



Radiolabeled Antibody Fragment for Preparation of ($^{177}\text{Lu-DOTA}$)_m-PAMAM G3.0-F(ab')₂ trastuzumab as a Radiopharmaceutical for Cancer Therapy

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ABSTRACT

Several radiolabeled monoclonal antibodies (mAbs) have been used as radioimmunotherapy (RIT) agents for cancer therapy. The use of mAbs as RIT agents is due to their ability to carry effectors, in the form of radionuclides which emit alpha (α) particles, beta (β) particles, or auger electrons, and bind specifically to cancer expressed receptor. This paper reports the preparation of radiolabelled trastuzumab in form of ($^{177}\text{Lu-DOTA}$)_m-PAMAM G3-F(ab')₂-trastuzumab, which will be expected as a potential RIT agent for therapy of breast cancer overexpressed human epidermal growth factor receptor 2 (HER2). Due to its reduced molecular weight, the use of F(ab')₂-trastuzumab on the aforementioned RIT agent candidate is expected to reach its target much faster compared to the intact trastuzumab. Meanwhile, the role of PAMAM G3 is to increase the specific activity of the radiotherapeutic agent of Lu-177 due to the ability of its 32 -NH₂ functional groups that are able to bind many DOTAs (≤ 31) which in turn can bind a large number of ^{177}Lu . The preparation was initiated by fragmentation of trastuzumab using pepsin enzyme in 0.02 M acetic acid buffer with a pH of 4.5 to produce F(ab')₂-trastuzumab with a purity of 95 % after purification with PD-10 column. The F(ab')₂-trastuzumab was then reacted with succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) to produce SMCC-F(ab')₂-trastuzumab. The next reaction was to conjugate SMCC-F(ab')₂-trastuzumab with DOTA-PAMAM G3.0-SH, which was prepared by reaction NHS-DOTA with PAMAM G3.0 and followed by reacting it with 2-iminothiolane to give (DOTA)_m-PAMAM G3.0-F(ab')₂-trastuzumab. Finally, the (DOTA)_m-PAMAM G3.0-F(ab')₂-trastuzumab was radiolabelled with ^{177}Lu to produce ($^{177}\text{Lu-DOTA}$)_m-PAMAM G3.0-F(ab')₂-trastuzumab, resulting in a radiochemical purity of 98 % after purification with PD-10 column.

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INTRODUCTION

GLOBOCAN is a project under the International Agency for Research on Cancer, World Health Organization, that estimates and presents information about the incidence of death, cancer, and its prevalence for countries and regions around the world. In 2012, GLOBOCAN estimated

that there were 14.1 million new cancer cases in the world and 8.2 million people died from them [1].

The increasing occurrence of new cancer cases each year requires effective treatments. Monoclonal antibody (mAb) has long been used for cancer therapy; the target is a tumor antigen. The mechanism of actions of antibodies in killing tumor cell are by inhibiting transduction signal, blocking the receptor, reducing proliferation, inducing apoptosis, using immune-mediated (T-cells, antibody dependent cell mediated

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cytotoxicity) and also by delivering conjugated antibody which is loaded with a toxin, radioisotope, or drug [2].

Trastuzumab was approved by FDA for human epidermal growth factor receptor type 2 (HER2)-positive breast cancer in 1998. Trastuzumab binds selectively to the extracellular domain of HER2 receptor. HER2 is one of the members of epidermal growth factor receptor (EGFR) family, which consists of HER1, HER2, HER3, and HER4. HER2 has been developed into a cancer therapeutic target because its level is closely related with tumorigenesis. HER2 is overexpressed approximately 30 % in breast cancer. Also, overexpressions of HER2 are found in other cancers such as the pancreas, ovarian, and colorectal cancers. The result of *in-vitro* and *in-vivo* studies showed that trastuzumab inhibit proliferation of HER2-positive tumor cell [3-5].

Radioimmunotherapy (RIT) can be used as an alternative cancer treatment in which a radioisotope is combined with a monoclonal antibody to create a tumor-specific targeting agent [6]. The trastuzumab used in this research was fragmented into a bivalent antibody fragment (F(ab')₂) using pepsin enzyme to reduce molecular weight. An F(ab')₂ antibody can be obtained by cleaving the carbonyl side of the residue under disulfide bond. Although F(ab')₂ fragments have a smaller size than the corresponding intact antibody, the fragments reportedly do not lose the ability to bind to the antigens and give a better tissue penetration [7,8]. Following the fragmentation, F(ab')₂ fragments were combined with bifunctional chelating agents (BFC) and dendrimer. Bifunctional chelating agents contain a reactive group for coupling to proteins (or other molecules) and a chelate for complexing to certain radioactive metals. Mono-N-hydroxysuccinimide ester 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (NHS-DOTA) is an example of bifunctional chelating agent [9] utilized in this research.

Dendrimers are monodisperse, homogenous, nano-sized macromolecules [10] that have a large surface functional group [11] like a tree branching lot. The structure of dendrimer that includes molecular weight, size, and the amount of surface functional groups can be modified. Therefore, dendrimers exist in the form of low to high generation namely dendrimer G1 (generation 1), G2, G3, and so on [11]. Poly(amidoamine) G3.0 (PAMAM G3.0) is a dendrimer that has been used as a drug delivery agent. It has 32 primary amine groups on its molecule surface. These groups can be used to bind both complex compounds such as ¹⁷⁷Lu-DOTA, and molecular carriers such as trastuzumab [12].

This study aims at developing a new radioimmunotherapeutic agent of (¹⁷⁷Lu-DOTA)_m-PAMAM G3-F(ab')₂-trastuzumab. (DOTA)_m-PAMAM G3-F(ab')₂-trastuzumab is synthesized through four reaction steps before being labeled with ¹⁷⁷Lu. The synthesis steps are: first, conjugation of ligand to the dendrimer; second, activation of NHS-DOTA-PAMAM G3.0; third, activation of the F(ab')₂-DOTA-trastuzumab with *succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate* (SMCC); and finally, conjugation of PAMAM G3.0 with F(ab')₂-trastuzumab-SMCC. The aim of this preliminary study is to synthesize and characterize (¹⁷⁷Lu-DOTA)_m-PAMAM G3-F(ab')₂-trastuzumab as a radiopharmaceutical for cancer therapy.

EXPERIMENTAL METHODS

One of the materials used in this study was trastuzumab/Herceptin, that was obtained from Roche. Another material, radionuclide ¹⁷⁷Lu, was prepared by irradiating ¹⁷⁶Lu in the G.A. Siwabessy Multi-Purpose Reactor and then processed at the Center for Radioisotope and Radiopharmaceutical Technology, Indonesian National Nuclear Energy Agency (PTRR-BATAN). The other materials were sulfo-NHS-DOTA (Macrocytic), PAMAM dendrimers G 3.0 (Sigma-Aldrich), pepsin from porcine gastric mucosa (Sigma-Aldrich), sulfo-SMCC (Thermo Fisher Scientific), and Traut's reagent of 2-iminothiolane (Thermo Fisher Scientific). Furthermore, bovine serum albumin, trizma base, monobasic sodium phosphate (NaH₂PO₄.H₂O), dibasic sodium phosphate (Na₂HPO₄.2H₂O), DMF, trizma base and tris-glycine were all purchased from Sigma, while EDTA, HCl, and NaOH were purchased from E-Merck. Further materials were protein standard and protein dye (Bio-Rad), dialysis cassettes 20000 Da MWCO (Thermo Scientific), Sephadex G25 M resin and PD-10 (Sephadex G25 Columns, GE Healthcare), and instant thin layer chromatography-silica gel (iTLC-SG) strips (Agilent). Equipment used for analysis were Gamma Counter (Nucleus), High Performance Liquid Chromatography (HPLC, Shimadzu) equipped with Size Exclusion Column (SEC column, Agilent), thermomixer and micropipette (Eppendorf), shaker (Heidolph Unimax 1010), micro spectrophotometer (BioTek), roller mixer (Multimix MMI Luckham), and dose calibrator (Capintec).

Preparation of F(ab')₂-trastuzumab

After dialysis process using dialysis cassette finished, an aliquot (100 µL) of digestion buffer was added to 0.75 mg pepsin. It was mixed until pepsin properly dissolved, followed by addition of 3 mL of dialyzed trastuzumab (5 mg/mL) [13]. The mixture was then incubated at 37 °C. An aliquot of TrisHCl 10 mM at pH 8.0 was added into each sample to stop fragmentation process. Purification of F(ab')₂-trastuzumab was carried out by using a PD-10 desalting column. The resulting eluate was collected in 0.25 mL fractions which were then analyzed by using HPLC. The fraction of F(ab')₂-trastuzumab with a purity of > 95 % was then pooled for use in further study [7,9].

Synthesis of DOTA-PAMAM G 3.0-F(ab')₂-trastuzumab

Conjugation of ligand to dendrimer

PAMAM G3.0 in 0.1 M phosphate buffer at pH 7.4 was added into sulfo-NHS-DOTA solution in 0.1 M phosphate buffer (mole ratio 1:96). The pH of the reaction was adjusted to 7.4. The mixture was then incubated at 4 °C for 24 hours while being rotated using a roller mixer. The ligand that did not react with the PAMAM G3.0 was separated using PD-10 column. All purification processes in this work used PD-10 column which was pre-blocked with BSA, equilibrated with the desired eluent, and then eluted with 0.01 M PBS at pH 7.4 (contains 5 mM EDTA). The eluates from a purification step of (DOTA)_m-PAMAM G3.0 conjugate were separated into 30 fractions of 0.25 mL each. Each fraction was taken (10 µL) and tested using protein dye (colorimetric assay). Fractions which gave a blue color indicating the presence of (DOTA)_m-PAMAM G3.0 were then pooled and used for further study.

Activation of (DOTA)_m-PAMAM G3.0 with Traut's reagent

Traut's reagent (2-iminothiolane) was dissolved in 0.05 M phosphate buffer of pH 7.4 containing 5 mM EDTA (1 mg/ mL). An aliquot of 2-iminothiolane was added into (DOTA)_m-PAMAM G3.0 conjugate with a mole ratio of 2:1 followed by incubation at room temperature for 1 h under nitrogen gas atmosphere to form (DOTA)_m-PAMAM G3.0-SH that was then purified using PD-10 column in similar to that of Sec. 2.1. The eluates were collected and separated into 40

fractions of 0.25 ml each. Each fraction was taken (10 µL) and tested using the dye-protein. Fractions, which gave a blue colour indicating the presence of (DOTA)_m-PAMAM G3.0-SH, were then retrieved and used for further study.

Activation of F(ab')₂-trastuzumab with SMCC

Sulfo-SMCC was dissolved in a small amount of DMSO/DMF, and then the concentration was adjusted to 1 mg/mL by addition of 0.1 M PBS at pH 7.4 (containing 5 mM EDTA). An aliquot of the sulfo-SMCC was added into F(ab')₂-trastuzumab (mole ratio 10:1) followed by incubation at room temperature for 30 minutes. Purification of F(ab')₂-trastuzumab-SMCC from byproducts was conducted using the PD-10 column (containing 5 mM EDTA) in a similar manner with Section 2.1. The eluates were collected and separated into 40 fractions of 0.25 ml each. Each fraction was taken (10 µL) and tested using the dye-protein. Fractions which gave a blue color indicating the presence of F(ab')₂-trastuzumab with SMCC were then retrieved and used for further study.

Conjugation of (DOTA)_m-PAMAM G3.0-SH with F(ab')₂-trastuzumab-SMCC

The activated (DOTA)_m-PAMAM G3.0-SH was added to the activated trastuzumab (F(ab')₂-trastuzumab-SMCC). The mixture was incubated while being rotated overnight at 4 °C followed by dialyzing, using dialysis cassette with a molecular weight cutoff (MWCO) of 20 kDa, with 0.25 M ammonium acetate at pH 7.4 containing 1.2 g Chelex-100. The dialysis was carried out at 4 °C for 72 hours with three buffer changes.

Labeling of DOTA-PAMAM-F(ab')₂-trastuzumab with ¹⁷⁷Lu

Preparation of ¹⁷⁷LuCl₃

The first step was to prepare a solution of ¹⁷⁷LuCl₃ by irradiating 0.3-0.4 mg of ¹⁷⁶Lu₂O₃ (64.30 % isotopically enriched) in the G.A. Siwabessy Multi-Purpose Reactor (National Nuclear Energy Agency of Indonesia) for four days. The irradiated target was dissolved by addition of 2 mL of 6 M HCl. The mixture was left to stand for 30 min after which 2 mL of H₂O₂ was added. The mixture was then heated to get dried and the residue was redissolved in 3 mL of HCl 0.05 M.

Labeling process

The solution of ^{177}Lu was diluted with 0.25 M ammonium acetate at pH 7.5 (1:3), and subsequently added into the (DOTA)m-PAMAM G3.0-F(ab')₂-trastuzumab. The pH of the solution was adjusted to 5.5 with 0.1 N NaOH, and then it was incubated for 90 min at 37 °C. An aliquot of 0.05 M EDTA (mol ratio EDTA : ^{177}Lu = 20:1) was added to the mixture, followed by incubation for 5 min at 37 °C. The reaction mixture from radiolabeling process was loaded into a PD-10 column. The column was then eluted using 0.01 M PBS at pH 7.4, and the eluent was collected and separated into 50 fractions (0.25 mL/fraction). The radioactivity of each fraction was measured using a dose calibrator. The fractions, which had high radioactivity, were then tested for their radiochemical purity using thin layer chromatography (ITLC-SG strips (1 cm×10 cm) and saline solution were used as a stationary and mobile phases, respectively [7]. The radiochemical purity of the (^{177}Lu -DOTA) m-PAMAM-F(ab')₂-trastuzumab was calculated based on the ratio between the count rate under the (^{177}Lu -DOTA)m-PAMAM-F(ab')₂-trastuzumab peak and the total count rate.

RESULTS AND DISCUSSION

The chromatogram of standard proteins that have passed the SEC column is shown in Fig. 1 (a). This chromatogram shows the five peaks of the five standard proteins with different molecular weights as indicated by their differing retention times (RT). Figure 1 (b) demonstrates the chromatogram of trastuzumab. Trastuzumab has a molecular weight of ~148 kDa [14]. Thus, when compared with the chromatogram of the standard protein, the RT of intact trastuzumab should be similar to the RT of γ -globulin (molecular weight of 158 kDa). The RT of γ -globulin is 11.243 minutes while the RT of the intact trastuzumab is 11.250 minutes for the percent area of 100 % (Fig. 1 (a) and 1 (b)). It is assumed that the data measured using the HPLC in this experiment agree with the reference data.

The chromatogram of fragmented trastuzumab in form of F(ab')₂-trastuzumab followed by purification using a PD-10 column is shown in Fig. 1 (c). When compared with the standard protein, peak of F(ab')₂-trastuzumab should be between the second peak (γ -globulin, MW 158 kDa) and the third peak (ovalbumin, MW 44 kDa). The RT of F(ab')₂-trastuzumab was 11.890 minutes with the percentage area of 95 %. The measured results demonstrate that the experimental data is in good agreement with the

reference. There is a small peak with a percentage area of 5 % in the retention time of 14.433 minutes, which is assumed to be the Fc portion that has not been removed completely through the column.

After obtaining high-purity trastuzumab fragments, the next step was to synthesize (DOTA)m-PAMAM G3.0-F(ab')₂-trastuzumab. This synthesis reaction was carried out in the four stages as described in the previous section. The reason of the use of NHS-DOTA instead of p-SCN-Bn-DOTA for preparing the conjugates of NHS-DOTA-PAMAM G3.0 was its low solubility of (DOTA-p-SCN-Bn)_n PAMAM G3.0 conjugate after the freeze dry process as evidenced in the previous studies.

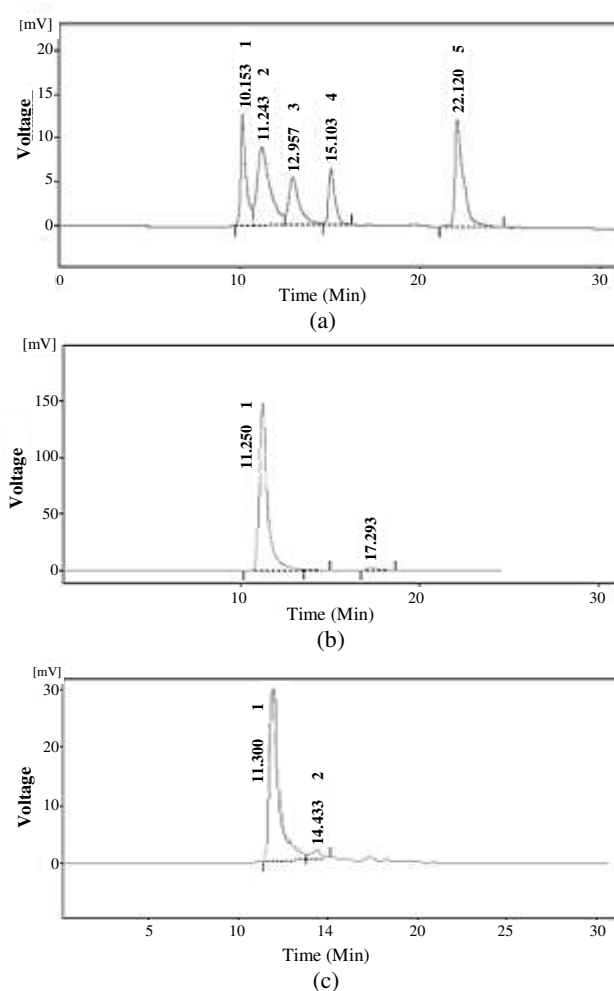


Fig. 1. Chromatograms: protein standard (a), intact trastuzumab with RT: 11.250 (b), and F(ab')₂-trastuzumab with RT: 11.907 (c). Standard protein consists of thyroglobulin (670 kDa, RT: 10.153 min), γ -globulin (158 kDa, RT: 11.243), ovalbumin (44 kDa, RT: 12.957), myoglobin (17 kDa, RT: 15.103), and Vitamin B₁₂ peak (1.35 kDa, RT: 22.120). Column: SEC, Eluent: 0.01 M PBS pH 7.4, Flowrate: 0.5 ml/min.

Therefore, it was impossible to reconstitute the conjugate completely before its labeling with ^{177}Lu [15]. The next step was the activation of dendrimer conjugate, (DOTA)m-PAMAM G3.0.

The activation of this conjugate with 2-iminothiolane was intended to provide a sulfhydryl group on the surface of PAMAM G 3.0 for further coupling. The resulted (DOTA)m-PAMAM G3.0-SH was purified from unreacted 2-iminothiolane and other by-products using a PD-10 column (eluted with eluent of phosphate buffer 0.05 M at pH 7.4 containing EDTA 5 mm).

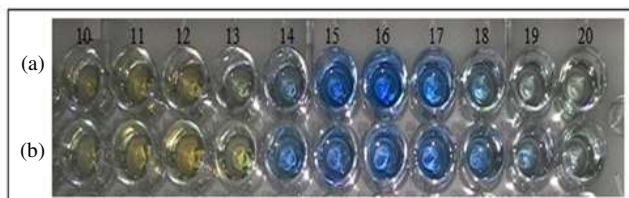


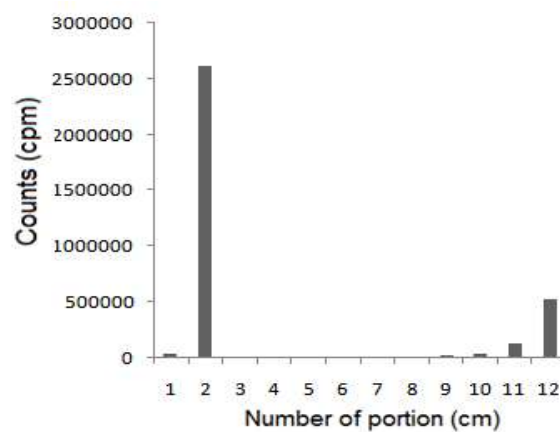
Fig. 2. Fractions of : (a). Activated DOTA-PAMAM G-3.0-sulfhydryl; and: (b). Activated F(ab')₂-trastuzumab-SMCC.

The eluates were collected and separated into 20 fractions of 0.25 mL each. Each fraction was taken (10 μ L) and tested using the dye protein. In this assay, the fraction containing DOTA-PAMAM G3.0-SH was blue, while the fraction containing no (DOTA)m-PAMAM G3.0-sulfhydryl was brown [16]. The positive fractions containing (DOTA)m-PAMAM G3.0-SH were fractions numbered 14-18 (Fig. 2(a)). The color changes in this colorimetric assay are due to the electrostatic interactions between the remaining primary amine groups of dendrimer and protonated one with one sulfo groups of Coomassie blue [12].

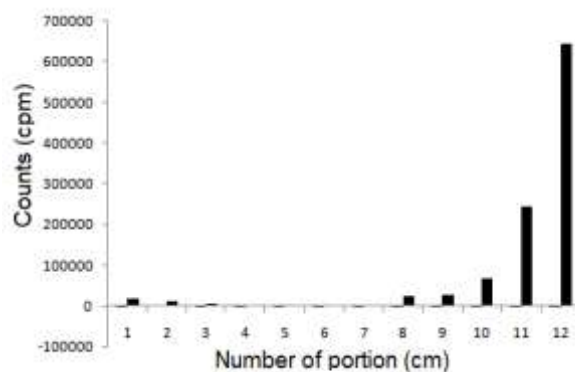
The subsequent step was the activation of F(ab')₂-trastuzumab by reacting it with maleimide to provide F(ab')₂-trastuzumab-SMCC that was then coupled specifically with the sulfhydryls of (DOTA)m-PAMAM G3.0-SH. The purification and testing for F(ab')₂-trastuzumab-SMCC were carried out in similar manners to that of (DOTA)m-PAMAM G3.0-sulfhydryl. The positive fractions containing trastuzumab-SMCC were fractions numbered 14-19. The result of a colorimetric assay of F(ab')₂-trastuzumab-SMCC is shown in Fig. 2(b). The last step was conjugation of DOTA-PAMAM G3.0-SH with F(ab')₂-trastuzumab-SMCC to form a DOTA-PAMAM G3.0-F(ab')₂-trastuzumab conjugate. The reaction mixture was incubated overnight, which was then dialyzed using Slide-A-Lyzer dialysis cassettes (20 kDa MWCO) with 0.25 M ammonium acetate at pH 7.2 containing 1.2 grams of Chelex-100.

The formation of the (¹⁷⁷Lu-DOTA)m-PAMAM G3.0-F(ab')₂-trastuzumab complex was performed by reacting a DOTA-PAMAM G3.0-F(ab')₂-trastuzumab conjugate with ¹⁷⁷Lu³⁺ at pH 5-5.5 with incubation time of 90 minutes. At the end of the incubation time, EDTA was added to the

reacting mixture to bind to the free ¹⁷⁷Lu³⁺, which was not bound to the DOTA-PAMAM G3.0-F(ab')₂-trastuzumab [16]. The labeling percentage of the (¹⁷⁷Lu-DOTA)m-PAMAM G3.0-F(ab')₂-trastuzumab before and after passing through the column was determined using thin layer chromatography (of ITLC-SG and saline solution as stationary and mobile phases, respectively). The determination of the labeling percentage before loading into the column is useful to determine the efficiency of the purification process.



(a)



(b)

Fig. 3. Radiochromatograms of (¹⁷⁷Lu-DOTA)m-PAMAM G3.0-F(ab')₂-trastuzumab: (a) before purification, and: (b) free ¹⁷⁷Lu (in the form of ¹⁷⁷Lu-EDTA).

Figure 3(a) is a radiochromatogram of (¹⁷⁷Lu-DOTA)m-PAMAM G3.0-F(ab')₂-trastuzumab before passing through the PD-10 column. The presence of free ¹⁷⁷Lu (in the form of ¹⁷⁷Lu-EDTA) was confirmed with a radiochromatogram of the ¹⁷⁷Lu-EDTA that was prepared separately and also spotted on ITLC-SG strips developed with saline solution (see Fig. 3(b)). It can be seen that there was free ¹⁷⁷Lu (R_f=1) in the reaction mixture that contain a main product of the (¹⁷⁷Lu-DOTA)m-PAMAM G3.0-F(ab')₂-trastuzumab (R_f=0) that has a radiochemical purity of 75.83 \pm 1.33 %.

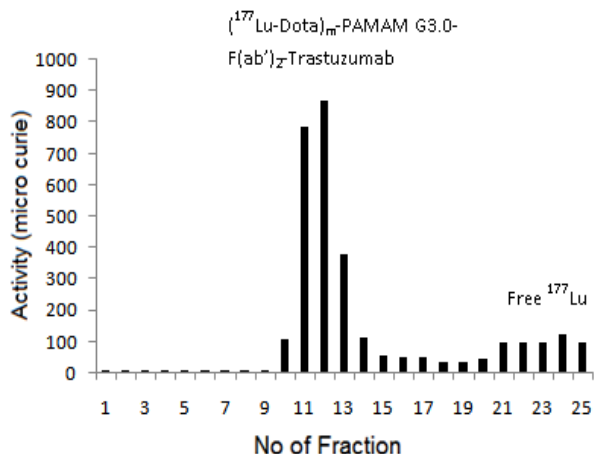


Fig. 4. One of the elution profiles of $(^{177}\text{Lu-DOTA})_m\text{-PAMAM G3.0-F(ab')}_2\text{-trastuzumab}$ after passing through the PD-10 column (Eluent: 0.01 M PBS pH 7.4).

The purification of $(^{177}\text{Lu-DOTA})_m\text{-PAMAM G3.0-F(ab')}_2\text{-trastuzumab}$ was carried out using the PD-10 column and its elution profile is shown in Fig. 4. It appears that based on the ^{177}Lu radioactivity there are two distinct peaks of $(^{177}\text{Lu-DOTA})_m\text{-PAMAM G3.0-F(ab')}_2\text{-trastuzumab}$ and $^{177}\text{Lu-EDTA}$, respectively. Fractions which have high radioactivities were then tested for their radiochemical purity using the ITLC-SG.

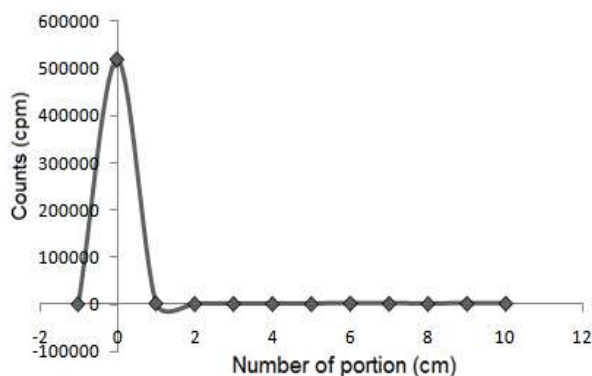


Fig. 5. Radiochromatogram of a fraction with the highest radioactivity. Stationary phase: ITLC SG, mobile phase: saline.

Figure 5 shows an ITLC-SG radiochromatogram of fraction with the highest radioactivity. The radiochemical purity of fraction as $(^{177}\text{Lu-DOTA})_m\text{-PAMAM G3.0-F(ab')}_2\text{-trastuzumab}$ in the ITLC-SG radiochromatogram is $98.36 \pm 0.25 \%$. The impurities in form of free ^{177}Lu (^{177}Lu which is not label to DOTA-PAMAM G3.0-F(ab')₂-trastuzumab) decrease from 24.17 % to 1.64 %.

CONCLUSION

The $(^{177}\text{Lu-DOTA})_m\text{-PAMAM G3-F(ab')}_2\text{-trastuzumab}$, which is expected to be a potential RIT agent for therapy of breast cancer overexpressed HER2, has been prepared. F(ab')₂-trastuzumab as a precursor was prepared by enzymatic digestion of intact trastuzumab using pepsin. The radiolabeling of $(\text{DOTA})_m\text{-PAMAM G3.0-F(ab')}_2\text{-trastuzumab}$ with ^{177}Lu at 37 °C and incubation time of 90 min results in $(^{177}\text{Lu-DOTA})_m\text{-PAMAM G3-F(ab')}_2\text{-trastuzumab}$ with the radiochemical purity of $98.36 \pm 0.25 \%$ after purification with PD-10 column. The stability and other tests of $(^{177}\text{Lu})_n\text{-(DOTA})_m\text{-PAMAM G3.0-F(ab')}_2$ will be reported shortly.

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