711.32 Da which indicated the formation of the DOTA-TOC with m/z of (1420.616+ 2 H)/2 Da (exact mass of literature 1420.616 Da). The radiolabel of conjugate with ^{177}Lu resulted in of ^{177}Lu-DOTA-TOC with radiochemical purity of 87%. These results showed that the DOTA-TOC conjugate was a promising precursor for preparation of the ^{177}Lu-DOTA-TOC.

Keywords: ligand, analog, targeted, radionuclide, chromatogram

ABSTRAK

Sintesis Konjugat DOTA-TOC sebagai Prekursor ^{177}Lu-DOTA-TOC untuk Terapi dan Diagnosis Kanker Positif Reseptor Somatostatin. *Reseptor somatostatin diekspresikan berlebih pada beberapa kanker seperti neuroendokrin,*

Vol. 19, No. 1, Juni 2016, hal: 1-14
ISSN 1410-7864

payudara, dll. Analog somatostatin, octreotida, yang ditandai dengan lutesium-177 (^{177}Lu) dapat beraksi sebagai ligan yang berfungsi sebagai agen diagnosis dan terapi. Pemilihan ^{177}Lu dikarenakan karakteristiknya yang disukai. ^{177}Lu mengemisikan partikel gamma dan beta yang berguna untuk fungsi yang sudah disebutkan di atas. Penelitian ini bertujuan untuk menyiapkan konjugat DOTA-tyr^3-octreotide (DOTA-TOC), prekursor dari ^{177}Lu-DOTA-Tyr^3-Octreotide, kandidat radiofarmaka yang nantinya bermanfaaat untuk diagnosis dan terapi kanker yang positif reseptor somatostatin. Konjugat DOTA-TOC disiapkan melalui dua langkah reaksi, yaitu konjugasi ester NHS-DOTA dengan BOC-TOC dan hidrolisis membentuk DOTA-TOC. Kromatogram dari konjugat DOTA-TOC menunjukkan puncak pada waktu reaksi 11,2 menit. Analisis ESI-MS memberikan hasil bahwa konjugat DOTA-TOC bermuatan +2 dan adanya puncak pada 711,32 Da. Hasil ini mengindikasikan pembentukan DOTA-TOC dengan m/z (1420.616+ 2 H)/2 Da (massa eksak literatur 1420.616 Da). Radiolabeling konjugat dengan ^{177}Lu menghasilkan ^{177}Lu-DOTA-TOC dengan kemurnian radiokimia 87 %. Hasil ini menyatakan bahwa konjugat DOTA-TOC merupakan prekursor yang menjanjikan dalam penyiapan radiofarmaka ^{177}Lu-DOTA-TOC.

***Kata Kunci**: ligand, analog, terarah, radionuklida, kromatogram*

Abbreviation:

DOTA	: N,N',N",N"'-tetraazacyclododecane tetra acetic acid
TOC	: tyr^3-octreotide
Lu	: Lutetium
NHS	: N-hydroxysuccinimide
ESI-MS	: Electron Spray Ionization
ITLC-SG	: Instant thin layer chromatography – silica gel

INTRODUCTION

Cancer has become a disease that causes a high number of death. Based on the data from the International Agency for Research on Cancer (IARC), there were 12.7 million new cancer cases in 2008 worldwide, of which 5.6 million occurred in developed countries and 7.1 million occurred in developing countries. IARC estimated that by 2030, there will be 13.2 million death cases out of 21.4 million new cancer cases [1]. It can be assumed that cancer is one of the most threatened diseases of our society nowadays.

Targeted radionuclide therapy using radiolabelled peptides or monoclonal antibodies have showed as an effective treatment for cancer therapy in the last decade. Some of radiolabelled peptides such as somatostatin analog has been widely used for both diagnostic and therapeutic

treatment for some cancers which overexpress somatostatin receptor such as neuroendocrine tumours, breast cancer, etc [2].

Somatostatin is neuropeptide that plays role in endocrine and exocrine secretion regulation. Treatment of cancer using somatostatin is limited to its short biologic half-life which is due to enzymatic degradation (less than 3 mins) as can clearly be seen in Figure 1 [3]. This result has stimulated the development for more stable somatostatin analog with a longer biologic half-life. A somatostatin analog that has been used clinically is octreotide that possesses two hours of biologic half-life [4]. Therefore, it can be hypothesized that somatostatin analog labelled with gamma and beta particle emitter radionuclide can act as a ligand that specifically (targeted) interact with somatostatin receptor in cancer cell surface. Gamma and beta particles from radionuclide are able to identify sites, size of cancer, and destroy cancers cell. Meanwhile somatostatin analog (octreotide) can control symptom caused from excessive hormone-released [4].

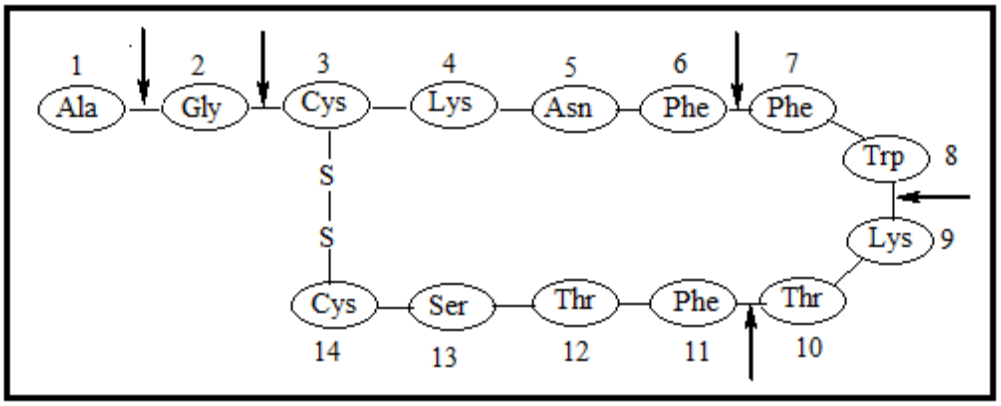

Figure 1. Structure of somatostatin-14 and key enzymatic degradation sites (marked with arrows) [3]with modification

There are two radiopharmaceuticals based-octreotide which have been approved by Federal Drug Administration (FDA) used for both diagnosis and therapy of cancers positive somatostatin receptor. These are Octreoscan™ (^{111}In-DTPA-octreotide) and Octrether™ (^{90}Y-DOTA-octreotide). Octreother™ has been reported to generate good response for relatively large mass of neuroendocrine cancer [5-7]. However, some drawbacks occurred in the utilization as the therapeutics radiopharmaceuticals after several administration cycles. It dramatically causes a decreasing in kidney function and bone marrow destruction, which is derived from high beta energy of ^{90}Y (2.2 MeV) [5]. In addition, deposition of this radiopharmaceutical on the target tissues cannot be detected using the available imaging modalities because ^{90}Y is pure beta

emitter particle [8]. In practice, Octreoscan™ is routinely administered together with Octrether™, so that its deposition and clearance can be monitored and evaluated.

Since characteristic of radionuclide ^{90}Y still needs a consideration to be used in therapeutic purpose, the chosen of therapeutic radionuclide which possess relatively lower energy of beta particle (compared to ^{90}Y) with an imageable gamma energy is much more preferable. Lu-177 has been considered as an ideal radionuclide therapy that offers many advantages over others. Beta particle energy of ^{177}Lu [E_{maks} 497 keV (78.6 %) and 176 keV (12.2 %)] is suitable for therapy**,** and its gamma [E_{maks} = 113 keV (6.4 %) and 208 keV (11 %)] also suit for *in vivo* imaging purposes using a gamma camera or SPECT [9].

Somatostatin analog, *i.e*. tyr3-octreotide (TOC), has been reported clinicaly potential for diagnostic and treatment of somatostatin receptor-expressing tumors [3]. The replacement of phenyalanine amino acid in third position by tyrosine amino acid, as can be seen in Figure 2, has been reported increase its binding affinity to somatostatin receptor in tumor cell [10].

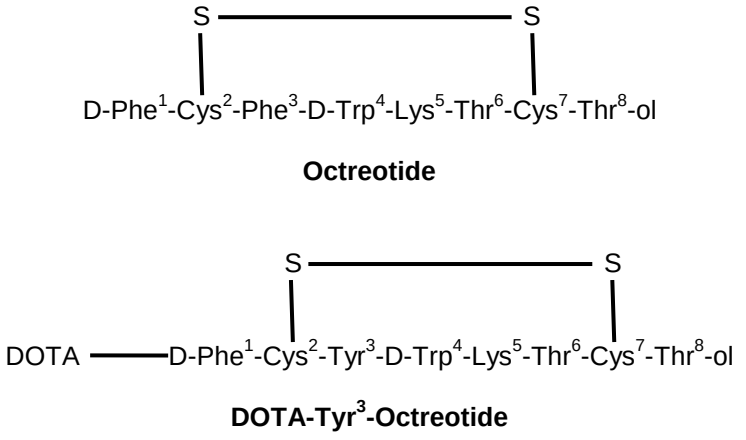

Figure 2. Structure of octreotide and DOTA-TOC **[3]** with modification

The application of radiopharmaceuticals based-somatostatin analog such as ^{177}Lu-DOTA-TOC is expected to meet the criteria for therapeutic and diagnosis radiopharmaceutical. In addition, there is no necessity to administer another radiopharmaceutical during the administration of ^{177}Lu-DOTA-TOC, since its gamma emitter can be used for imaging or diagnosis purpose. Since ^{177}Lu-DOTA-TOC can be used for both therapeutic and diagnosis purpose, it is expected that the use this agent would be cost effective for cancer treatment in Indonesia. Therefore, the continous availability of that such radiopharmaceuticals is important. Radionuclide of

^{177}Lu can be easily produced with high specific activity due to their high cross reaction (Lu-176 (n,γ) Lu-177] (2100 barn). This research aims to prepare a conjugate of DOTA-tyr3-octreotide (DOTA-TOC) that can be used as a precursor of ^{177}Lu-DOTA-Tyr3-octreotide for diagnosis and therapy of somatostatin receptors positive cancers.

EXPERIMENTAL METHOD

Materials

All chemicals were used as received without further purification. Hhydroxisuccinimide-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (NHS-DOTA) was supplied by Macrocyclics. [Tyr-3, Lys(Boc)5]-Octreotide-TFABOC-TOC (BOC-TOC) was supplied by Bachem. Other chemicals such as dimethyl formamide anhydrous, methanol anhydrous, ethanol anhydrous, hydrazine hydrate, trifluoroacetic acid (TFA) and acetonitrile were supplied by Sigma Aldrich. H_2O (18 Mohm) was obtained from a purification water system (Merit). Nitrogen gas was supplied by local supplier. Thin-layer chromatography plate (TLC-Silica Gel $60F_{254}$) and normal hexane, iodine were supplied by Merck.

Preparation of $^{177}LuCl_3$

$^{177}LuCl_3$ was prepared by irradiating ~0.3 mg of ^{176}Lu ($^{176}Lu_2O_3$, 60,60 % enriched) at RSG-GAS (neutron flux: 1.26 x 10^{14} n cm^{-2} det^{-1}) for 4 days. The irradiated target was removed to a beaker glass and 2 mL of HCl 6 M was then added. The mixture was allowed to stand for 30 mins before addition of 2 mL H_2O_2. The mixture was heated until dried and redissolved in 3 mL of HCl 0.5 M. The resulted $^{177}Lu^{3+}$ was non-carrier free and contained non-radioactive $^{176}Lu^{3+}$ close to the mass of the irradiated target.

Preparation of DOTA-TOC Conjugate

DOTA-NHS was reacted with [Tyr^3, Lys(Boc)5]-Octreotide-TFABOC-TOC (BOC-TOC) in a solution of dimethyl formamide (DMF) anhydrous to form DOTA-BOC-TOC. Molar ratio of BOC-TOC to NHS-DOTA was 1 : 4. The mixture was stirred for 30 mins. The solvents were then evaporated. The methanol anhydrous was added to the residue and the result suspension was centrifuged. An aliquot portion of ethanol anhydrous was added into the filtrate, and it was then evaporated in vacuo. Deprotection DOTA-BOC-TOC was carried out by introducing 1 mL of DMF containing 2 % of hydrazine hydrate. The mixture was incubated in room temparature for about 10 mins then redried under vacuo [11].

Radiolabeling of DOTA-TOC with Lutetium-177

DOTA-TOC conjugate (5.6 nmol) was labelled with ^{177}Lu with molar ratio of 1 : 1. Lu-177 was preconditioned with ammonium acetate buffer pH 7.5 (1 : 1) prior to its addition into the solution of DOTA-TOC conjugate. The pH of solution was subsequently adjusted to 4.5 and was followed by heating the solution at 80^{o}C for 20 mins [10]. The radioconjugate (^{177}Lu-DOTA-TOC) was analyzed using TLC system (radiochromatography). Then, the ^{177}Lu-DOTA-TOC was purified using Sep-Pack C-18 cartridge.

Characterization of DOTA-TOC Conjugate

The characterization of DOTA-TOC conjugate was carried out using HPLC and LC-MS MALDI TOF. The HPLC was conducted using C-18 column, eluent of TFA 0.1 % and Acetonitrile with gradient system and flow rate of 1 ml/min), whereas LC-MS MALDI TOF was analyzed in BPPT Biotechnology Centre.

Characterization of ^{177}Lu-DOTA-TOC (Radioconjugate)

Characterization of radioconjugate and the radiochemical purity of the resulted ^{177}Lu-DOTA-TOC were carried out by thin layer chromatography system using a ITLC-SG strips and EDTA 0.01 mM as stationary phase and mobile phase, respectively. Purification of ^{177}Lu-DOTA-TOC was conducted using a Sep-Pack C-18 cartridge with H_2O and ethanol 96 % as eluent. The ^{177}Lu-DOTA-TOC solutions were counted with dose calibrator and analyzed using TLC system using ITLC-SG and EDTA 4 mm as stationary and mobile phase, respectively.

RESULTS AND DISCUSSION

It has been reported that there are several methods for conjugating 1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetraacetic acid (DOTA) to peptide [12]. One of them involves a reaction of NHS ester derivative of DOTA a with primary amino groups at either the N-terminus or on the lysine side chains of the peptide [12]. This method has advantages over the others because it uses the available reagents and allows conjugation different varieties of both peptide and protein to their ligands [12]. The scheme reaction can be seen in the Figure 3. The reactions consist of two steps. The first step is the conjugation of NHS-DOTA to BOC-tyr^3-octreotide (BOC-TOC) and the second one is deprotection or hydrolisis of its BOC to form DOTA-TOC conjugate.

DOTA was used as a bifunctional chelating agent (BFC) that plays role as a linker to attach some biomolecule, such as monoclonal antibody,

peptide, etc. In addition, DOTA is capable to act as bifunctional chelator that provides a standard amidation reactions from an activated NHS ester [13].

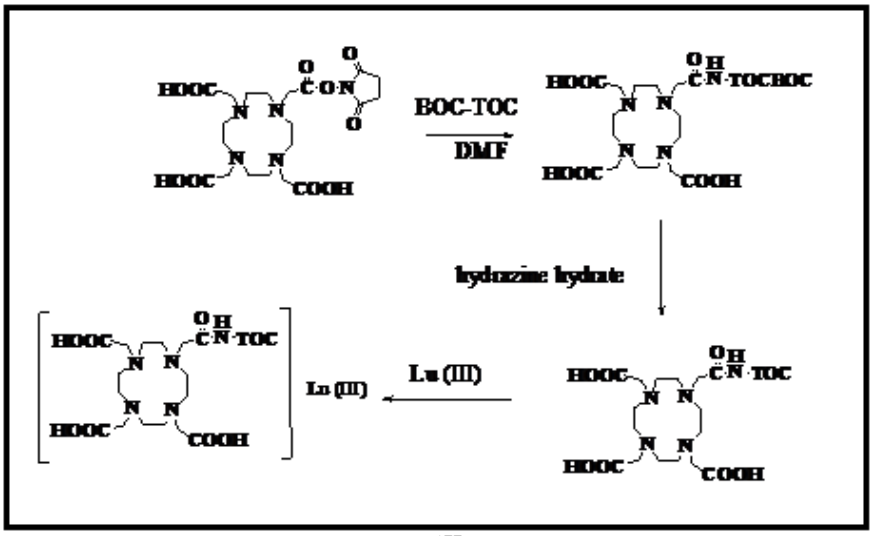

Figure 3. Reaction scheme of ^{177}Lu-DOTA-TOC preparation

NHS-DOTA was chosen because of its ability to attach to BOC-TOC, with a coupling moiety that was attached to the macrocycle at the position of one carboxylate arm [14]. This ability may due to the presence of electrophilic active group that can be easily coupled to primary amine of BOC-TOC to form a stable amide bond [15]. The scheme reaction of conjugation of peptide through NHS ester can be seen in Figure 4.

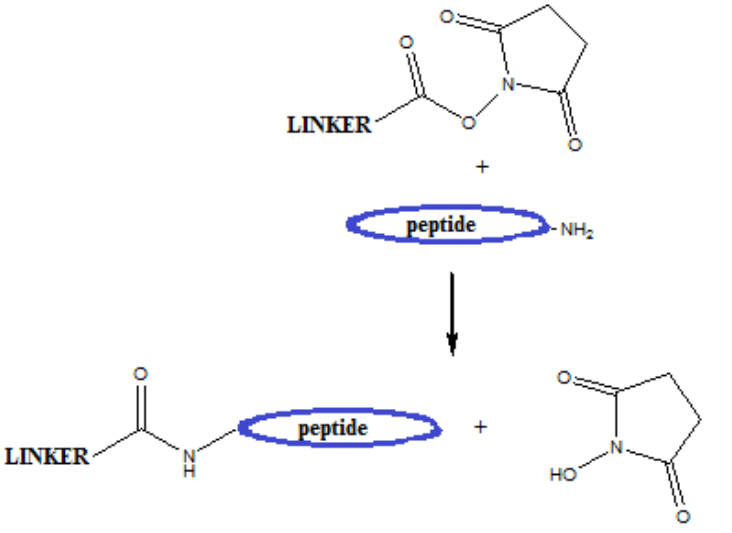

Figure 4. Coupling peptide through NHS-ester groups, **[16]** with modification

The chromatogram of DOTA-TOC which was overlayed with the chromatograms of its precursor and by product, such as NHS-DOTA, BOC-TOC, NHS-DOTA-BOC-TOC are shown in Figure 5. These chromatograms showed that the conjugate of DOTA-Tyr^3-Octreotide (DOTA-TOC) had been formed. This was confirmed by its retention time (11.2 min) which was assumed as a peak of DOTA-TOC conjugate.

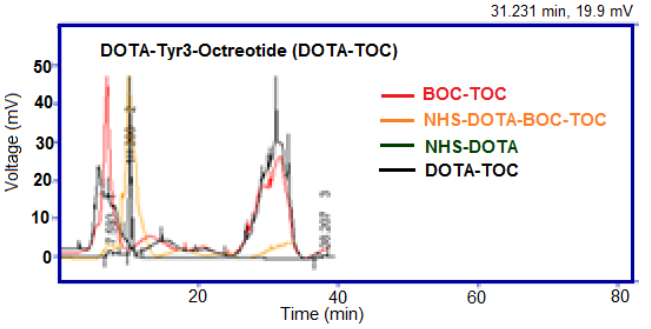

Figure 5. HPLC Chromatogram of DOTA-TOC using C-18 column TPB 201 (8 x 250) mm, mobile phase : A TFA 0.1 % and B. ACN, Gradient system, flow rate 1 ml/min, detector: UV/Vis 280 nm

Analysis of Electron Spray Ionization (ESI MS) is found to be useful for peptide and protein determination [17]. This is not only because of its compatibility with both liquid chromatography and tandem mass analyser, but also it gives a high sensitivity (subpicomole with nano ESI) [17]. Furthermore, ESI allows multiple charging of the samples that enables them to be used in protein analysis with limited m/z range [17]. DOTA-TOC can be as +1, +2, +3 charges. In order to determine the possible charges that were formed, it is important to see from the pattern of the isotope and observing the possible charge states, as well. ESI-MS chromatogram of DOTA-TOC conjugate can be seen in Figure 6. This chromatogram revealed that DOTA-TOC conjugate was found to have a retention time and m/z of 3.43 mins and 711.32, respectively. Based on the commercially available DOTA-TOC shows the exact mass of 1420.616 [18]. Mass spectra measures mass to charges ratio (m/z). The pattern of the isotope as can be seen in Figure 6 showed that the possible charge of the conjugate was +2. The analysis of ESI MS of DOTA-TOC conjugate in this research gave value of 711.32 Da. It was calculated from mass-to-charges ratio which follows the equation of $(1420.616 + 2H)^{2+}$ which means that m/z of theoretical was 1422.616/2, giving m/z of 711.308 Da. Therefore from the m/z that was obtained, it might be asssumed that the DOTA-TOC conjugate was successfully synthesized.

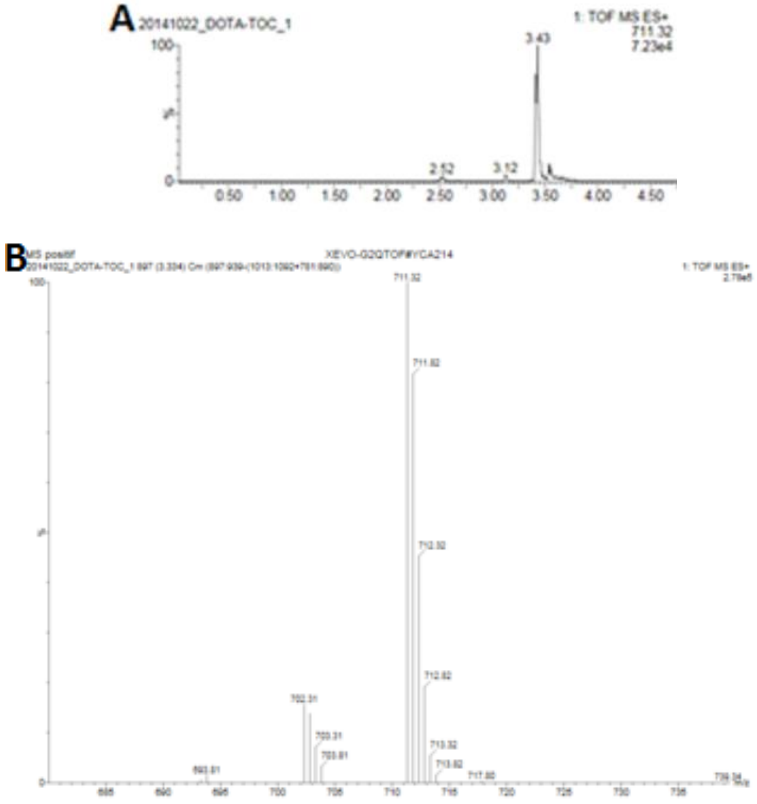

Figure 6. LC-MS chromatogram of DOTA-TOC conjugate

Peptide is a small size molecule, so that introducing of such bulky agents, for instance in a radiolabelling process, might change its binding affinity to its particular receptor [3]. Therefore, selection of BFC or linker needs to be well-considered. DOTA is a widely used BFC in the field of radiopharmaceuticals preparation that exploits Y-90 and Lu-177 radionuclides. DOTA complex gives high thermodynamic stability compared to DTPA and EDTA complex. The order of thermodynamic stability complex is DOTA> DTPA> EDTA. Peptide Receptor Radionuclide Therapy (PRRT) has exploited DOTA due to its versatility compared to others BFC [19]. DOTA is also suitable for linking trivalent metallic radioisotopes (^{177}Lu, ^{90}Y) to biomolecules, such as peptide [20]. The schematic of radiotherapy using TOC peptide can be seen in Figure 7.

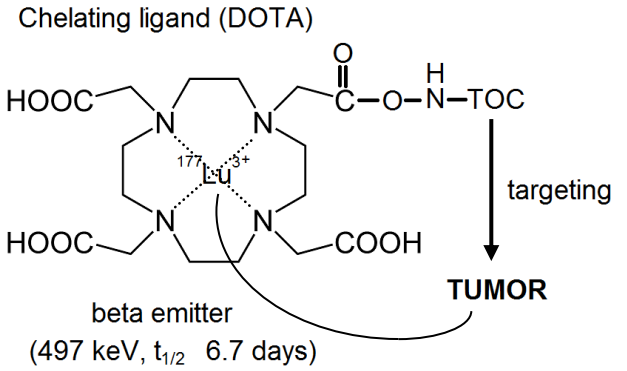

Figure 7. Schematic of radiotherapy using TOC peptide [21] with modification

There is a need to result a high specific activity of radiopharmaceuticals which are going to be used for therapy purposes. Several factors which determine the specific activity of particular radiopharmaceutical are specific-activity of radionuclide use for radiolabeling, temperature, pH, and incubation time [10]. The radionuclide use in the radiolabeling of DOTA-TOC was ^{177}Lu which was produced by iradiating ~ 60.00 % encriched of $^{176}Lu_2O_3$ in G.A. Siwabessy Reactor. The resulted ^{177}Lu was carrier added with specific activity of 1.579 Ci/ mg. The radiolabeling of ~6 nmol of DOTA-TOC would only need ~6 nmol Lu with correlated with ~7.42 mCi of ^{177}Lu. Therefore a high specific activity of ^{177}Lu-DOTA-TOC which was prepared from the above-mentioned ^{177}Lu would not be expected. The only way to obtain a high specific activity of ^{177}Lu-DOTA-TOC is by radiolabeling of DOTA-TOC with non-carried added of ^{177}Lu. Unfortunately, the non-carried added of ^{177}Lu has not yet available in our laboratory.

The radiolabeling of DOTA-TOC with ^{177}Lu was carried out at pH 4.5. This pH has been reported that the reaction kinetic was found to be optimal at pH 4-4.5 [10]. The time incubation and temperature also effects the reaction kinetics of radiolabeling DOTA-TOC with ^{177}Lu. The condition of radiolabeling was incubated at 80^0C for 20 mins [10, 22].

In this research,the radiochemical purity of ^{177}Lu used for radiolabeling was 97% as it was reprensented by its radiochromatogram (Figure 8). In this research the radiolabeling of DOTA-TOC conjugate with Lu-177 has been successfully carried out, although the radiolabeling yield still lower than 95%. The radiochemical yield of ^{177}Lu-DOTA-TOC resulted in from the above-mentione radiolabeling was 52% (Figure 9) prior to purification. The ^{177}Lu-DOTA-TOC radioconjugate was then purified with pre-activated Sep-Pack C-18 which resulted in ^{177}Lu-DOTA-TOC with a radiochemical purity of 87% (Figure 10).

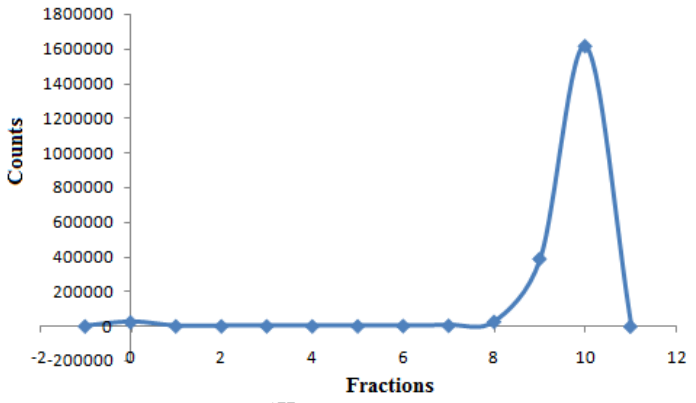

Figure 8. Chromatogram of ^{177}Lu, TLC system (eluent: 4mM of EDTA) Rf= 0 (^{177}Lu-DOTA-TOC) , Rf ~ 1 (Lu-EDTA) species

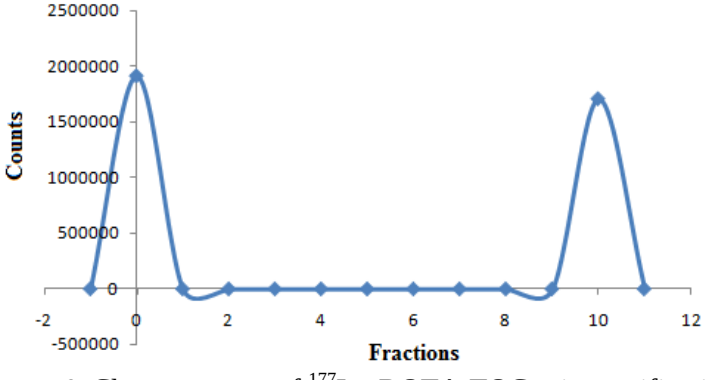

Figure 9. Chromatogram of ^{177}Lu-DOTA-TOC prior purification TLC system (eluent: 4mM of EDTA) Rf= 0 (^{177}Lu-DOTA-TOC); Rf ~ 1 (Lu-EDTA) species

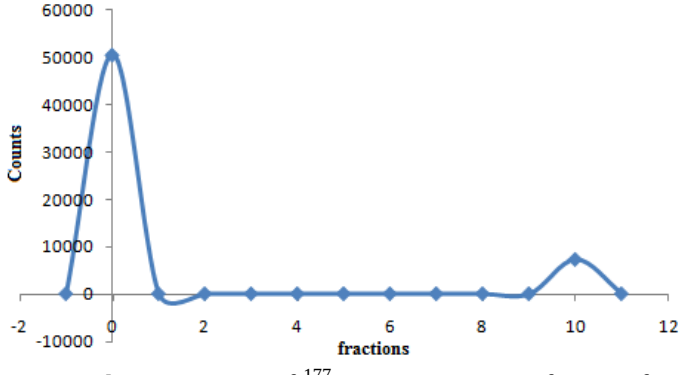

Figure 10. Chromatogram of ^{177}Lu-DOTA-TOC after purification TLC System (eluent: 4mM of EDTA) Rf= 0 (^{177}Lu-DOTA-TOC) ; Rf ~ 1 (Lu-EDTA) species

CONCLUSION

DOTA-TOC was succesfully synthesized. The synthesis consist of two steps, first conjugation of NHS-DOTA to BOC-TOC, then followed by the hydrolisis or deprotection to form DOTA-TOC conjugate. Chromatogram from HPLC analysis gave a peak with retention time of 11.2 mins which was closely similar to the one reported on a literatur. The ESI-MS MALDI TOF showed that DOTA-TOC conjugate had m/z of 711.32 Da. The radiolabeling yield revealed that the DOTA-TOC conjugate was form and was able to be labeled with Lutetium-177 which resulted in ^{177}Lu-DOTA-TOC with a yield 52 % prior purification. After the purification of pre activated sep-pack C-18 gave the radiochemical purity of 87 %.

In the future there are several works, such as the optimization of labeling condition, stability test, *in vitro* and *in vivo* test, needs to be performed in obtaining ^{177}Lu-DOTA-TOC which is conformed to requirement of a good radiopharmaceutical which is suitable for diagnosis and therapy of somatostatin receptor positive cancer.

ACKNOWLEDGEMENT

We thank to Hambali and Abidin for their assistance related to ^{177}Lu irradiation at RSG-GAS. We also would like to thank to Siti Darwati, Rohadi Awaludin, and Adang Hardi Gunawan for their advice in this research.

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