**REPORT OF THE COORDINATED RESEARCH PROJECT for 2014**
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# *Establishment and standardization of a technology for ready to use production of cold kit formulation of DOTA-Rituximab and peptide based radiopharmaceuticals for labeling with lu-177 and Y-90*

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**Development and preclinical evaluation of therapeutic radiopharmaceuticals based on Lu-177 and Y-90 labeled monoclonal antibodies**

## Introduction

The purpose of this investigation was to introduce the available technology for ready to use preparation of cold kit freeze-dried formulation of conjugated Rituximab and peptide based radiopharmaceuticals for labeling with Lu-177 and Y-90 and to establish and to standardize the methods identification and quality control of obtained product.
The method of labeling was the same for introducing cold and radioactive Lutetium and Yttrium following the calculated proportion of the amount of both elements. This was important to receive the same results as when the patient is treated with the maximal dose of conjugated antibodies labeled with Lutetium-177 or with Yttrium-90.
Our work was focused on investigation of ready to use freeze dried rituximab immunoconjugates in order to increase the stability and higher efficiency and lower toxicity. Three bifunctional chelating agents (BFCA), p-SCN-Bn-DOTA, p-SCN-Bn-DTPA and 1B4M-DTPA where compared.

## Standardization of the protocol for freeze drying of antibodies

The proposed and established procedure for freeze drying following the proposed protocol was standardized for three used conjugates to obtain the final kit formulation for the antibody, ready for labeling.
The goal of the procedure that we used and standardized was to provide stable formulation identically available for preclinical investigation as the freshly prepared solution, to have the same immunoreactivity of the conjugated antibody before conjugation and after conjugation in the liquid formulation.

**Freeze Drying Procedure**

The freeze dried process was performed using Labconco Free Zone Stoppering Tray Dryer, (USA) using protocol described by Park et al., in 2013, modified to our experience. Briefly, the liquid immunoconjugates were filled in 10 mL type I glass tubing vials using a fill volume of 1 mL and were partially stoppered with corresponding lyophilization stoppers and were equally distributed on the shelves of the freeze-dryer. In order to monitor the temperature variation, thermocouples were placed in the center of each shelf using a blank vial. The samples were loaded at shelf temperature 5°C. The temperature was then decreased to -40°C at 0.40°C/min and held for 3 h. An annealing step was included, at -15 °C, to allow complete crystallization and to obtain the desired cake structure, thus completing the freezing step in 10 h. The primary drying was performed at temperature of -10°C for 25 h and the secondary drying at shelf temperature 25°C for 11 h. Upon finishing the process, the vials were stoppered and kept at 4°C until analysis.

Shelf temperature of $40^0$C for 10 hours (increase 15-20 hours) - (ramp rate $1^0$C/min)

**Conjugation of Rituximab**

BFCA's were dissolved in 0.1 M PBS (pH 8.0) to final concentration of 10 mg/mL. Calculated amounts of BFCA required to give a 20-fold molar excess over the amount of Rituximab (10 mg/mL) were added to the purified monoclonal antibody in 0.1 M PBS (pH 8.0). The mixture was incubated overnight, at 4°C with gentle shaking. Purification of the conjugates was made with ultrafiltration (Ultracel® - 30K, Millipore, Ireland), by washing the immunoconjugates with 0.05 M ammonium acetate, pH 7.0, until the absorbance in the ultrafiltrate set at 280 nm was nearly zero (meaning that there is no unbound chelating agent in the immunoconjugate solution).

**Concentration determination of antibody immunoconjugate**

Concentration of the antibody/immunoconjugate was determinate using UV spectrophotometer (Jenway UV/VIS spectrophotometer 6715), and semi-micro UV polypropylene tubes with 0,1M PBS pH=8.0, at 280nm in trilicate.

The concentration of freeze died preparation was measured using the same method and after complete reconstitution in 1 mL 0,9% NaCl.

The concentration of the antibody was determined using the standard coefficient of extinction for antibodies, 1.4 mL/mg/cm.

**Absorbance determination at 410nm**

Freeze dried simples after the reconstitution with 1 mL 0,9% NaCl were measured at 410nm in triplicate.

**Separation with Gel chromatography using Sephadex G25 column**

The Sephadex G25 column was pretreated with 30 mL 1% Bovin serum albumin (BSA) dissolved in PBS, pH 7,4 and used for separation of the immunoconjucate in the fractions of 500µL.

The fractions were analyzed with UV spectrometry (for not radioactive imunoconjucate) and using Dose calibrator (Talete SA, Comecer) for radioactive immunoconjucate.

**Protein Integrity Test Using SDS-PAGE**
SDS-PAGE was performed according to Laemmli protocol. About 5 µL of sample was mixed with 10 µL of sample buffer. The samples were boiled for 10 min at 100° C. Approximately 5 µL of each preparation was applied per lane in 12% bisacrylamide under reducing conditions. Coomassie staining (Coomassie Brilliant Blue R-250, Sigma) was performed according to the manufacturer's instructions. All chemicals used were reagent and HPLC grade. As molecular marker Low molecular weight marker (Amersham GE Healthcare) was used.

**Protein Characterisation by MALDI-TOF MS**
Both characterization of the conjugates and determination of the average number of BFCA attached to each antibody molecule is performed by MALDI-TOF mass spectrometry. A representative procedure can be outlined as follows: A volume (10 µL) of the solution of the conjugated BFCA's was diluted (1:10) with a matrix solution of 3,5-dimethoxy-4-hydroxycinnamic acid [10 mg/mL dissolved in a mixture of acetonitrile (50%)/TFA (1%), Sigma] to a concentration of about 10 pmol/µL. An aliquot (1-2 µL) of the final solution was applied to the sample target prior to insertion into the high vacuum chamber of a mass spectrometer. Operational conditions for the MALDI-TOF apparatus were set as follows: mode of operation, linear; polarity, positive; acceleration voltage, 20000 V; delayed extraction time, 100 nsec; cquisition mass range, 140000-170000 Da.

**Obtained results during the period from the last report:**

**Freeze Drying Procedure**
Comparison of two applied protocols for freeze dying was performed with the variation of the temperature during the process of freeze drying.

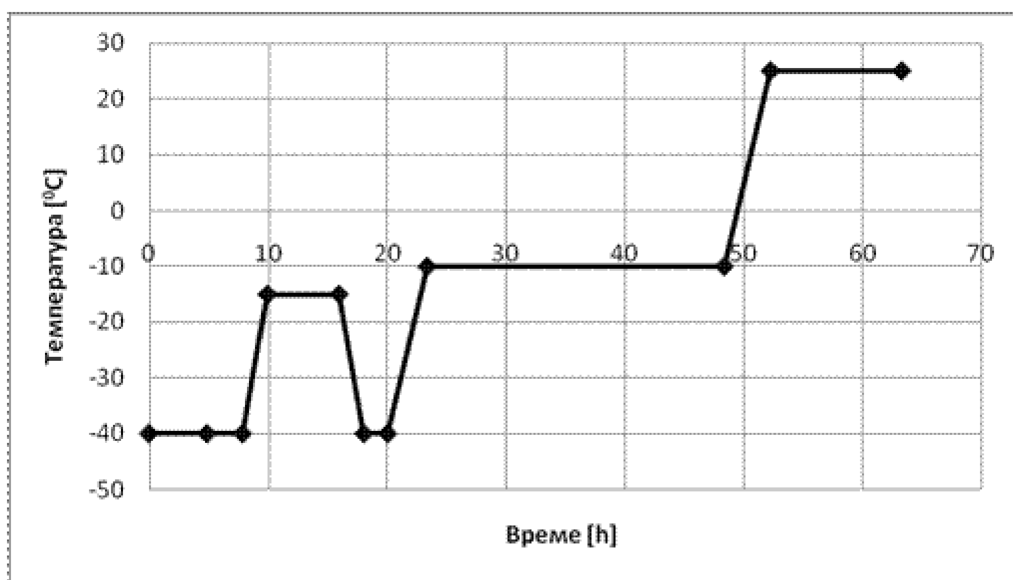


Figure 1. Protocol 1 – direct freezing at -40°C

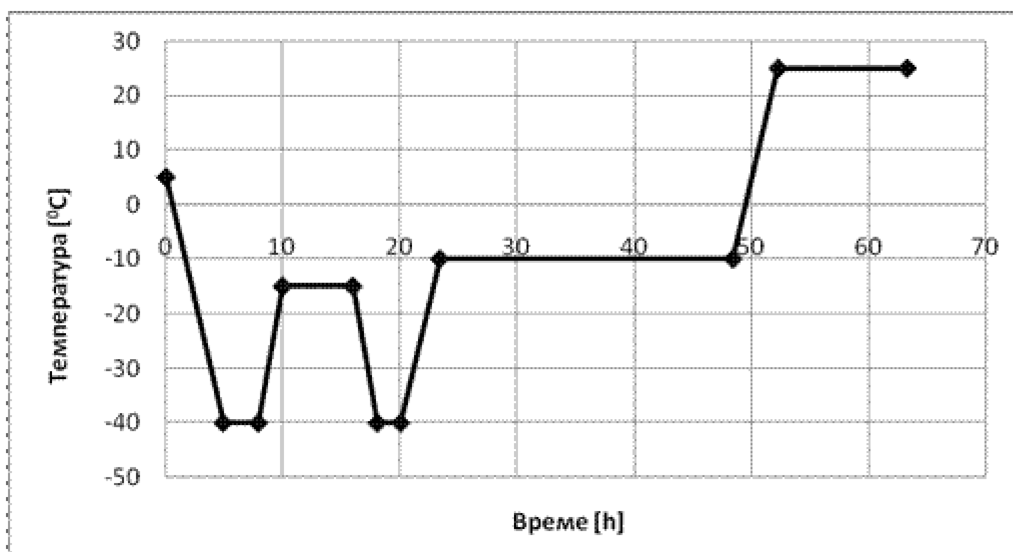

Figure 2. Protocol 1 – slowly freezing with increasing the temperature from 5 to -40˚C.

**Velocity of the reconstruction for freeze dried product**

In all freeze dried products containing mannitol as cryo protectant using both freeze drying protocols, the time of the total reconstruction was measured after adding 0.9% NaCl.

In the table below we can see that there is no significant difference in the time required for total dissolution of the freeze dried product. All simples were completely dissolved within 2 minutes.

| Simple | Time of reconstruction (s) | Abs. 410 nm |
|---|---|---|
| | | |
| Rituximab – DTPA 1B4M without mannitol Protocol 1 | 25 - 40 | 0.219 |
| Rituximab – DTPA 1B4M with mannitol Protocol 1 | 30 - 45 | 0.396 |
| Rituximab – DTPA 1B4M without mannitol Protocol 2 | 30 - 45 | 0.225 |
| Rituximab – DTPA 1B4M with mannitol Protocol 2 | 50 - 70 | 0.474 |

**Determination of the aggregate**

Instability of the protein containing pharmaceutical formulation is a common problem with the formation of the aggregate. Aggregates can be present in a form of the liquid or solid form and they can be easily dissolved or stay in a not dissolved form (Rathore and Rajan, 2008). To obtain one objective consideration of the presence of aggregate and colloid particles, enough big to obtain visible opalescent liquid, we measured the absorbance of the dissolved preparation at 410 nm. According to the results showed in the table, we can conclude that the

simples containing cryo preservatives (mannitol) show the higher absorbance using both protocols. These results corresponding also with the visible form of the freeze dried simples after reconstruction.

**Protein Integrity Test Using SDS-PAGE**

In order to demonstrate the integrity of the protein and purity after conjugation and lyophilization, gel electrophoresis was performed on SDS-PAGE gels using 12% bisacrylamide gel. The loaded samples were Rituximab (1 mg/mL, commercial sample), conjugated *p*-SCN-Bn-DOTA, *p*-SCN-Bn-DTPA in a form of liquid and reconstituted lyophilizated kit, both in concentration of 1 mg/mL. Figure below shows the SDS-PAGE patterns for BFCA conjugates, compared to unconjugated rituximab as control sample.

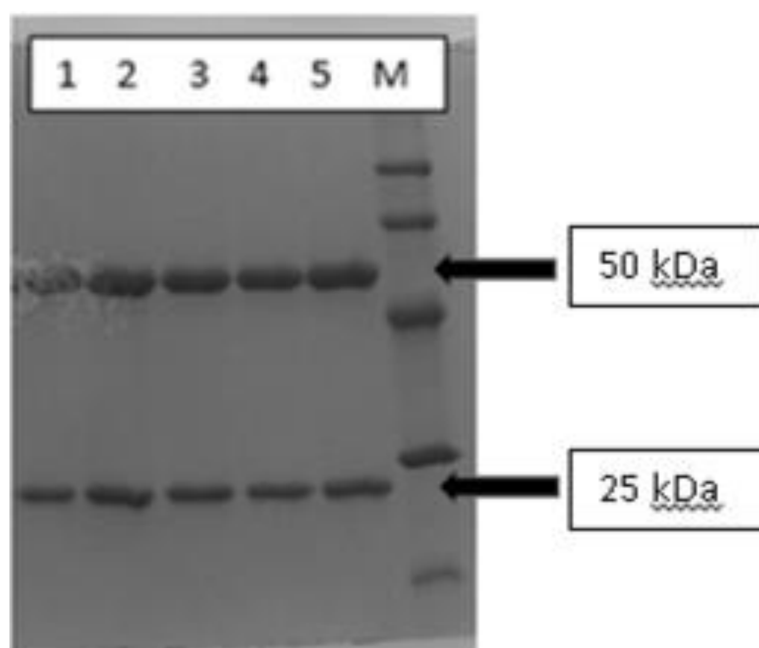


Figure 3. Reducing SDS-PAGE lane patterns for rituximab (1) (1 mg/mL), DOTA-rituximab conjugate, before lyophilization (2), DTPA-rituximab conjugate, before lyophilization (3), DOTA-rituximab conjugate, after lyophilization (4) and DTPA-rituximab conjugate, after lyophilization (5); M is molecular marker;

All BFCA-rituximab conjugates (before and after lyophilization) were resolved in two distinct Mr species which migrated in two bands (upper band at ~50 kDa and lower band at ~25 kDa) confirming the migration behavior typical for IgG antibodies which are composed of two identical subunits each composed by two polypeptide chains: two heavy and two light chains, linked via disulfide bonds. The obtained fragments correspond to molecular masses of rituximab heavy and light chain given at the corresponding literature.

As it is shown in Figure 3, the reducing SDS-PAGE patterns for rituximab, and BFCA-rituximab immunoconjugates were with very similar intensity. The reducing SDS-PAGE results, compared to the result of commercially available rituximab sample, showed no clear indication for antibody degradation. Similar results were obtained for one month integrity test on stored BFCA-rituximab immunoconjugate products.

**Protein Characterisation by MALDI-TOF MS**

One of the most important quality attributes of immunoconjugates is the average number of chelator molecules that are conjugated because this determines the drug distribution and the amount of “payload” that can be delivered to the tumor cell and can directly affect both safety and efficacy.

Characterization of the conjugates and determination of the average number of BFCA attached to each antibody molecule was performed by MALDI-TOF MS. This is a rapid and sensitive technique for the characterization of peptides and proteins. Used in a variety of modes, MALDI-MS provides information such as the molecular weight of an intact protein, peptide mass mapping from a tryptic digest, and peptide sequencing. MALDI-TOF MS as a “soft” ionization technique is suitable for thermolabile, nonvolatile compounds, especially those of high molecular mass and is used successfully in biochemical and biotechnological areas for the analysis of therapeutic proteins, peptides, glycoproteins, complex carbohydrates and oligonucleotides. One of the first reports of mass spectroscopic characterization of immunoconjugates described use of a UV MALDI-TOF instrument. Mass spectra of intact antibodies conjugated through lysine residues or through antibody carbohydrates with chelating agents (DTPA, macrocycle 12N4) or with drugs (calicheamicin, methotrexate, mito-xantrone) were compared with the corresponding unconjugated antibodies.

MALDI-TOF results for DOTA-rituximab conjugate, after lyophilization (shown on Figure 4), revealed the presence of two major peaks corresponding to a MW of 146491 Da (unconjugated mAb), and 149873 Da (conjugated mAb) which corresponds to an average of 6.1 groups of *p*-SCN-Bn-DOTA per molecule of rituximab. No structural changes in terms of appearance of additional peaks were observed.

MALDI-TOF results for DTPA-rituximab conjugate, after lyophilization (shown on Figure 5), revealed the presence of two major peaks also, corresponding to a MW of 146477 Da (unconjugated mAb), and 151246 Da (conjugated mAb) which corresponds to an average of 8.8 groups of *p*-SCN-Bn-DTPA per molecule of rituximab.

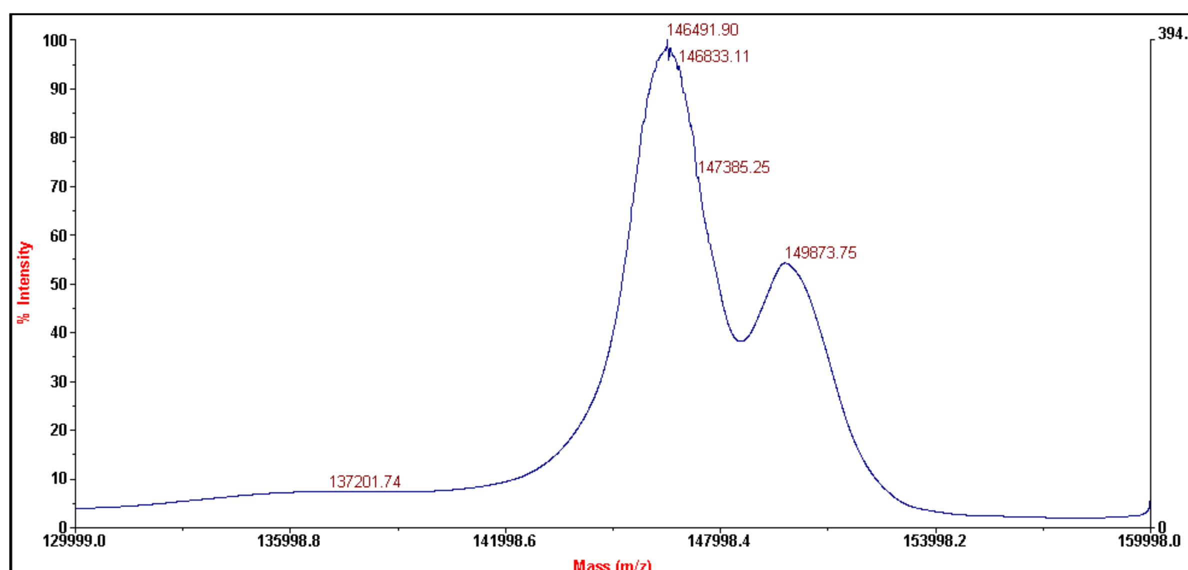


Figure 4. MALDI-TOF results for DOTA-rituximab conjugate.

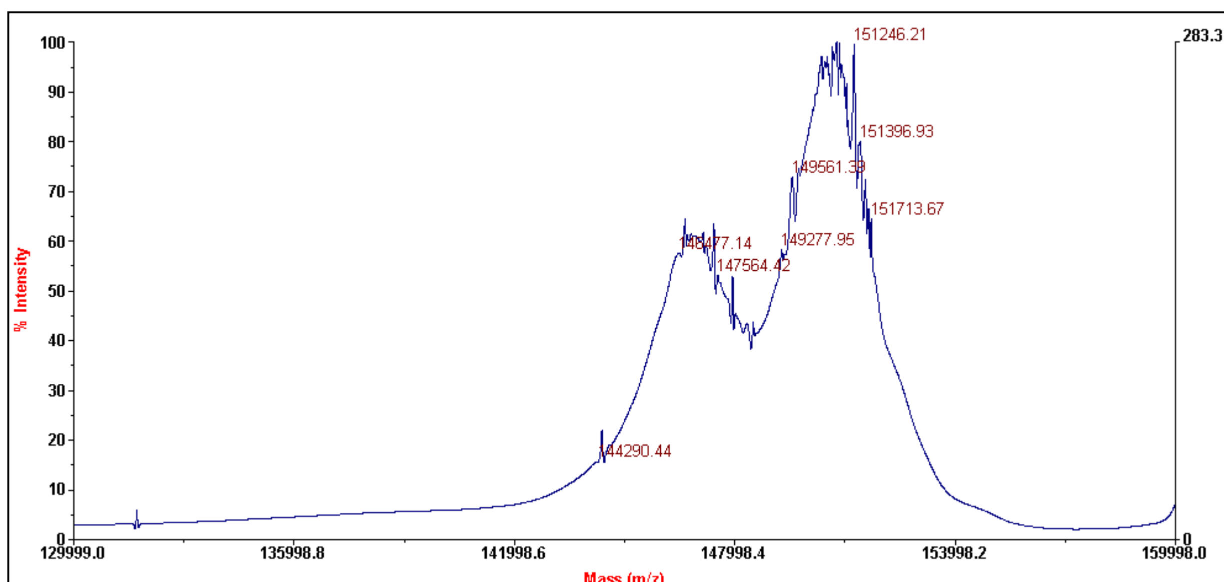


Figure 5. MALDI-TOF results for DTPA-rituximab conjugate.

According to literature data, 4.25 ± 1.04 DOTA-SCN molecules attached to each antibody molecule are found to be sufficient for prompt subsequent labelling with radioisotope. This is result for investigated molar ratio 1:50 (antibody:chelator). Our results of average of 6.1 groups of *p*-SCN-Bn-DOTA and 8.8 groups of *p*-SCN-Bn-DTPA per molecule of rituximab, pointed that this number can be increased using different molar ratios for conjugation, as 1:20 in this case. In another study, up to five DOTA molecules were conjugated to MORAb-003, with no apparent loss of immunoreactivity. Highly DOTA-substituted anti-tumor antibody leads to the formation of immunoconjugates with high specific activity and excellent *in vivo* behavior which is a valuable option for radioimmunotherapy and potentially antibody-drug conjugates.

**Radiolabeling of freeze dried immunoconjucate with Lutetium-177**

Radiolabeling of freeze dried immunoconjucate was performed after reconstitution in 0.9% NaCl, in presence of acetate ions at pH 7.0 with Lutetium-177, at room temperature for Rituximab-DTPA-1B4M and Rituximab-DTPA-SCN and in $40^0$C for Rituximab-DOTA-SCN. The obtained radiolabeled immunoconjucates were with the high radiochemical yield. The radiochemical purity was determined using ITLC and HPLC.

**Determination of the radiochemical purity using Instant Thin Layer Chromatography (ITLC)**

Determination of the radiochemical purity using ITLC method and auto radiographic analysis was performed for all three immunoconjucates (picture below). The Rf value of 0.1 – 0.15 corresponding to the radiolabeled antibody, and the rest of the radioactivity can be result of the low molecular fragments or free bifunctional chelating agent. The rest of the radioactivity (Rf = 0.8 – 1.0) is corresponding to the free Lutetium-177.

These results is corresponding with all our previous results and conclusion that the process of freeze drying is not reflecting to the quality of the radiolabeled conjugated antibody, e.g. the process of freeze drying doesn’t provoke any chemical changes in the structure of the immunoconjucate. We can conclude that the new Rituximab complexed immunoconjucate

containing chelating agents (p-SCN-Bn-DOTA, p-SCN-Bn-DTPA and 1B4M-DTPA) are stable after labeling with Lu-177 at pH 8.0.

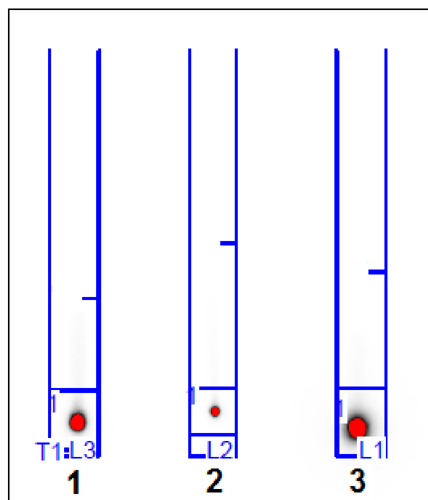


Figure 6. ITLC chromatogram for determination of the radiochemical purity of $^{177}$Lu-DOTA-rituximab (1),$^{177}$Lu-DTPA- rituximab (2) and $^{177}$Lu-1B4M-DTPA – rituximab (3).

**Determination of the radiochemical purity using Size-exclusion High Performance Chromatography (SE-HPLC)**

Representative SE-HPLC profiles of the radiolabeled immunoconjucate are showed in the figure . From the obtained results we can see only presence of one pick that is corresponding to the radiolabeled antibody. These results confirm the results obtained from ITLC.

The radiochemical purity of all immunoconjucates is more than 95%.

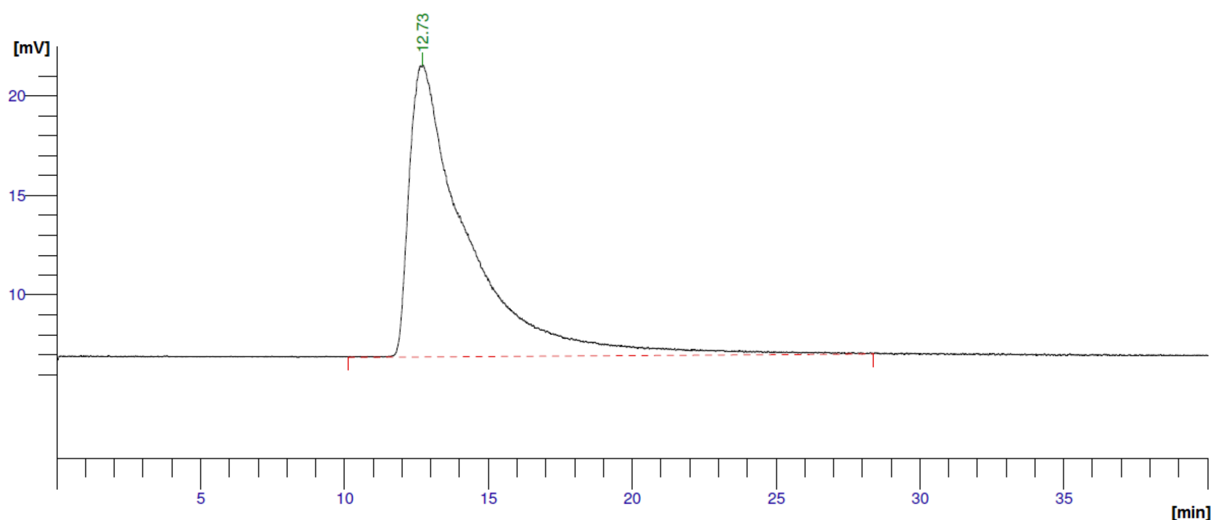


Figure 7 . SE-HPLC of $^{177}$Lu-DOTA-rituximab.

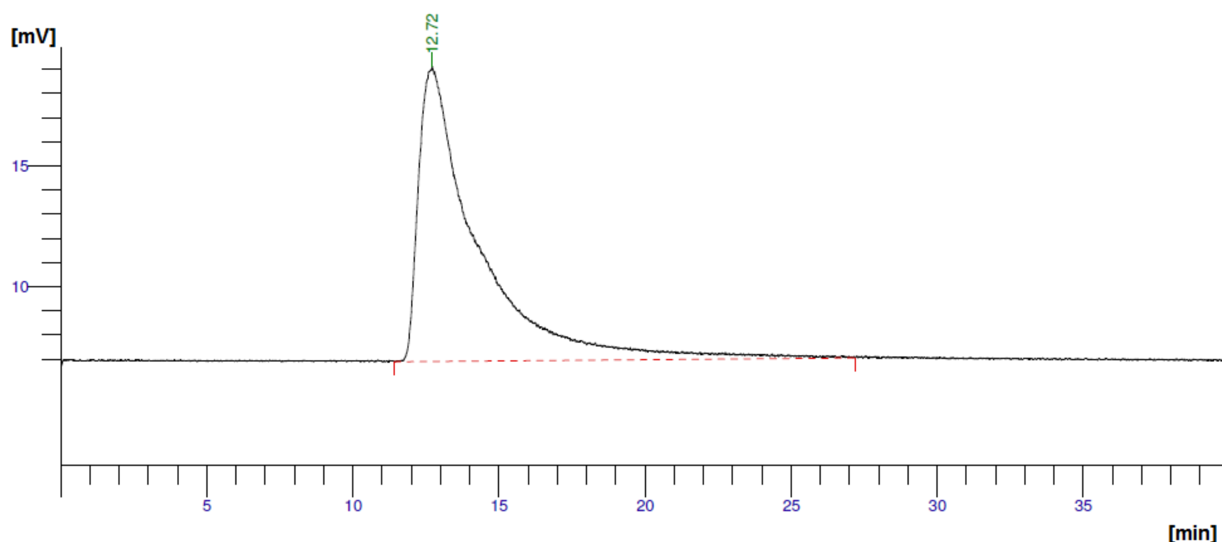


Figure 8. SE-HPLC of $^{177}$Lu-DTPA-rituximab

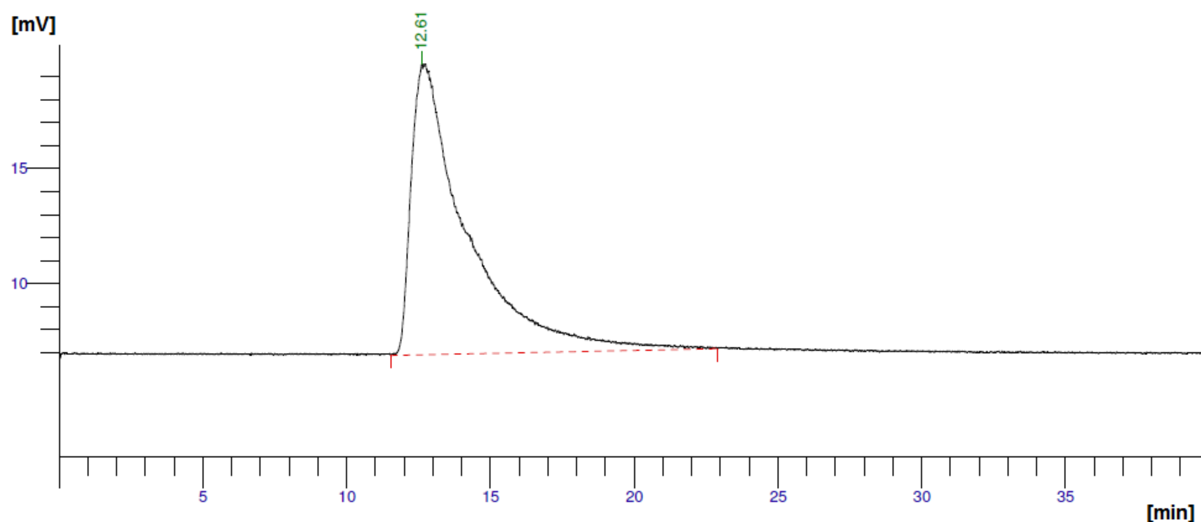


Figure 9. SE-HPLC of $^{177}$Lu-1B4M-DTPA -rituximab

**Conclusion**

All our results show that all three immunoconjucates after labeling provide high radiochemical yield and high radiochemical purity up to the expected values.

The choice of the most appropriate immunoconjucate for labeling we expect to obtain after planned biodistribution studies in animal model and after stability studies that we are planning to perform and check within next period.

The toxicological studies of radiolabeled immunoconjucates will be performed first after immunohistological evaluation of cold prepared products and their comparison with effects of the rituximab already known. The confirmation of the results will be completed also with the chemical identification of the complexes using RAMAN spectroscopy and IR spectroscopy.