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Production and Preclinical evaluation of the ^{67}Ga-CHX-A-DTPA-trastuzumab for HER2+ breast cancer SPECT Imaging

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HIGHLIGHTS

- Study of ^{67}Ga-CHX-A-DTPA-trastuzumab were performed in this work.
- The radioimmunoconjugate was prepared with a radiochemical purity of higher than 98±0.5% (ITLC).
- The ^{67}Ga-CHX-A-DTPA-trastuzumab showed high immunoreactivity towards HER2/neu antigen and $SkBr_3$ cell line.
- In vitro stability of the labeled product was more than 91% in PBS and 81.8±1.8% in human serum over 48 hr.
- It is a potential compound for molecular imaging of SPECT for diagnosis and follow-up of HER2 expression in oncology.

ABSTRACT

Radiolabeled monoclonal antibodies have shown great promise for cancer diagnosis and therapy. Breast cancer continues to pose a significant challenge in the field of healthcare, despite the remarkable strides made in cancer research. Approximately 15% to 20% of breast cancers exhibit an overexpression of a growth-promoting protein known as HER2. In the present study, we prepared Trastuzumab as an anti-HER2 antibody via identical chelator, CHX-A-DTPA (p-SCN-Bn-CHX-A-DTPA) labeled with Ga-67 [$T_{1/2}$ = 78.3 h, EC, E_γ = 93 (40%), 184 (24%), 296 (22%) and 388 (7%) keV] and performed preliminary biodistribution studies in female Sprague Dowley rat. Trastuzumab was conjugated with CHX-A-DTPA (Macrocyclics B-355), the average number of the chelator conjugated per mAb was calculated and total concentration was determined by spectrophotometrically. CHX-A-DTPAtrastuzumab was labeled with Ga-67 (10 mCi, 375 MBq) then Radiochemical purity and immunoreactivity by $SKBR_3$ cell line and serum stability of ^{67}Ga-CHX-A-DTPA-trastuzumab were determined. The biodistribution studies and radioimmunoscintigraphy were performed in female Sprague Dowley rat (^{67}Ga-CHX-A-DTPA-trastuzumab i.v., 200 microl, 200 ± 20 mCi, 35 ± 5 μg mAb, 4, 24, 48 and 72 h). ^{67}Ga-CHX-A-DTPA-trastuzumab was prepared (RCP>98% ± 0.5, Specific activity 4.1 ± 0.7 μCi.μg^{-1}). Conjugation reaction of chelator (50 molar excess ratio) to antibody resulted in a product with the average number of chelators attached to a mAb (c/a) of 4.1 ± 1.2. Labeling yield with Ga-67 in 400 μg concentration of bioconjugate was 92.5% ±2.1. Immunoreaction of ^{67}Ga-CHX-A-DTPA-trastuzumab complex towards HER2 antigen was determined by RIA and the complex showed high immunoreactivity towards HER2. In vitro and in vivo stability of radioimmunoconjugate was investigated respectively in PBS and blood serum by RTLC method. In vitro stability showed more than 91% ±2.6 in the PBS and 81% ±1.8 in the serum over 24 h. The Immunoreactivity of the radiolabeled anti-HER2 towards SKBR3 cell line was done by using Lindmo assay protocol. Under these conditions, the immunoreactivity of the radioimmunoconjugate was found to be 0.84. The biodistribution of ^{67}Ga-CHX-A-DTPA-trastuzumab complex in normal Sprague Dowley rat at 4, 24, 48, and 72 h after intravenous administration, expressed as percentage of injected dose per gram of tissue (%ID/g). Biodistribution studies at 24 and 48 h post-injection revealed the similar pattern to the other radiolabeled anti-HER2 immunoconjugates.

KEYWORDS

HER2
Gallium-67
Trastuzumab
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1 Introduction

The HER2/neu antigen is a transmembrane receptor (Schechter et al., 1984) overexpressed in 25-30% of breast cancers (Pauletti et al., 2000). The overexpression has been implicated in the carcinogenesis of breast cancer and is an independent prognostic indicator of survival in patients (Slamon et al., 1989). Trastuzumab is a humanized IgG1 monoclonal antibody (mAb) recognizing an epitope in the extracellular domain of the receptor and is used for immunotherapy for HER2/neu-positive tumors (Leonard et al., 2002). Breast cancer radioimmunoscintigraphy targeting HER2/neu expression has been proposed by different research groups and could allow direct assessment of the receptor status of primary and metastatic lesions suggesting the effectiveness of Herceptin therapy. Herceptin and its fragments have been radiolabeled and used in the imaging of HER2/neu-positive tumors using In-111 (Tang et al., 2005), Y-90 (Blend et al., 2003), Y-86 (Garmestani et al., 2002), Br-76 (Winberg et al., 2004), and Zr-89 (Dijkers et al., 2009). Ga-67 [$T_{1/2}$ = 78.3 h, EC, E_γ = 93 (40%), 184 (24%), 296 (22%) and 388 (7%) keV, allows for SPECT imaging and follow up of the radionuclide therapy (Firestone, 1996). Due to the superior bioconjugation of DTPA bi-functional ligands p-SCN-Bn-CHX-A-DTPA was used as a bi-functional ligand (Chappell et al., 2003). This ligand has already shown good biological performance when used in protein conjugation of various radioisotopes such as Ac-225 (McDevitt et al., 2002), Lu-177 (Smith et al., 2003) and lead radioisotopes (Chappell et al., 2000). In order to develop Herceptin radioimmunoconjugates for using in imaging studies, CHX-A-DTPA -trastuzumab (Herceptin) was labeled by Ga-67 chloride for preliminary biodistribution studies in rats.

2 Experimental

Enriched zinc-68 chloride with a purity of more than 95% was obtained from Ion Beam Separation Group at Agricultural, Medical and Industrial Research School. Ga-67 was produced in Radiation Application Research School, Karaj, Iran, by ^{68}Zn$(p,2n)^{67}$Ga reaction. p-SCN-Bn-CHX-A-DTPA (B-355) was purchased from Macrocyclics (NJ, USA). Trastuzumab (Herceptin) was a pharmaceutical sample purchased from Roche Co. Fetal Bovine Albumin (FBS), RPMI-1640 medium, and L-Glutamine were bought from Gibco Co. (Dublin, Ireland). PD10 Desalting column was inquired from Amersham Pharmacia Biotech; additional chemicals were purchased from Sigma Chemical Co. (MO, USA). Sprague-Dawley rats were obtained from Pasteur Institute (Tehran, Iran). A Bioscan AR-2000 radio TLC scanner instrument (Bioscan, Paris, France) was used for Radio-chromatography purposes. A p-type coaxial high-purity germanium (HPGe) detector (model: EGPC 80-200R) coupled with a multichannel analyzer card system and a dose calibrator ISOMED 1010 (Dresden, Germany) were utilized for the measurement of the activity. Calculations were carried out based on the 184 keV peak for Ga-67. The United Kingdom Biological Council's Guidelines on the Use of the Living Animals in Scientific Investigations, 2nd edition was used to determine the framework of animal experiments. Achieved results are displayed as mean ± standard deviation (Mean ± SD), and Students T-test was used to compare the data based on statistical significance defined as $P < 0.05$.

2.1 Conjugation of pSCNBn-CHX-A″DTPA with the trastuzumab

The final concentration of trastuzumab mAb was measured by means of a biophotometric assay at 280 nm. About 4.0 mg of the antibody in a 1000 μl bicarbonate buffer was then added to 3 borosilicate vials containing 0.3 (1:25), 0.5 (1:50), and 1.0 mg (1:80) of pSCNBn-CHX-A″DTPA and 400 μl bicarbonate buffer following gentle pipetting for 20-30 times. Afterward, each vial was shacked for 12 h at room temperature. Then, the mixture was transferred on a Vivaspin2 cutoff filter (30 kDa) and centrifuged at 4000 rpm for 15 min. To terminate the conjugation step and providing appropriate conditions for radiolabeling, the upper fraction of the filter was washed by 2 mL of 2.0 M ammonium acetate buffer (pH = 5.5) In the last step, ammonium acetate buffer (1 ml) was added to the upper fraction to dissolve the conjugated antibody, followed by gentle pipetting for 10-20 times for the purpose of immunoconjugate dissolution. Then, the filter was centrifuged at 2.684 × g for 5 min upsidedown to detach the conjugated antibody from the filter. The concentration of conjugated antibody was finally measured utilizing a biophotometer (Eppendorf) at OD = 280 nm.

2.2 Determination of the average chelator: mAb ratio

The number of pSCNBn-CHX-A″DTPA conjugated to each mAb was determined utilizing the Pippin method and in accordance with the previous researches (Pippin et al., 1992). 1.6 μM yttrium (III), 0.15 M sodium acetate buffer (pH 4.0), and 5.0 μM Arsenazo (III) were used to prepare Arsenazo yttrium (III) complex (2:1, 1 ml). The optical density of the prepared complex was measured by means of a biophotometer at 652 nm. A standard solution of 3 mM pSCNBn-CHX-A″DTPA in 0.15 M sodium acetate buffer solution (pH 4.00) was supplied. Then, 15 μl of the standard solution was added to the Arsenazo yttrium (III) complex and the optical density was measured and documented. This procedure (adding the standard solution and measuring the optical density) was repeated four times. In the next step, the optical density of the 1:2 yttrium (III) complex of Arsenazo (1 mL) was measured at 652 nm in the presence of conjugation product to investigate the pSCNBn-CHX-A″DTPAantibody attachment.

2.3 Radiolabeling of the pSCNBn-CHX-A″DTPAtrastuzumab conjugate with Ga-67

Certain amounts of the pSCNBn-CHX-A″DTPAtrastuzumab conjugate (200, 400, and 600 μg) were added to the vials comprising 74 MBq of Ga-67 adjusted to the pH of 5.5 using acetate buffer. The mixture was incubated at 25 °C for 1 h. Then, the radiochemical purity of the complex was

investigated by instant thinlayer chromatography (ITLC) using Whatman No. 2 and 1 mM DTPA as the stationary and mobile phase, respectively. Whenever the optimum concentration was specified, experiments were performed to determine the suitable time for labeling. Ultimately ethylene diamine tetra acetic acid solution (10 μl, 10 mM) was added to the labeling mixture and incubated for 10 min in order to scavenge the unlabeled In-111 cation. The mixture was then passed through a disposable PD10 desalting column to remove the impurities with small molecular weight and elevate the radiochemical purity. Finally, the mAbcontaining solution was stored at 4 °C to be used in further in-vitro and in-vivo studies.

2.4 Stability studies of the radiolabeled compound in PBS buffer and human serum

About 150 μL of the radioimmunoconjugate (with approximately 250 μCi (9.5 MBq activity) was added to the 500 μL of the PBS buffer and freshly prepared human serum while keeping at 4 °C and 37 °C, respectively. Samples were taken from the complex at 0, 12, 24, and 48 h after preparation, and the radiochemical purity was assessed by means of the ITLC method using Whatman No. 2 and 1 mM DTPA solution.

2.5 Reactivity of the radiolabeled compound toward HER2 antigen using radioimmunoassay method

Three rows of eight wells of ELISA were selected. In the eight wells of the first row, 100 μl of BSA with the concentration of 0.6 μg/100 μl and in another eight wells, 100 μl of HER2 at 0.3 μg/100 μl were poured and then were incubated overnight at 37 °C. The wells were washed three times with 10 mM phosphate buffer (containing tween 20) and after complete removal of water, all wells were filled with bubble solution and incubated for 45 min at 37 °C. ^{67}GaCHX-A″-DTPAtrastuzumab was then added with the concentration of 10 μg in 100 μl and incubated for 2 h and the wells were washed six times by washing buffer, after full water drainage, each of the wells was removed from the plate by breaking, and their activation was counted in the gamma counter. Each measurement was performed 3 times.

2.6 Immunoreactivity of radiolabeled mAb trastuzumab toward $SKBR_3$ and CHO cell lines

Distinct numbers (5×10^6, 2.5×10^6, 1.25×10^6, 0.62×10^6, 0.31×10^6, and 0.15×10^6) of CHO and $SKBR_3$ cells were put into the tubes and mixed with 30,000 cpm of the radiolabeled mAb trastuzumab. The tubes were incubated at 4 °C for 3 h and the radioactivity of each tube was measured by means of a γcounter. In the centrifuge of the tubes in 3000 × g, the supernatant was disposed and respective radioactivities of the cell debris were determined. The immunoreactivity was ascertained utilizing a Lineweaver-Burk plot. Lindmo method was applied to analyze the data.

2.7 Biodistribution studies of ^{67}Ga-CHX-A″-DTPAtrastuzumab in normal Sprague Dowley rat

For biodistribution studies, ^{67}Ga-CHX-A″-DTPAtrastuzumab was administered to normal Sprague Dawley rats separately. A volume (150-200 ml) of final radioactive solution containing 200 ± 10 μCi radioactivity and 35 ± 5 μg of trastuzumab was injected intravenously to rats through their tail vein. The total amount of radioactivity injected into each rat was measured by counting the 1 ml syringe before and after injection in a dose calibrator with a fixed geometry. Biodistribution studies were performed using groups of 3 rats sacrificed at 4, 24, 48 and 72 hours after injection of each radiolabeled mAb trastuzumab. At each time point, rats were sacrificed using CO_2 gas and normal organs (lungs, stomach, small and large intestine, spleen, blood, heart, kidneys, bone, feces, skin and liver) were excised. Organs were weighed and gamma counted with an HPGe detector counting the area under the curve of the 184 keV peak. The percentage of injected dose of radioisotope per gram (% ID/g) organ was calculated (after correcting for radioactive decay using an aliquot of the injected at each time point). Mean values and standard errors for each tissue and time point were plotted.

2.8 Statistical analysis

The experiments were performed three times for each test and final values were presented as the mean ± SD. Statistical analyses were performed using IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp, U.S.A. 10.0 considering $P < 0.05$ as statistically significant. The paired ttest was used to compare the results of the two experiments.

3 Results and Discussion

3.1 Conjugation of trastuzumab with pSCNBn-CHX-A″DTPA and radiolabeling of trastuzumab with Cu-64

In order to overcome the effect of excipients and producing appropriate acidity for conjugation step the pharmaceutical sample was purified by ultra-filtration using cut-off filters followed by determination of the antibody concentration using spectrophotometry. In order to improve the conjugation step alkaline pH is necessary, thus bicarbonate buffer was used to reconstitute the antibody. The use of polymer tubes and other synthetic materials in the conjugation and labeling step interfered with the conjugation reaction, while borosilicate vials were the appropriate vessels. In order to remove the leftover of Bi-Functional Chelator in the reaction and concentrate the antibody, the cut-off filter was used once more (30 kDa). At this stage, a pH 5.0 buffer was used to recover the antibody in order to terminate the conjugation step and provide the suitable radiolabeling pH, and for final fraction the quantity of the antibody was measured at OD = 280 nm. In order to estimate the number of CHX-A-DTPA prosthetic group on each antibody molecule, the arsenazo yttrium

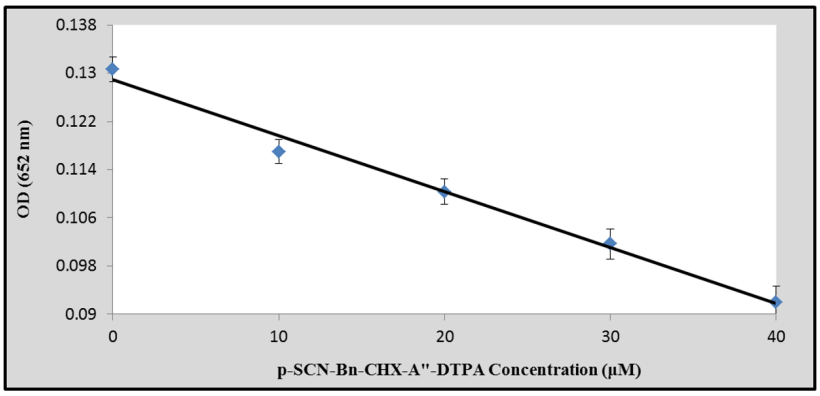

Figure 1: The standard curve of the absorption of Arsenazo yttrium (III) complex against the concentration of p-SCN-Bn-CHX-A″-DTPA ($n = 3$).

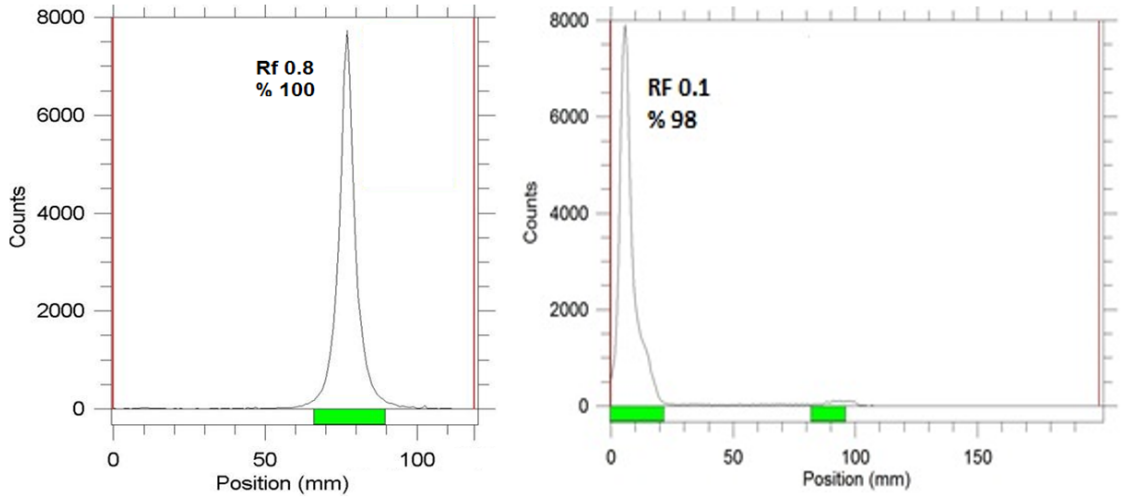

Figure 2: Radiochromatogram of free Ga-67 (left) and ^{67}GaCHX-A″-DTPAtrastuzumab (right) using Whatman No. 2 in 1 mM DTPA, pH 5.0 ($n = 3$).

complex [Y(AAIII)2] method was used. The absorbance of Y(AAIII)2 at 652 nm decreases upon the addition of CHX-A-DTPA-trastuzumab while the corresponding absorbance of AAIII at 538 nm increases (Fig. 1). The Y(AAIII)2 and arsenazo III are the only absorbing species in solution; neither CHX-A-DTPA-trastuzumab nor its Y(III) complex have any absorbance in this wavelength region. The isosbestic point observed at 585 nm is consistent with only two absorbing species for reaction. The data demonstrated the CHX-A-DTPA:antibody ratio of 4.1±1.2:1 (Table 1).

Kukis et al. observed that coupling of too many ligands to antibody leads to impairment of immunoreactivity. As described for the Lym-1 antibody (Kukis et al., 1995). Zimmermann et al. also found that unfavorable biodistribution were a more sensitive marker for over-substitution of the mAb than in vitro immunoreactivity. Whereas substituting 18 ligands per antibody molecule lead to an about 25% lower in vitro immunoreactivity, in vivo tumor uptake was decreased by 80%. At the same time accumulation of radioactivity in the liver was increased about 40%. A c/a ratio up to 11 seemed to be the limit for substituting mAbs without damage to immunoreactivity and effects on biodistribution (Zimmermann et al., 2003). Thus, in this study we optimized conjugation procedure to obtain a chelate-to-mAb molar ratio less than 11 (4.1±1.2:1 for instance), in order to minimize any immunoreactivity loss and unwanted liver uptake.

3.2 Radiochemical purity determination

By direct radiolabeling of the Conjugate in a simple and rapid manner, the radioimmunoconjugate was easily prepared in radiochemical purity of higher than 98%. The DTPA-trastuzumab conjugate was labeled with Ga-67 at Specific activity 4.1 ± 0.7 μCi/μg, and the radiolabeled mAb exhibited high in vitro serum stability and minimal loss of immunoreactivity. The effect of time on RCP was also studied and 30 min was considered as the best time. ITLC chromatogram of the radiolabeled compound and free Ga-67 are indicated in Fig. 2. As a result of the experiments, the best conditions for radiolabeling were as follows: 400 μg of the bioconjugate was added to a vial containing 74 MBq of Ga-67 radionuclide and pH of the reaction mixture was arranged to 5.5 as keeping at 25 °C for 30 min.

Table 1: Average numbers of chelators bound to each antibody (c/a) in different molar of chelator added to conjugation reaction ($n = 3$).

Bifunctional chelator	Conjugate	Molar excess of chelator added to conjugation reaction	Average number of Chelators/mAb
pSCNBn-CHX-A″DTPA	CHX-A″-DTPAtrastuzumab	25	2.2 ± 0.4
		50	4.1 ± 1.2
		80	7.2 ± 0.9

4 Stability of Radiolabeled Protein in presence of human serum and PBS

Table 2 presents the in vitro stability of radiolabeled mAb by using size exclusion chromatography using G-25 Sephadex at 37 °C after 0, 12, 24, and 48 h of incubation (in buffer and human serum). The results showed that After incubation of ^{67}Ga-CHX-A″-DTPAtrastuzumab (300 μCi) with freshly prepared human serum at 37 °C for up to 48 h, more than 78% of total radioactivity eluted in the same position as ^{67}GaCHX-A″-DTPAtrastuzumab. Cole et al. reported that deacreases of stability in human serum resulted from mild transchelation of Ga-67 to serum components such as albumin (Cooper et al., 2012), while Cooper et al. reported the high stability of macrocyclic immunoconjugates such as DOTA-mAb, DO3A-mAb and NOTA-mAb in serum over 48 h (Tolmachev et al., 2010) which is in agreement with our data.

Table 2: Stability of ^{67}Ga-CHX-A″-DTPAtrastuzumab in PBS at 4 °C and human blood serum at 37 °C at different times postlabeling ($n = 3$).

Time post labeling (h)	Radio-Chemical Purity (RCP) (%)	
	PBS (4 °C)	Human Serum (37 °C)
0	98.0 ± 0.5	98.0 ± 0.5
12	95.5 ± 0.7	91.0 ± 1.1
24	91.1 ± 2.6	81.8 ± 1.8
48	90.5 ± 1.1	78.3 ± 0.9

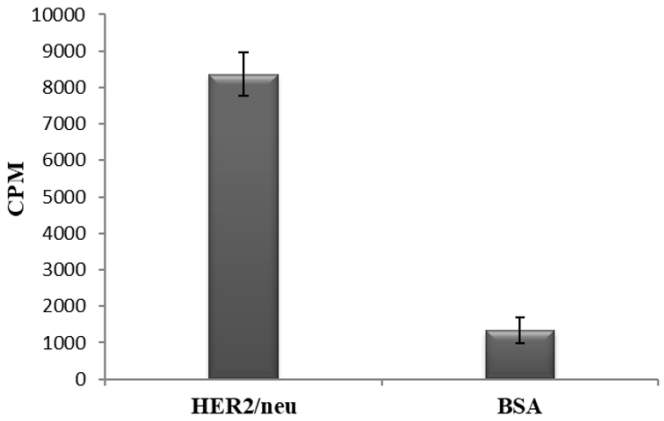

Figure 3: Reactivity of ^{67}Ga-CHX-A″-DTPA-trastuzumab (10 μg/well) toward HER2 antigen (0.3 μg/well) and BSA (0.6 μg/well) using radioimmunoassay method ($n = 3$).

4.1 *Reactivity of radiolabeled trastuzumab towards HER2/neu antigen by RIA*

Reactivity of radiolabeled mAb trastuzumab towards the HER2/neu antigen (0.2 μg/well) and BSA (0.2 μg/well) was shown in Fig. 3. The ^{67}GaCHX-A″-DTPAtrastuzumab showed high immunoreactivity towards HER2/neu antigen. This result also showed that the chemical binding of Ga-67 to mAb had no adverse effects on the immunoreactivity of antibody towards HER2/neu antigen.

4.2 *Immunoreactivity of radiolabeled trastuzumab towards SkBr$_3$ cell line*

Zimmermann et al. reported that moderate increase of chelator to mAb ratio leads to slight decrease in immunoreactivity. Lewis et al also reported this slight decrease in immunoreactivity is likely due to non-specific conjugation of chelators to mAb, which possibly attaches a chelator in the region of the antigen binding site (Spiridon et al., 2004). The Immunoreactivity of the radiolabeled trastuzumab (^{67}Ga-CHX-A″-DTPAtrastuzumab) towards SkBr3 cell line was determined under conditions of antigen excess in HER2/neu antigen-expressing SkBr$_3$ human breast carcinoma cells by using a Lineweaver-Burk plot. Under these conditions, the immunoreactivity of radioimmunoconjugate was found to be 0.84 which is suitable for further imaging studies in animal models and possibly human trials. It seems that exact optimization of c/a ratio has led to such suitable immunoreactivity. Thus, conjugation and complexation procedures did not affect the affinity of mAb toward its antigen in this work (Fig. 4).

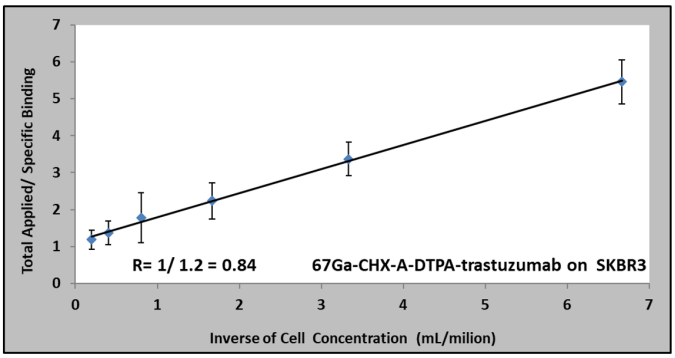

Figure 4: Immunoreactivity of ^{67}Ga-CHX-A″-DTPA-trastuzumab (total applied/specific binding) toward SKBR$_3$ cell line at different cell concentration ($n = 3$).

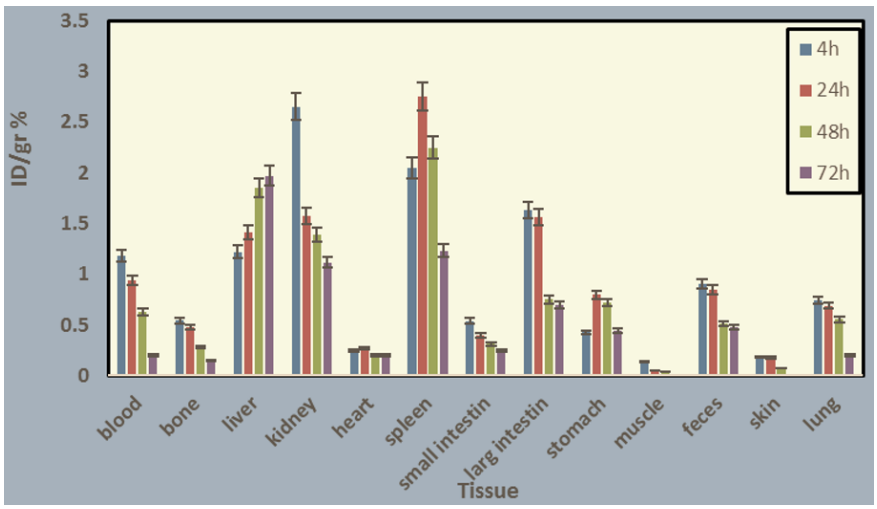

Figure 5: Percentage of injected dose per gram (% ID/g) of ^{67}Ga-CHX-A″-DTPA-trastuzumab in normal Sprague-Dawely rat at 4, 24, 48, and 72 h postinjection ($n = 3$).

4.3 ^{67}Ga-CHX-A″-DTPAtrastuzumab biodistribution in normal Sprague-Dawley rat tissues

Kokai et al. showed that during late gestation (Embryonic day 18) and during the early postnatal period (PND-1 and PND-5), two types of tissue expressed HER2/neu antigen (p185) in normal rat. Strong immunoreactivity was detected on epithelial cells of the intestinal villi, the skin, pulmonary bronchioles, and proximal renal tubules. Also, faint staining of connective tissue was observed. Staining in the nervous system could no longer be detected. In adult animals, immunoreactive p185 was limited to epithelial tissues, including the basal layer of skin, the mucosal epithelium of intestine, bronchiolar epithelium of lung, and proximal tubular epithelium of kidney. Connective tissue no longer expressed p185 (Costantini et al., 2007). As shown in Fig. 5, biodistribution studies in adult normal Sprague-Dawely rats after injection of ^{67}Ga-CHX-A″-DTPAtrastuzumab demonstrated that uptake in intestine, skin and lung organs significantly increased during the study time also high uptake in kidney organ during the time study were observed which is due to the presence of HER2/neu antigens in mentioned tissues also leading to high colon and feces activity content, which is in agreement with the findings of Kokai and co-workers. This has been already shown by other groups, working with ^{125}I-anti-HER2 probes (Kokai et al., 1987). A significant kidney uptake of radiolabeled mAb trastuzumab is observed all time intervals, in Fig. 5 possibly due to the presence of HER2/neu antigen. As shown in Fig. 5, accumulation of radiolabeled mAb trastuzumab in spleen and liver increases by the time up to 24 h. This behaviour has been already shown to be consistent with half life of intact mAb in circulation like other radiolabeled mAb anti HER2 probes.

5 Conclusions

Radiolabeling, stability testing, radiochemical purity, reactivity, immunoreactivity and biodistribution study of ^{67}Ga-CHX-A″-DTPA-trastuzumab were determined followed by biodistribution studies in normal Sprague-Dawley rat tissues (200 ± 20 μCi, 4, 24, 48, and 72 h p.i.). The radioimmunoconjugate was prepared with a radiochemical purity of higher than 98 ± 0.5% (ITLC). The average number of chelators per antibody (c/a) for the conjugate used in this study was 4.1±1.2:1. The ^{67}Ga-CHX-A″-DTPA-trastuzumab showed high immunoreactivity towards HER2/neu antigen and $SkBr_3$ cell line. In vitro stability of the labeled product was found to be more than 91% in PBS and 81.8 ± 1.8% in human serum over 48 h. The accumulation of the radiolabeled mAb in liver, skin, intestine, lung, spleen, kidney and other tissues demonstrates a similar pattern to the other radiolabeled anti-HER2 immunoconjugates.

These data validate this radiopharmacutical for further clinical testing. Therefore ^{67}Ga-CHX-A″-DTPA-trastuzumab is a potential compound for molecular imaging of SPECT for diagnosis and follow-up of HER2 expression in oncology.

Conflict of Interest

The authors declare no potential conflict of interest regarding the publication of this work.

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