

PREPARATION AND INTEGRITY EXAMINATION OF FREEZE DRIED KIT OF TRASTUZUMAB-IMMUNOCONJUGATES AND COLD LABELED IMMUNOCONJUGATES BY APPLYING SDS-PAGE ELECTROPHORESIS

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Radioimmunoconjugates are promising agents in diagnostics and treatment of different types of cancers. The aim of this study is formulation of stable freeze dried kits of trastuzumab with three types of bifunctional chelators for further radiolabeling. The integrity of the antibody in formulated conjugates was examined with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Trastuzumab is a humanized monoclonal antibody used in the therapy of aggressive HER2 positive breast cancer. Conjugation of trastuzumab was made with various chelators: p-SCN-Bn-DTPA (1:10, 1:20, 1:50), p-SCN-Bn-DOTA (1:20), and 1B4M-DTPA (1:10, 1:20, 1:50). The purified immunoconjugates were lyophilized by applying two day protocol in order to produce the stable freeze dried kits. Cold labeling with nonradioactive isotopes LuCl_3 and YCl_3 was performed to examine the possible modifications of secondary structure after radioactive labeling. SDS-PAGE electrophoresis was used to estimate the purity and integrity of the antibody before and after conjugations, lyophilization and labeling under reducing conditions.

The obtained results show that there is no degradation of the examined antibody. The trastuzumab-conjugates and cold labeled formulations migrated in two bands (~50 kDa and ~25 kDa), in the same way as IgG1 antibodies and unmodified trastuzumab.

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Key words: Bifunctional chelators, conjugations, electrophoresis, trastuzumab

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Introduction

The human epidermal growth factor receptor (HER2) is overexpressed on a surface of the cells in aggressive breast cancer. HER2 positive breast cancers without anti-HER2 therapy lead to metastasis and very low survival rates of the patients (1). Trastuzumab is the first approved humanized monoclonal antibody. It's a potent anti-HER2 antibody which originates from murine 4D5 antibody (2). Carter et al., 1992 (3) cloned hypervariable regions from 4D5 in plasmids which are encoding formation of constant regions from

human IgG1 antibody. A vector that is encoding formation of chimeric antibody, which is additionally humanizes, was successfully generated. Trastuzumab is binding to the IV subdomain of the receptor and manifests the effect through antibody-dependent cellular cytotoxicity (4).

Conjugated antibodies provide high expectation for development of cancer-specific cytotoxic reagents. Each immunoconjugate has an enhanced therapeutic specificity due to the conjugation with antibody which shows specificity to a particular antigen on the surface of tumor cells (5). Radioimmunoconjugates are step forward in diagnostic and treatment of different types of cancers (6). Until now, there is no FDA registration of trastuzumab-radioimmunoconjugates for commercial use. A series of clinical and preclinical trials of radioimmunoconjugates for treatment and imaging of breast cancer with different radionuclides ^{111}In (7-9), $^{99\text{m}}\text{Tc}$ (10), ^{67}Ga (11), ^{90}Y , ^{86}Y (12), ^{177}Lu (13, 14) have been made in the past few years. Creation of stable immunoconjugate with bifunctional chelators (BFCAs) is required in order to obtain successful labeling. This chelator allows binding to the antibody on the one side and coordinative binding of radioisotopes on the

opposite. The commonly investigated chelators are: DOTA (1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid), DTPA (diethylene triamine pentaacetic acid), DTPA derivate 1B4M-DTPA (2-(4-isothiocyanatobenzyl)-6-methyl-diethylene-triamine-pentaacetic acid) and EDTA (ethylenediamine tetraacetic acid) (15). Due to the easy chemical and physical degradations, monoclonal antibodies are not stable in water solutions. The commonly processes of preparations are denaturation, deamidation, oxidation, deglycolisation, aggregation, precipitation and adsorption. In order to prolong the life of protein pharmaceuticals, it is necessary to remove the water from the product. The commonly used method is lyophilization which obtains stable freeze dried kits (16).

Examinations with non-radioactive isotopes (Lu and Y) are done with intention to confirm that there are no changes in the secondary structure of the antibody after coordinative binding of the metal, because these studies are impossible with radioactive isotopes (17). Reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is important for the determination of molecular mass (Mr) as well as the possible degradations of trastuzumab, trastuzumab-immunoconjugates and cold labeled kits too (18).

The aim of this study was formulation of stable freeze dried kit of trastuzumab with p-SCN-Bn-DOTA (2-(4-isothiocyanatobenzyl)-1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid), p-SCN-Bn-1B4M-DTPA (2-(4-isothiocyanatobenzyl)-6-methyl-diethylene-triamine-pentaacetic acid) and p-SCN-Bn-DTPA (2-(4-isothiocyanatobenzyl)-diethylenetriamine-pentaacetic acid) and subsequently labeling with cold isotopes (Lu and Y). Protein integrity determination and possible aggregations were investigated by using reducing one-dimensional SDS-PAGE.

Material and methods

Purification of trastuzumab. Trastuzumab was purified from Herceptin® (Hoffmann-La Roche, Basel,

Switzerland) with Amicon® Ultra-4 (Sigma Aldrich, Missouri, USA), 30 KDa for ultrafiltration for 1 hour at 5000 rpm, by washing the antibody in six cycles with 0.1 M PBS, pH = 8, using HuMax 4k (Human, Wiesbaden, Germany) centrifuge.

Conjugation of trastuzumab. The conjugations were made by mixing the antibody with 10 mg/mL solution of BFCAs in different molar ratio (p-SCN-Bn-DTPA – 1:10; 1:20; 1:50, 1B4M-DTPA – 1:10; 1:20; 1:50 and p-SCN-Bn-DOTA – 1:20) and 18 hours incubation on 4 °C with gentle shaking. The immunoconjugates were purified with six cycles of ultrafiltration (Amicon® Ultra-4, 30 KDa), by rinsing with 0.05 M ammonium acetate, pH = 7. The concentration was adjusted to 1 mg/mL using the 6715 UV/Vis Spectrophotometer Jenway® (Staffordshire, UK).

Lyophilization of trastuzumab-immunoconjugates. The volume of 1 mL of the solution was transferred to type I glass vials and lyophilized to solid state. The immunoconjugates were lyophilized with cryoprotectant and bulking agent, 1 % mannitol (50 µL in each vial). The lyophilization was performed with Labconco Free Zone Stoppering Tray Dryer (Kansas City, Missouri, USA), using the protocol by Gjorgieva Ackova et al., 2014 (19), with several modifications. The samples were equally placed on the shelves after reaching the temperature of 4 °C. The first step was freezing the samples. The temperature was decreased at -40 °C, 1 °C/min (rate of cooling, from 4 °C to -40 °C) and the time of keeping was 5 hours. The second phase of preparation was primary drying at the temperature of -10 °C, 0.15 °C/min (rate of heating, from -40 °C to -25 °C) for 28 hours and pressure of 0.133 mBar. The third part was secondary drying on 25 °C, 0.2 °C/min (rate of heating, from -25 °C to 25 °C) for 14 hours. The whole protocol of lyophilization is shown in Figure 1. After completing freeze drying, the vials were closed and kept at 4°C in order to perform the following examinations.

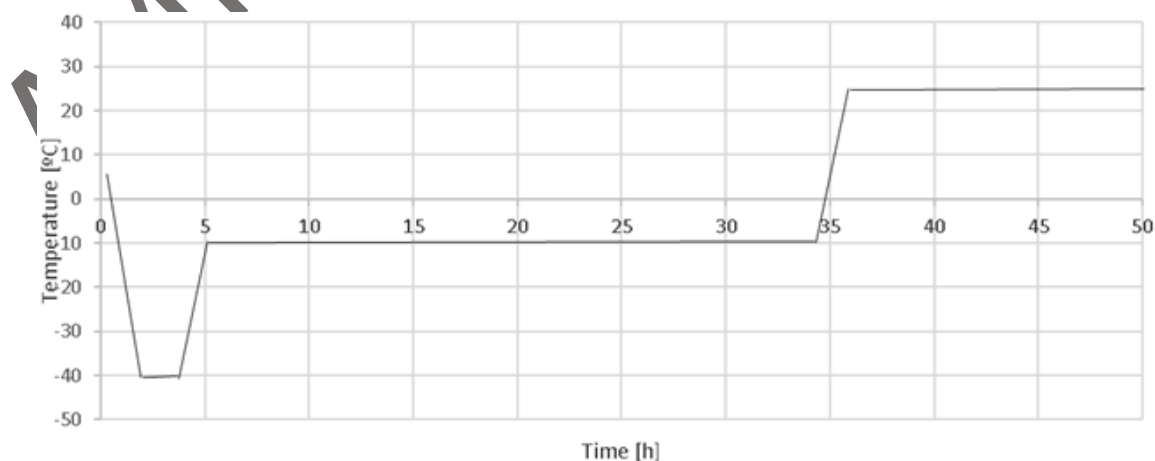


Figure 1. Lyophilization protocol

Labeling trastuzumab-immunoconjugates with non-radioactive Lu and Y. The solutions of non-radioactive Lu and Y were prepared by dissolving of LuCl₃ to concentration of 1.0709 µg/µL (equivalent to maximum tolerated dose (MTD) of ¹⁷⁷Lu (4377,1 MBq)) and YCl₃ to concentration of 1.1555 mg/µL (equivalent to MTD of ⁹⁰Y (1049,69 MBq)). The immunoconjugates were dissolved with sterile 0.9 % solution of NaCl and then labeled with 1 µL of prepared solution of cold Lu and Y to total volume of 1 mL. The labeled DTPA and 1B4M-DTPA conjugates were incubated for 30 min at 25 °C as well as DOTA conjugates were incubated for 1 hour at 40 °C.

Integrity examinations with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Gel electrophoresis was performed in reduced conditions with Enduro® Modular Vertical Gel Electrophoresis System (Labnet, Edison, NJ, USA), using mercaptoethanol. In this process, 12 % separating acrylamide/bisacrylamide gel and 4 % stacking gel was prepared according to the protocol of the manufacturer manual instructions. Electrophoresis was performed three times, in the same conditions. Concentration of each sample was 1 mg/mL. At the first gel, unmodified trastuzumab and freeze dried immunoconjugates were applied after reconstitution with 0.9 % NaCl. At the second and the third gel, immunoconjugates were applied labeled with non-radioactive Lu and Y. A mass of 30

µg of sample was added in 10 µL of loading buffer. Then the mixtures were heated 5 min at dry bath at 98.5 °C and centrifuged for 5 min at 5000 rpm. 25 µL of each sample and 10 µL of SigmaMarker™, Wide Range, Molecular Weight (Saint Louis, MO, USA) were applied in wells of stacking gel. According to our previous studies, constant voltage at 150 V and approximately current at 23 mA has been used as optimum condition. After finishing the entire process (which lasted approximately an hour and a half), the gel was placed for Coomassie staining for 20 hours. Discoloration was observed in the solution composed of water, methanol and acetic acid for 7-8 hours. All used reagents were from Sigma-Aldrich (Missouri, USA). The prepared gels were scanned with Glite 900 BW Gel Scanner (PacificImage, Torrance, CA).

Results

Stable freeze dried kits of trastuzumab-immunoconjugates were formulated. The structures of used BFCAs are shown in Figure 2. There are various groups (anhydride, bromoacetamid, isothiocyanate) that can be attached to the BFCAs molecules, to achieve an easier conjugation (17). The chelators have 4-izothiocyanatobenzyl groups in their structures which participate in the process of binding to the antibody (Figure 3).

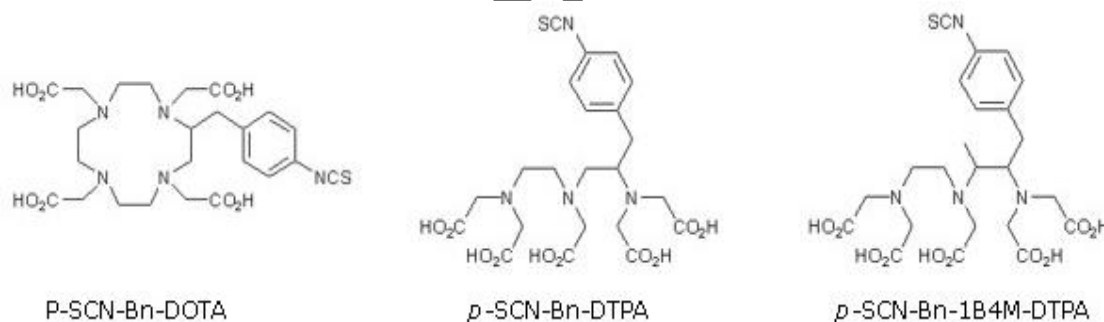


Figure 2. Structure of BFCAs

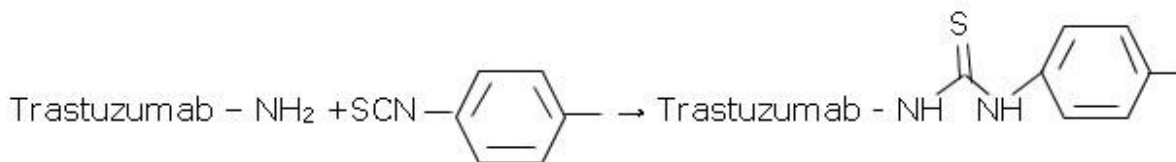


Figure 3. Reaction of conjugation

Thiourea linkage is created due to reaction between amino groups of lysine residues of trastuzumab and isothiocyanate groups of chelators (20). To remove the water and increase the stability,

the immunoconjugates were lyophilized using the previously described protocol. The whole process includes freezing of the sample and then removing the water by sublimation in a vacuum environment.

After performing the freeze drying process solid formulations with a homogeneous structure were obtained. For further examinations the stable freeze dried cakes are used. The integrity of the protein, purity and possible formation of aggregates after lyophilization and after cold labeling were established with SDS-PAGE. For comparison, unmodified

trastuzumab in a concentration of 1 mg/mL was used. The electrophoresis was performed under reducing conditions. Disulfide bonds were reduced with 2-mercaptoethanol and antibody was separated in two fragments: two heavy chains ~50 kDa and two light chains ~25 kDa (Figure 4) (21).

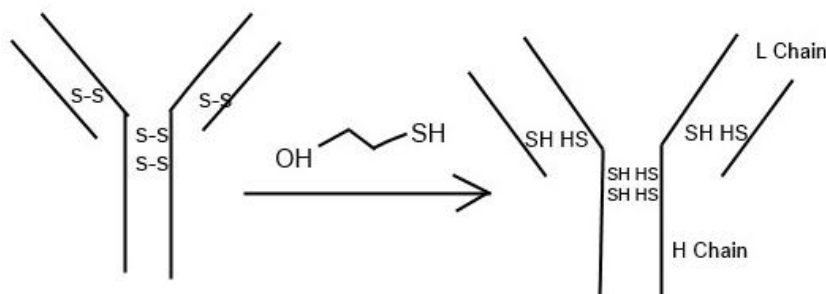


Figure 4. Reducing SDS-PAGE

The gel with unmodified trastuzumab and trastuzumab-immunoconjugates with different molar ratio of BFCAs is shown in Figure 5. The gels with

cold labeled immunoconjugates are shown in Figure 6 and Figure 7, as well.

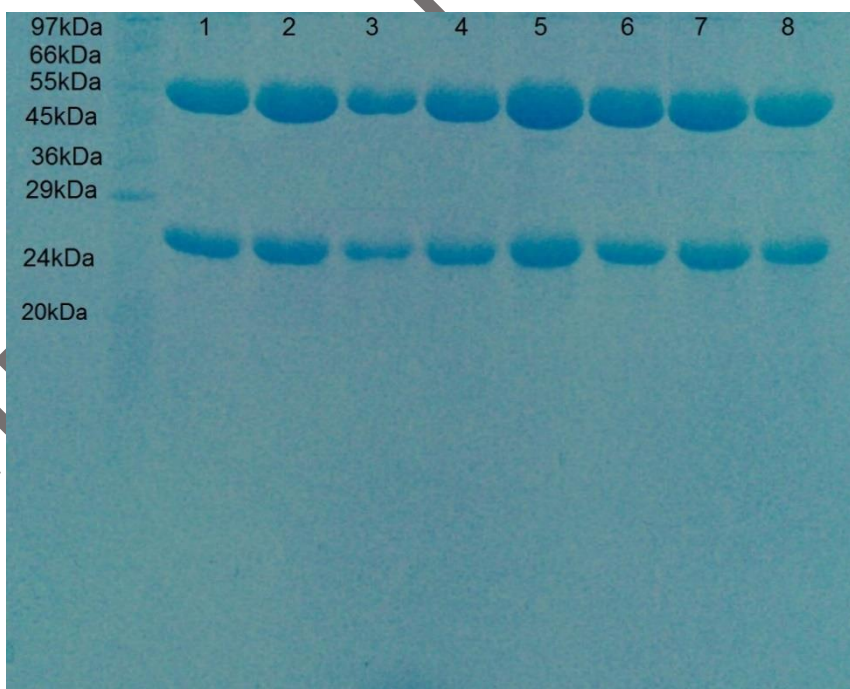


Figure 5. Reducing SDS-PAGE of Trastuzumab 1 mg/mL (1); DTPA-Trastuzumab (1:10) (2); DTPA-Trastuzumab (1:20) (3); DTPA-Trastuzumab (1:50) (4); DOTA-Trastuzumab (1:20) (5); 1B4M-DTPA-Trastuzumab (1:10) (6); 1B4M-DTPA-Trastuzumab (1:20) (7); 1B4M-DTPA-Trastuzumab (1:50) (8).

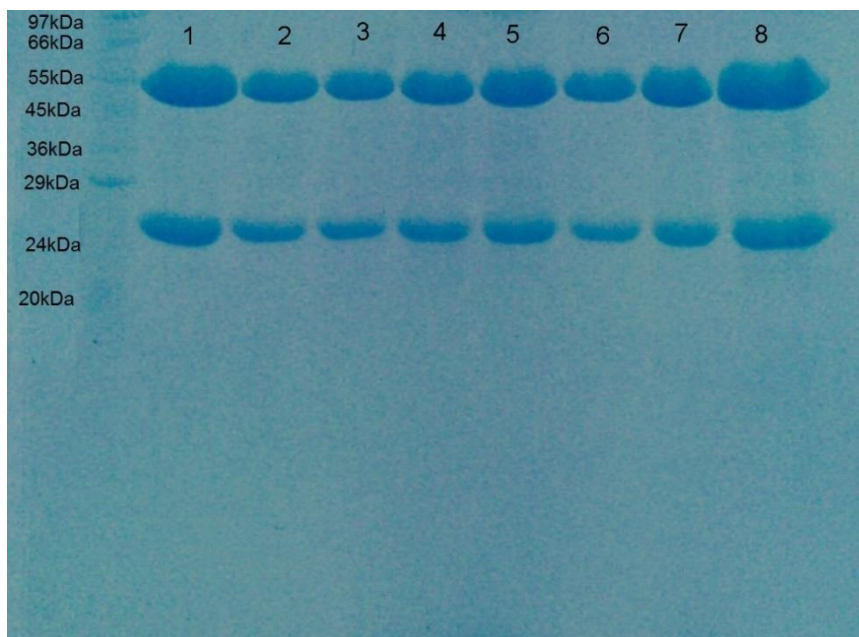


Figure 6. Reducing SDS-PAGE of Y-DTPA-Trastuzumab (1:10) (1);
 Lu-DTPA-Trastuzumab (1:10) (2);
 Y-DTPA-Trastuzumab (1:20) (3);
 Lu-DTPA-Trastuzumab (1:20) (4);
 Y-DTPA-Trastuzumab (1:50) (5);
 Y-DTPA-Trastuzumab (1:50) (6);
 Y-DOTA-Trastuzumab (1:20) (7);
 Lu-DOTA-Trastuzumab (1:20) (8).

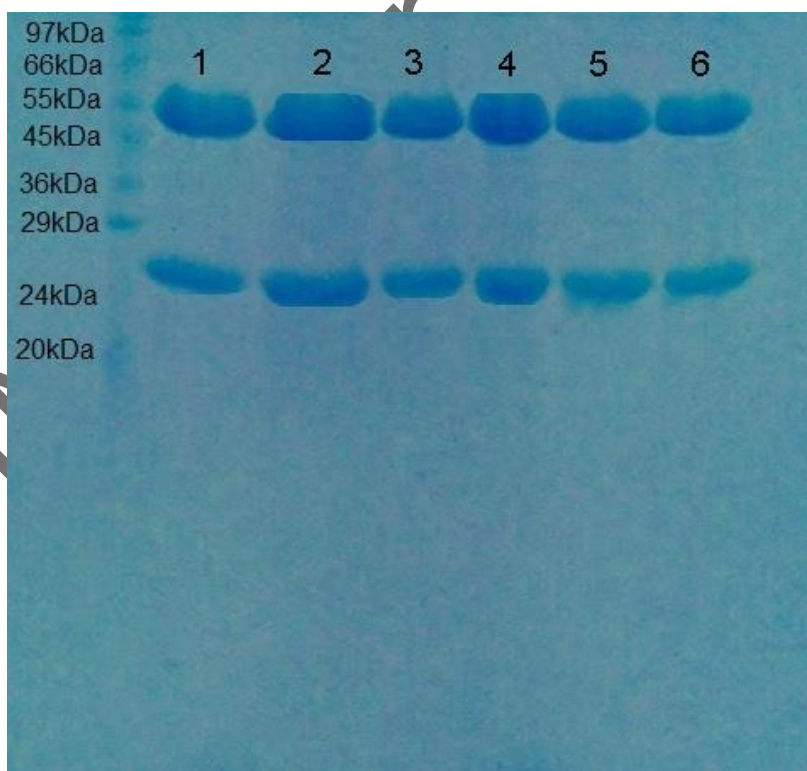


Figure 7. Reducing SDS-PAGE of Y-1B4M-DTPA-Trastuzumab (1:10) (1);
 Lu-1B4M-DTPA-Trastuzumab (1:10) (2);
 Y-1B4M-DTPA-Trastuzumab (1:20) (3);
 Lu-1B4M-DTPA-Trastuzumab (1:20) (4);
 Y-1B4M-DTPA-Trastuzumab (1:50) (5);
 Lu-1B4M-DTPA-Trastuzumab (1:50) (6).

Discussion

Lyophilization has a major significance in the process of formulation of stable pharmaceutical products (vaccines, monoclonal antibodies and protein formulation). Properly selected conditions are essential for complete removal of water and retention of the structural integrity of the proteins. They are necessary procedures for obtaining stable, homogeneous cakes with flat surfaces which retain the properties after reconstitution. The first step, freezing, is important to the process of separation of the water from the solution and leads the protein to crystalline state, which is more stable. Control of crystallization and size of crystals during the freezing are critical parameters and the appropriate selection of cooling rate is very important. It is already known that slow cooling provides larger crystals, while the faster freezing provides smaller crystals. On the other side, the faster cooling can lead to aggregations and denaturation of the antibody. During preparation of aqueous formulations, it is necessary to place them on the cooled shelves to 4-5 °C (22). With intention to improve the crystallization, the annealing step is often included (23). Our research team has already some experience with freeze drying of monoclonal antibody rituximab. Namely, Gjorgieva Ackova et al., 2014 (19) used a three day protocol with included annealing process. The obtained results were promising, since stable cakes with no aggregates and denaturation of the antibody were produced. But, our goal was to shorten the length of procedures in two days with exclusion of the annealing step. Also we tried to show that with the application of cooling rate of 1 °C/min we could obtain cakes with the same quality. The choice of the mentioned cooling rate was done based on results of the study of Tang and Pikal, 2004 (24). Our improvement of procedure of a super cooling without phase of separation and a uniform of ice structure was reached with the same rate of freezing.

The primary drying is the second step of lyophilization when the majority of frozen bulk water is removed by sublimation in a vacuum environment. Instead of 25 hours, the time was prolonged to 28 hours. Also, the secondary drying time was extended to 14 hours. At this phase some bound unfrozen water is removed at temperatures higher than 25 °C (23). Many sugars (glucose, mannitol or trehalose) can be used like cryoprotectants, to protect the proteins from denaturation and inactivation during the process of freezing (25). Nounou et al., 2005 (26) have shown in their study that obtained freeze dried cakes without any cryoprotectant possessed a compact structure but also demonstrated difficulties in process of reconstitution. This is probably the results of using sugars as bulking agents in production of fluffy cakes for easy reconstruction. In our studies to protect the antibody from stress temperature and to obtain elegant cakes for easy reconstitution 1 % mannitol was used. After the dissolving of the cakes with 0.9 % NaCl for less than a minute clear to opalescent solutions were obtained without presence of visible solid particles and colloids.

The processes of conjugation and freeze drying of the antibodies can lead to changes in protein integrity (aggregations and fragmentations). It is important to show that after cold labeling with Y and Lu, the metal does not change the structure of antibody. One of the most important methods for examination of protein integrity and purity is SDS-PAGE in reducing conditions (size-based method, where separation is based on the size of the molecules) (17, 27). Many stability studies of trastuzumab were made using a SDS-PAGE in reducing or non-reducing conditions. Under reducing conditions, trastuzumab was migrated as two bands a ~50 kDa and ~25 kDa (Mr of heavy and light chain), while under non-reducing conditions only one band at ~150 kDa was observed (Mr of whole antibody) (18, 28, 29). Our group has already used the applied method of reducing electrophoresis for integrity examinations of rituximab after conjugation and lyophilization (17, 19).

Almost the same conditions were used for examination of trastuzumab, with several modifications. In order to achieve reducing conditions, instead dithiothreitol, it was used 2-mercaptoethanol. The SDS-PAGE examinations were performed after six months storage of the freeze dried kits at temperature of 4 °C. The cold labeling was done before starting the process of electrophoresis. The obtained results of migration of formed samples correspond to already published results for other IgG1 monoclonal antibodies (18, 28). Under reducing conditions migration of the trastuzumab provided separation of two bands of fragments with molecular weight of 25 kDa for light chain and 50 kDa for heavy chain, proven with the Wide Range, molecular weight marker. The same intensity of the fragments of lyophilized and labeled conjugates with the fragments of pure commercial (unmodified) trastuzumab indicated that there was no degradation of the antibody. The results have shown that the conjugation, lyophilization and labeling are successful, without any damage to the antibody, with absence of fragmentation, denaturation and aggregation of the trastuzumab.

Conclusion

The results of electrophoretic examination have shown that stable freeze dried conjugates of trastuzumab were formulated with BFCAs in different molar ratios. The integrity and purity of the antibody were retained after cold labeling with Y and Lu. Therefore, these kits will be used for further characterization of the secondary structure of the antibody and determination of the number of the chelators with Infra Infrared Spectroscopy (IR), Raman Spectroscopy and Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometer (MALDI-TOF-MS). Moreover, immunoconjugates are good basis for radiolabeling with application of ¹⁷⁷Lu and ⁹⁰Y and therefore further researches will be done in order to evaluate the potential role of these conjugates in diagnostics and therapy of metastatic breast cancer.

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AMM Paper Accepted

PRIPREMA I ISPITIVANJE INTEGRITETA LIOFILIZIRANIH KITOVA TRASTUZUMAB-IMUNOKONJUGATA I HLADNO OBELEŽENIH IMUNOKONJUGATA PRIMENOM SDS-PAGE ELEKTROFOREZE

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Radioimunokonjugati su agensi za koje se smatra da imaju veliko potencijal u dijagnostici i lečenju različitih tipova kancera. Cilj ove studije je formulacija stabilnih liofiliziranih kitova trastuzumaba korišćenjem tri vrste bifunkcionalnih helatora, radi daljeg radioaktivnog obeležavanja. Integritet antitela u formulisanim konjugatima je ispitivan primenom natrijum dodecil sulfat poliakrilamid gel elektroforeze (SDS-PAGE).

Trastuzumab je humanizovano monoklonsko antitelo koje se koristi u terapiji agresivnog HER2 pozitivnog kancera dojke. Konjugacija trastuzumaba je izvršena korišćenjem različitih helatora: p-SCN-Bn-DTPA (1:10, 1:20, 1:50), p-SCN-Bn-DOTA (1:20), and 1B4M-DTPA (1:10, 1:20, 1:50). Prečšćeni imunokonjugati su liofilizirani primenom dvodnevnog protokola kako bi se dobili stabilni liofilizirani kitovi. Hladno obeležavanje neradioaktivnim izotopima LuCl₃ i YCl₃ je izvršeno da bi se uočile potencijalne modifikacije sekundarne strukture nakon radioaktivnog obeležavanja. SDS-PAGE elektroforeza u uslovima redukcije je korišćena za utvrđivanje čistoće i integriteta antitela pre i posle konjugacije, liofilizacije i obeležavanja.

Na osnovu dobijenih rezultata nije uočena degradacija ispitivanog antitela. Trastuzumab-konjugati i hladno obeležene formulacije migriraju u dve trake (~50k Da i ~25 kDa), na isti način kao IgG1 antitela i nemodifikovani trastuzumab.

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Ključne reči: Bifunkcionalni helatori, konjugati, elektroforeza, trastuzumab