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Kit formulation for 99mTc-labelling of recombinant anti-HER2 Affibody® molecules with a cysteine engineered at the C-terminal

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Author **Kristofer Andersson**	
Title (English) **Kit formulation for ^{99m}Tc-labelling of recombinant anti-HER2 Affibody® molecules with a cysteine engineered at the C-terminal**	
Title (Swedish)	
Abstract Affibody® molecule $Z_{HER2:2395}$-C has previously been shown to allow for site-specific radiolabelling. The aim of this project was to optimize a one-vial kit formulation for ^{99m}Tc labelling of $Z_{HER2:2395}$-C allowing it to be introduced into hospital pharmacy. Verification that the ^{99m}Tc-$Z_{HER2:2395}$-C conjugate was stable were performed in mouse plasma. Conformation that $Z_{HER2:2395}$-C kept its high specificity and affinity were performed on the HER2 overexpressing cell line, SKOV-3. The kit was evaluated over 28 days to show that no deterioration occurred. Results from this test showed that the labelling yield was 96.4 % ± 1.7 and the RHT content 0.35 ± 0.45. Cell studies showed that $Z_{HER2:2395}$-C kept its high affinity and specificity.	
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Kit formulation for ^{99m}Tc-labelling of recombinant anti-HER2 Affibody® molecules with a cysteine engineered at the C-terminal

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Sammanfattning

Bröstcancer är idag den vanligaste cancerformen hos kvinnor. Idag finns ett växande antal tumörmarkörer vilket underlättar val av lämplig behandling. En av dessa markörer är human epidermal growth factor (HER2) som överuttrycks i 20-30 % av alla bröstcancerfall. HER2 används idag som måltavla för behandling med den monoklonala antikroppen trastuzumab (Herceptin®). För att kunna välja patienter till denna behandling krävs att HER2-uttrycket kan detekteras.

Idag diagnostiseras HER2-uttrycket genom test på tumörvävnad. Detta har dock sina brister. Ett kirurgiskt ingrepp innebär alltid vissa risker och det finns undersökningar som tyder på att cirka 20 % av bröstcancerfallen feldiagnostiseras med avseende på HER2-status. Ytterligare problem är att primärtumören inte nödvändigtvis uppvisar samma HER2-status som dess dottertumörer. Av dessa anledningar vore det attraktivt att kunna ställa diagnos genom helkropps-imaging med en målsökande radioaktivt inmärkt molekyl.

Detta projekt syftade till att möjliggöra reproducerbar inmärkning av en målsökande Affibody® molekyl i sjukhusfarmaci. För att uppnå detta har sammansättningen av ett kit optimerats. Verifiering av att inmärkningen var stabil samt bevarade sina målsökande egenskaper utfördes.

Med hjälp av detta kit kan en Affibody® molekyl stabilt och reproducerbart märkas inmärkas med technetium. Detta kan potentiellt tillåta att ett överuttryck av HER2 upptäcks med större säkerhet och mindre obehag för patienter. Detta kan leda till en bättre och mer individanpassad behandling.

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1. Introduction

1.1 Breast cancer

Breast cancer is the most common cancer amongst women today (WHO fact sheet N°297, 2006). Even though the first diagnosis is relatively straightforward the nature of cancer, being a heterogeneous disease, makes the treatment more difficult. Therefore, diagnosis of what subclass a specific tumour belongs to is of interest, so the patient can receive best possible treatment. Today there are a growing number of tumour markers available both for treatment and diagnosis. One of these is the human epidermal growth factor receptor type 2 (HER2) that is overexpressed in 20-30 % of all breast cancer and is associated with an aggressive course of disease (Andrulis *et al.*, 1998; Slamon *et al.*, 1987)

HER2 belongs to a family of transmembrane receptors with four members (HER1, 2, 3 and 4). At present there is no known ligand to HER2. However, it plays an important role in heterodimerization, primarily with HER3 but also 1 and 4. The cellular signalling occurs after dimerization of the receptors. This leads to autoposphyrylations and various protein kinase signalling cascades. Overexpression of these receptor proteins induces a vast number of onco-related cell states e.g. increased proliferation, angiogenesis and decreased apoptosis (Prenzel el al, 2001; Yarden *et al.*, 2001).

Today both the American Society of Clinical Oncology and the European Group on Tumor Markers recommends that the HER2-status is determined for every new or recurrent breast cancer. If the tumour is HER2-positive treatment with e.g. trastuzumab (Herceptin®) (Molina *et al.*, 2005; Wolff *et al.*, 2007) should be initiated. Diagnosis for HER2 positive tumours is today performed using immunohistochemistry (IHC) and/or fluorescent in situ hybridization (FISH) test of biopsy samples. This works fairly well when primary tumours are concerned. However, it has been suggested that 20 % of the HER2 test are inaccurate (Wolff *et al.*, 2007). Additionally a HER2 negative primary tumour may have HER2 positive metastases. Other problems related to biopsies are that the location of the metastases might make it impossible to take a biopsy or the existence of metastasises might be unknown. Furthermore, the biopsy procedure is an invasive method with associated risks for the patients and possibilities of

sampling errors. This makes imaging, using a radionuclide labelled molecule with affinity for HER2, an attractive choice.

1.2.1 Tracers

For a radiolabelled tracer to be of interest for diagnosis it need to be accurate. To achieve this the tracer needs to be both specific and sensitive. Besides any natural ligand, there are several types of molecules that could be used.

Antibodies and antibody fragments could be used. However, these are mostly relatively large molecules (IgG ~ 150 kDa, Fab fragment ~ 55 kDa), this result in slow tissue penetration and blood clearance. For imaging this means that it will be harder to obtain a good contrast. Of the antibody fragments the single chain Fv fragments (25 – 27 kDa) and other fragments, in this size or smaller, would provide the best contrast.

Small binding peptides, around 10 amino acids, would allow a rapid tumour localization and fast blood clearance. However, their small size makes it hard to achieve an affinity in the low nanomolar range when radiolabelled.

Small scaffold proteins combine properties from both small peptides and antibody fragments. They possess favourable size and have a defined structure. The structure is usually robust, allowing for harsh labelling conditions. One example of such a protein is the Affibody® molecule.

1.2.2 Affibody® molecules

Affibody® molecules are derived from one of the five IgG-binding domains of staphylococcal protein A. Upon modification, aimed to increase its stability, the modified domain was renamed from B to Z. The Affibody® molecule consists of three anti-parallel α-helices consisting of 58 amino acids (~ 6.5 kDa). Thirteen of the surface exposed amino acid residues have been randomized, to create a library of approximately 3×10^9 molecules. From this library it is possible to obtain an Affibody® molecule with affinity to a vast range of proteins. The selection of Affibody® molecule is done by phage display (Nilsson *et al.*, 1987; Nord *et al.*, 1995).

Previously an Affibody® molecule with affinity to (dissociation constant, K_D = 50 nM) HER2, $Z_{HER2:4}$ have been isolated (Wikman *el al.,* 2004). The next generation of Affibody® molecule, $Z_{HER2:342}$, produced by affinity maturation, reached an affinity of 22 pM (K_D). Labelling of $Z_{HER2:342}$ with nuclides such as ^{111}In and ^{125}I enabled visualization of HER2-expressing xenografts in mice. (Orlova *et al*., 2006). Further engineering resulted in $Z_{HER2:2395}$-C, an Affibody® molecule with a cysteine incorporated at the C-terminal (Ahlgren *et al*., 2008). It have previously been shown that ^{99m}Tc can be stably and site-specifically bound to an Affibody® molecule via a N_3S cheleate formed by the last three amino acids (see Figure 1) (Ahlgren *et al*., 2008).

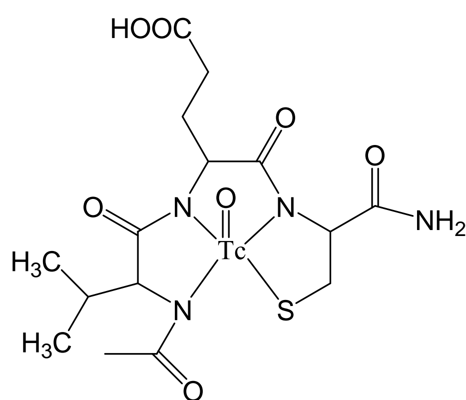

Figure 1. Hypothetical structure of the Tc-N_3S cheleate formed by the last three amino acid residues, VDC, closest to the C-terminal.

1.3 Kit formulation for ^{99m}Tc labelling

Metastable technetium isotope 99 (^{99m}Tc) decays through isomeric transition emitting gamma-quanta of 140 keV ($t_{1/2}$ = 6.01 h) to its isomer ^{99}Tc, a β^- emitter ($t_{1/2} = 2.12 \times 10^5$ years). The energy of 140 keV is well suited for gamma-imaging purposes, allowing the radiation to be detected with a gamma camera while exposing the patient for a low dose burden. The ^{99}Mo/^{99m}Tc generator allows a relatively simple logistic of ^{99m}Tc production. Aluminium bound ^{99}Mo-molybdate allows elution of its daughter nuclide, ^{99m}Tc, as pertechnetate, using saline. The favourable gamma energy, simple logistic and low price have made ^{99m}Tc the preferred and most used nuclide for gamma imaging.

The relatively short half-life of ^{99m}Tc means that the labelling must be made at the site of use. For this reason the labelling procedure must be easy to perform. This generated a concept of so-called ‘labelling kits’. These kits contain an optimal concentration of all reagents necessary for labelling. To minimize the risk of human errors the number of manipulations should be minimal. Ideally, the kit formulation should be a ‘one-vial kit’ i.e., containing all the ingredients but radionuclide in one vial. This would allow labelling by adding a pre-defined amount of radionuclide, possible heating and quality controls. A procedure like this would allow for untrained personnel to perform the labelling.

In hospital pharmacy a ^{99m}Tc labelled compound may be used if the labelling yield is above 90 % i.e., 90 % of the added radioactivity is bound to the tracer. However, a higher yield promises higher contrast. Furthermore, the formation of reduced hydrolysed ^{99m}Tc (RHT, colloids) must be below 2 %. RHT forms colloids from free technetium and accumulate to the liver, spleen and lungs.

To achieve a high labelling yield and low level of reduced hydrolysed ^{99m}Tc (RHT) the kit composition was optimised, primarily with respect to tin(II)chloride, glucoheptonate and EDTA. Tin(II)chloride is needed to reduce technetium from non-reactive +7 to reactive +5 oxidation state. Glucoheptonate aids in incorporation of ^{99m}Tc to the cheleate by forming a weak intermediate complex with ^{99m}Tc and stabilising it in its +5 state. EDTA forms complexes with metals and is therefore added to avoid that small amounts of other metals, besides technetium, to form complexes with the N_3S cheleate.

2. Aim of the project

The aim of the project was to develop a robust composition of a freeze-dried kit, allowing reproducible labelling of anti-HER2 Affibody® molecule, $Z_{HER2:2395}$-C, with ^{99m}Tc.

To verify that the ^{99m}Tc-$Z_{HER2:2395}$-C conjugate functioned in biological settings, various tests were performed. To evaluate the stability of the labelling in vitro tests in mouse plasma were conducted. Verifications that $Z_{HER2:2395}$-C preserves its specificity and high affinity to HER2 were performed on the HER2 overexpressing cell line, SKOV-3. A study in mice was also performed to show stability and verify a favourable biodistribution.

3. Material and methods

3.1 Chemicals

α-D-gluconic acid sodium salt (glucoheptonate) was acquired from Acros organic and Sigma. Etylenediamine-Tetraacetic acid (EDTA) was from Sigma. Acetic acid, disodium phosphate dihydrat (Na_2HPO_4) and dithiohereitol (DTT) was purchased from Merck. Tin(II)-chlorid dihydrat ($SnCl_2$) and pyridine was purchased from Fluka Chemika. 1× phosphate buffered saline, pH 7.4 (PBS) was produced, in house, at the Rudbeck laboratory. For preparations of solutions, high-quality Milli-Q water (resistance higher than 18 MΩ/cm) was used. $Z_{HER2:2395}$-C was produced as previously described (Ahlgren *et al.,* 2008). ^{99m}Tc was obtained as pertechnetate from an Ultra-TechneKow generator (Tyco) eluated with sterile 0.9 % sodium chloride (Mallinckrodt BV, Petten, The Netherlands).

3.2 Instrumentation

An automated gamma counter with a 3 inch NaI(Tl) detector (1480 WIZARD™, Wallac Oy, Turku, Finland, further referred to as gamma counter) was used to measure radioactivity. The Cyclone Storage Phosphor System and the OptiQuant image analysis software (Perkin Elmer, Wellesley, MA, USA, further referred to as PhosphorImager) were used to measure and analyze the radioactivity on silica gel impregnated glass fiber sheets for instant thin-layer chromatography strips (ITLC-SG, Gelman Schiences Inc.). NuPAGE® 10% Bis-Tis gels 1.0 mm × 10 well (Introgen™) was used to analyze conjugate stability, the radioactivity

distribution was analyzed on PhosphorImager. Cells were counted in an electronic cell counter (Beckman Coulter, Fullerton, CA, USA).

3.3 Cell culture

In this study SKOV-3 cells were used. This is a human ovarian cancer cell line that overexpresses HER2. The cells was cultured in McCoy’s medium (Flow Irvine, UK) in a humidified incubator with 5% CO_2. The medium was supplemented with 10% fetal calf serum (Sigma, USA), 2 mM L-glutamine and PEST (penicillin 100 IU/ml and 100 µg/ml streptomycin), all from Biochrom AG (the supplemented medium is further referred to as complete medium and as incomplete medium if it was not supplemented). Cells were detached by scraping or using trypsin–EDTA solution (0.25% trypsin and 0.02% EDTA) from Biochrom AG.

3.4 Freeze drying of kits

All solvents were degassed by sonication before use. Kits were made by dissolving $SnCl_2$ and EDTA in water (except for kit 100ZG5Na where 0.2 M Na_2HPO_4 was used). $SnCl_2$ was dissolved in 0.01 M HCl. The final concentrations (see Table 1) were adjusted with water and for one-vial kits, $Z_{HER2:2395}$-C, was added. Siliconized eppendorf vials (1.7 ml) containing portions of the kit formula were frozen under Argon atmosphere at - 80°C, for a minimum of 20 min and freeze-dried over night.

The kit RG5 differ in the production from the rest of the kits containing $Z_{HER2:2395}$-C in that pre-reduced $Z_{HER2:2395}$-C was used. To break spontaneously formed intermolecular disulfide bonds between two cysteines the protein was pre-treated with DTT. A 0.5 M DTT-solution was made by dissolving DTT in water. The DTT-solution was mixed with $Z_{HER2:2395}$-C for a final concentration DTT of 30 mM. The $Z_{HER2:2395}$-C-DTT-mixture was incubated at 37°C during 2 h prior to purification on a NAP-5 size-exclusion column (Amersham Biosciences, Uppsala, Sweden). The column was pre-equilibrated with water and the $Z_{HER2:2395}$-C-DTT mixture was loaded onto the column and eluated with 900 µl water. $Z_{HER2:2395}$-C and a mixture of glucohepotonate, EDTA and $SnCl_2$ (see Table 1) was quickly mixed and freeze-dried as above.

Table 1. Composition of kit formulations

Kit	Glucoheptonate (mg)	EDTA (µg)	$SnCl_2$ (ug)	$Z_{HER2:2395}$-C (µg)	0.2 M Na_2HPO_4 (µl)
S1	5	100	50	0	0
S2	5	100	25	0	0
S3	5	100	100	0	0
S4	5	100	12	0	0
S5	5	100	5	0	0
S6	5	100	0.5	0	0
S7	5	100	0	0	0
S8	5	100	200	0	0
S9	5	100	400	0	0
S10	5	100	75	0	0
S11	5	100	150	0	0
G1	2.5	100	75	0	0
G2	10	100	75	0	0
G3	1	100	75	0	0
G4	20	100	75	0	0
G5	3.75	100	75	0	0
G6	7.5	100	75	0	0
E1	3.75	50	75	0	0
E2	3.75	200	75	0	0
E3	3.75	0	75	0	0
E4	3.75	25	75	0	0
E5	3.75	400	75	0	0
ZG5	3.75	100	75	100	0
RG5	3.75	100	75	100	0
ZG5	3.75	100	75	100	0
75ZG5	2.8125	75	56.25	100	0
100ZG5	3.75	100	75	133	0
100ZG5Na	3.75	100	75	133	82
75ZG5Na-2	2.8125	75	56.25	100	18.75

Amount of Glucoheptonate, EDTA, $SnCl_2$, $Z_{HER2:2395}$-C and Na_2HPO_4 per kit.

3.5 Labelling of $Z_{HER2:2395}$-C using kits

For kits containing no protein (two-vial kits), labelling was performed by dissolving content from one kit in PBS. The solution was thereafter mixed with $Z_{HER2:2395}$-C, for a total volume of 100 µl. For kits already containing protein (one-vial kits) no PBS was added. From this point both type of kits, those containing protein and those that does not, were treated in the same manner. ^{99m}Tc-pertechnetate was added, mixed and sealed under argon before incubated at 90°C during 60 min. The solution was allowed to cool for 15 min in room temperature. Two ITLC-SG strips were loaded with 1 µl of the solution and run in PBS or a mixture of pyridine, acetic acid and water (ratio 5:3:1.5), respectively.

In PBS free technetium and ^{99m}Tc-glucoheptonate complex moves with the front, protein and colloids stay at the origin. In the pyridine, acetic acid and water mixture, protein and free technetium moves with the front and colloids stay at the origin. The ITLC-SG strips were analysed using PhosphorImager.

3.6 In vitro specificity test

Petri dishes (diameter = 30 mm) were seeded with 250k cells 1-2 days prior to the experiment. Typically a dish contained 400k cells on the day of experiment. The dishes were emptied from medium and washed with incomplete medium. For blocking a 0.5 ml solution (0.5 ml complete medium and non-labelled $Z_{HER2:342}$, ratio $Z_{HER2:342}$ to HER2 roughly 100:1) was added to three of the dishes. To the other three dishes a compensating volume (0.5 ml) of complete medium was added. A 0.5 ml solution of ^{99m}Tc-$Z_{HER2:2395}$-C conjugate (a ratio of Affibody® molecule to HER2 of 1:1) was added to all dishes and incubated at 37°C during 1 h. The medium was collected and the dishes washed with medium. The cells were incubated in a 0.5 ml trypsin-EDTA solution until they detached (approximately 10 min). A volume of 0.5 ml complete medium was added and the cells were collected. The radioactivity was measured using a gamma-counter and corrected for background i.e. radioactivity measured by the gamma-counter without samples loaded. The cell-associated radioactivity was calculated as $R_{cell}/(R_{cell}+R_{med}) \times 100\%$ where R_{cell} is the radioactivity in the cell sample and R_{med} is the radioactivity in the medium sample.

3.7 Antigen binding capacity (ABC)

Bottles containing growing cells were emptied from medium and new complete medium were added so that the cells could be scraped from their growing surface (the cells were scraped instead of using trypsin-EDTA solution to preserve their receptors). Six million cells were transferred to three different falcon tubes. The tubes were centrifuged gently (5 min, 1k rpm) and all but approximately 1 ml of the supernatant was removed. This allowed the cells to be transferred to eppendorf tubes and further centrifugation (2.5 min, 5k rpm). The supernatant was completely removed and a 1 ml solution of ^{99m}Tc-$Z_{HER2:2395}$-C conjugate and medium (ratio Affibody® molecule to HER2 of 1:100) was added. After being carefully re-suspended the cells was incubated at 4°C during 4 h, under slight shaking. Centrifugation (10 min, 14k rpm), to allow the cells to form a pellet, was performed prior to the transfer of 0.5 ml

supernatant to new vials (B). The activity of (B), cells and remaining medium (A) was measured on a gamma counter, corrected for background and ABC was calculated as (A-B)/(A+B) × 100%.

3.8 In vitro plasma stability test

A volume of 100 µl mouse plasma was mixed with 2 µg ^{99m}Tc-labelled conjugate and incubated at 37°C during 2 h. A mixture of 100 µl PBS and 2 µg ^{99m}Tc-labelled conjugate was handled in the same way. As a control, free technetium pertechnetate was diluted to have the same activity as the other two samples. All three samples were further diluted using PBS (20 µl sample to 1 ml PBS) and controlled to have similar activity using a gamma counter. Diluted free technetium pertechnetate (20 µl), ^{99m}Tc-$Z_{HER2:2395}$-C conjugate incubated in mouse plasma (20 µl) and ^{99m}Tc-$Z_{HER2:2395}$-C incubated in PBS respectively (20 µl) were mixed with 20 µl 40 × LDS buffer. The three mixtures were incubated at 70°C during 10 min. The gel was set up in an Xcell Surelock Mini-Cell according to manufacturers instructions using 1×NuPage® running buffer in both upper and lower chamber (20 × MES buffer diluted 20 times with deionized water). A sample from each mixture (10 µl) were loaded onto the gel, run for approximately 25 min at 200 V and analysed on PhosphorImager.

3.9 Biodistribution in Naval Medical Research Institute (NMRI) Mice

The animal experiments were planned and performed in accordance with national legislation on laboratory animals' protection. The animal study plans have been approved by the local Ethics Committee for Animal Research in Uppsala. The study was aimed at study the biodistribution ^{99m}Tc labelled $Z_{HER2:2395}$-C using kit 75ZG5 and performed in four NMRI mice. The mice were injected in their tail vein with a 100 µl solution of labelled $Z_{HER2:2395}$-C. Labelling was performed as described above and diluted in PBS for a final solution of 1 µg $Z_{HER2:2395}$-C and an activity of 20 kBq. Four hours post injection (p.i.) the mice were euthanized with an intraperitoneal injection of ketamine HCl (Ketalar, Pfizer) and xylazine HCl (Rompun, Bayer) mixture. Blood, lungs, liver, spleen, stomach, kidneys, salivary gland, and a piece of muscle were collected in pre weighted plastic bottles, reweighted and measured on a gamma counter. For these samples percentage of injected activity per gram tissue (% IA/g) were calculated ((injected activity – activity in syringe – activity in the tail) / gram tissue) × 100%). The thyroid, intestine with content, tail and carcass were also collected and

measured on a gamma counter for these the activity were expressed as percentage injected activity per whole sample (% IA).

4. Results

4.1 Influence of concentration $SnCl_2$, Glucoheptonate and EDTA respectively on labelling yield

The kits concentration of $SnCl_2$, glucoheptonate and EDTA was varied one at a time, freeze-dried and analysed as described above.

For $SnCl_2$ (Figure 2) it is clearly seen that for concentrations between 50 µg/kit and 100 µg/kit the labelling yield is above 90% peaking at approximately 75 µg/kit. At these concentrations the formation of RHT was negligible. For higher concentrations of $SnCl_2$ there was a significant formation of RHT.

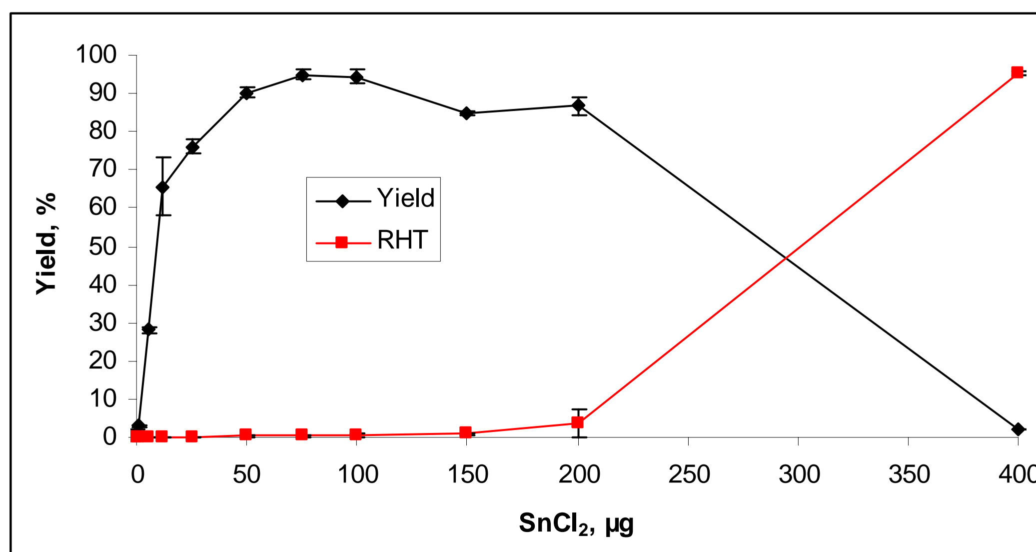

Figure 2. Labelling yield and RHT formation as a function of concentration $SnCl_2$. The concentrations of $SnCl_2$ as µg/kit are: 0 (n = 2), 0.5 (n = 2), 5 (n = 2), 12 (n = 2), 25 (n = 2), 50 (n = 4), 75 (n = 8), 100 (n = 6), 150 (n = 4), 200 (n = 4) and 400 (n = 2). Error bars represent one standard deviation.

For glucoheptonate concentrations between 2.5 mg/kit and 7.5 mg/kit the yield was stable above 90% peaking somewhere around 2.5 - 3.75 mg/kit (see Figure 3). The RHT formation was small if existent at these concentrations of glucoheponate. For lower concentrations of glucoheptonate a decrease in yield and an increase of RHT is evident.

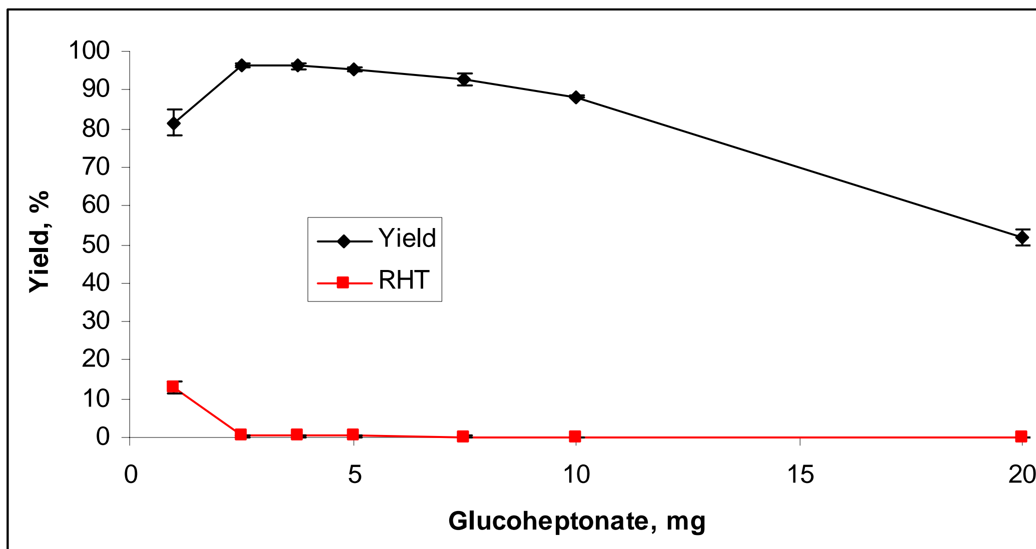

Figure 3. Labelling yield and RHT formation as a function of concentration glucoheptonate. The concentrations of glucoheptonate as mg/kit are: 1 (n = 2), 2.5 (n = 4), 3.75 (n = 9), 5 (n = 7), 7.5 (n = 2), 10 (n = 2) and 20 (n = 2). The error bars represent one standard deviation.

The result in labelling efficiency due to changes in EDTA concentration can be seen in figure 4. Between 25 µg/kit and 200 µg/kit the yield was above 90% with a plateau between 25 µg/kit and 100 µg/kit. There seems to be a small decrease (not significant) between 50 µg/kit and 25 µg/kit.

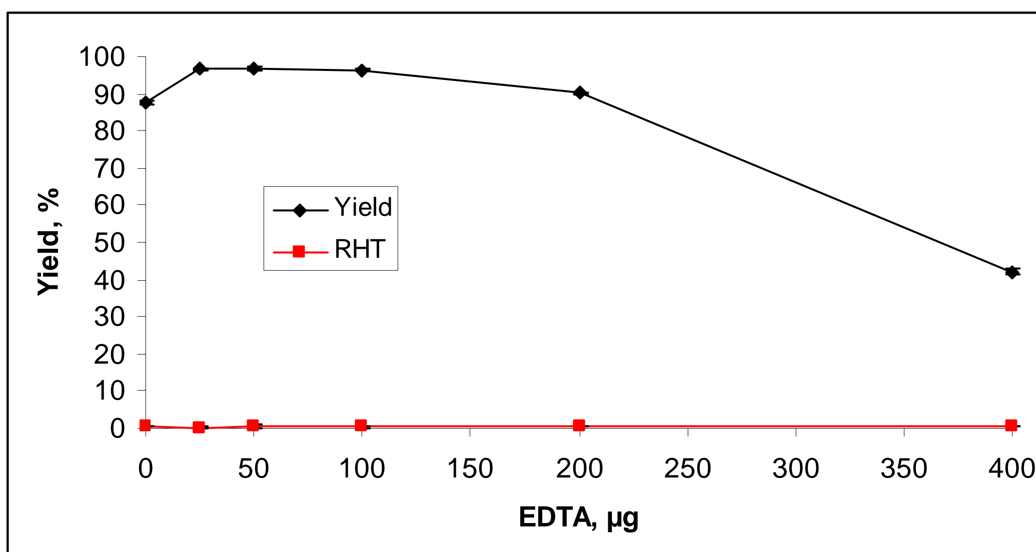

Figure 4. Labelling yield and RHT formation as a function of concentration EDTA. The concentrations of EDTA as µg/kit are: 0 (n = 2), 25 (n = 2), 50 (n = 5), 100 (n = 6), 200 (n = 2) and 400 (n = 2). The error bars represent one standard deviation.

4.2 Influence of protein concentration on labelling yield

The dependency of $Z_{HER2:2395}$-C concentration for the labelling yield was evaluated using kit S10 (see Table 1). This kit was chosen due to being the most optimised kit at the time of the experiment (i.e. after $SnCl_2$ was optimised but before glucoheptonate). As shown in Figure 5, there is a clear correlation between concentration $Z_{HER2:2395}$-C and yield. For concentrations higher than 100 µg $Z_{HER2:2395}$-C per kit increased concentration of protein has no major importance for the yield, evident from the beginning of a plateau.

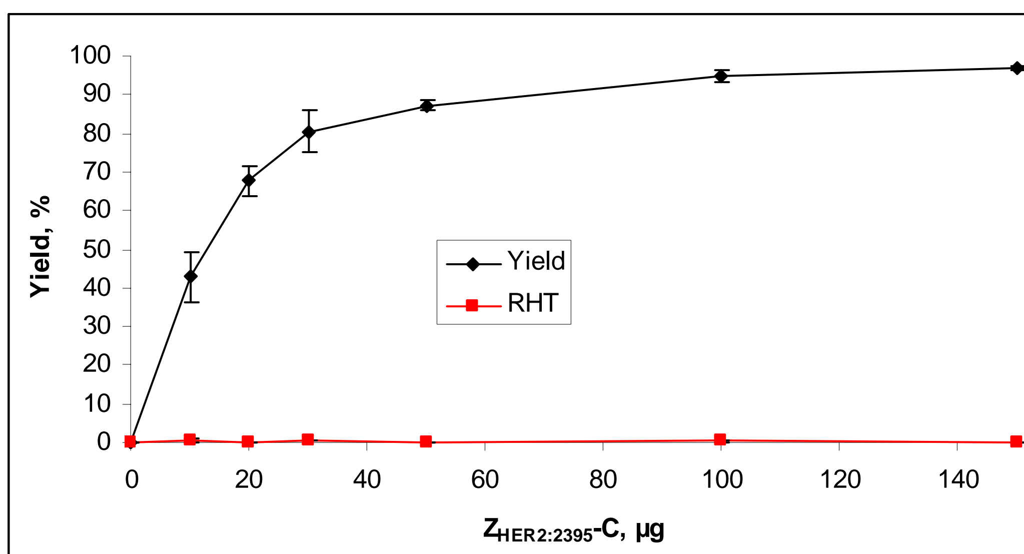

Figure 5. Labelling yield and RHT formation as a function of concentration of $Z_{HER2:2395}$-C concentration. The different concentrations of $Z_{HER2:2395}$-C as µg/kit are: 0 (n = 2), 10 (n = 2), 20 (n = 2), 30 (n = 2), 50 (n = 2), 100 (n = 6) and 150 (n = 2). The error bars represent one standard deviation.

4.3 Influence of pertechnetate volume on labelling yield

To evaluate the influence of perthechnetate volume on labelling yield kit S3 (see Table 1) was chosen. The choice of this kit is due to it being the kit that gave the highest yields at the time of experiment. Labelling with 100 µl, 500 µl and 1 ml technetium pertechnetate was made and results are shown in Figure 6. It can be seen that a five times increase in pertechnetate volume (compared to the 'normal' 100 µl) cause no decrease in yield. Increment by a factor 10 results in a small decrease in labelling efficiency. Labelling of kit 75ZG5 using 500 µl pertechnetate a labelling yield of 89.2 % and 2.6 % colloid formation (n = 1).

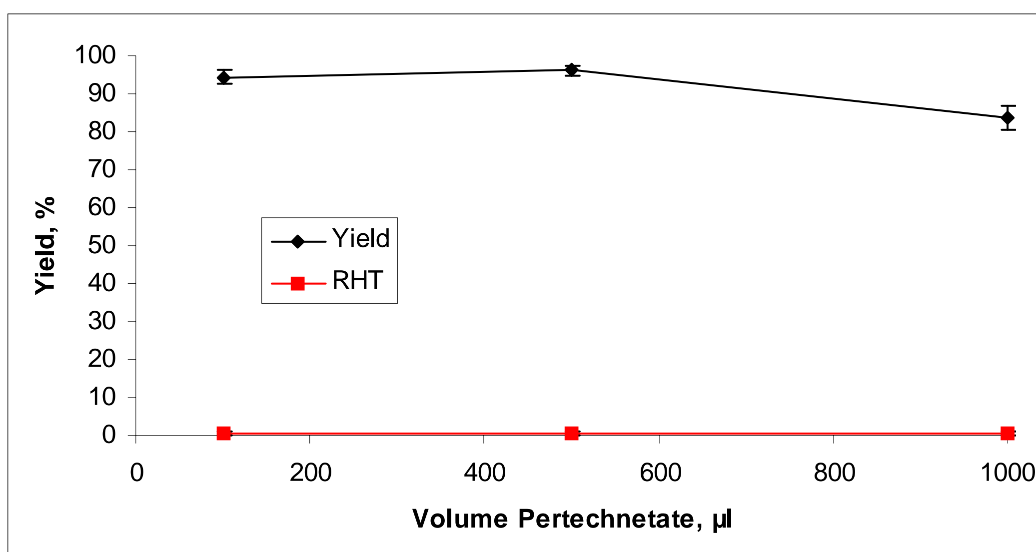

Figure 6. Labelling yield and RHT formation as a function of volume ^{99m}Tc pertechnetate. The different volumes of pertechnetate in µl are: 100 (n =6), 500 (n = 2) and 1000 (n = 2). The error bars represent one standard deviation.

4.4 One-vial kits

Results from the analysis of kit ZG5 shows a slight decrease yield (mean = 92.8 % ± 1.2, n=4) compared to its counterpart, without $Z_{HER2:2395}$-C pre added (kit G5, mean = 96.3 % ± 0.7, n = 6, three values excluded). Tests with pre-reduced protein (kit RG5, mean = 93.1 % ± 1.0, n = 4) indicate no difference compared to its counterpart kit with non pre-reduced protein (kit ZG5).

Decreasing the concentration of all components in the kit but $Z_{HER2:2395}$-C, with regard to kit ZG5, resulted in an increase of a few percentage units in labelling efficiency (mean = 96.4 % ± 1.7, n = 23, six values excluded).

Attempts were made with increased $Z_{HER2:2395}$-C concentrations, compared to kit ZG5, however no increase in yield could be seen. Adjustment in pH, using Na_2HPO_4 were also done, the results were however lower then corresponding kits with pH unadjusted.

4.5 Plasma stability test

In vitro stability test performed in mice plasma and PBS showed no indication of the Affibody® molecules forming dimers. Small amounts of free pertechnetate could be seen,

however the labelling yield for this particular labelling was 95.7 %, therefore small amounts of free pertechnetate is expected. Analyses of amount free pertechnetate after incubation in plasma and PBS respectively were not performed. This was due to insufficient separation between free pertechnetate and ^{99m}Tc-$Z_{HER2:2395}$-C conjugate due to high background.

4.6 In vitro specificity test

The test was performed on ^{99m}Tc-$Z_{HER2:2395}$-C conjugates made using three different kits S10, G5 and 75ZG5. The tests showed that when the HER2 where blocked using Affibody® molecule $Z_{HER2:342}$ only a low level of radioactivity where associated with the cells. When no blocking was pre-added, significantly higher levels of radioactivity could be seen associated to the cells (see Figure 7). From this it could be seen that the ^{99m}Tc-$Z_{HER2:2395}$-C conjugates still binds specific to the HER2.

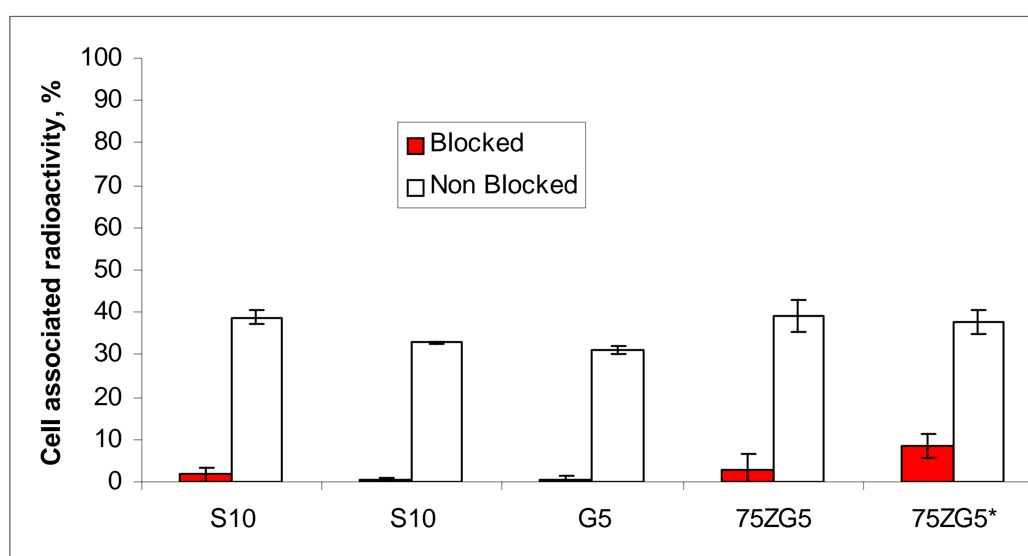

Figure 7. The figure shows percentage of ^{99m}Tc labelled $Z_{HER2:2395}$-C, using kit S10, G5 and 75ZG5, that is associated with the cell sample in this test, i.e. binds to HER2 on SKOV-3 cells. Open bars shows percentage for non-blocked cell association and red bars (shaded) shows percentage after treatment with a block, Affibody® molecule $Z_{HER2:342}$. All tests were performed in triplicates. The error bars indicate one standard deviation. The p-values, blocked compared to non-blocked, are (from left to right) 2.5×10^{-6}, 1.5×10^{-8}, 4.9×10^{-7}, 9.6×10^{-4}, 8.2×10^{-4}. *The uncharacteristically high values for the block are due to failure to add enough blocking solution to compensate for an unusual high number of cells.

4.7 Antigen binding competent test

ABC-tests for ^{99m}Tc labelled $Z_{HER2:2395}$-C, using kit G5, E1 and 75ZG5 respectively gave an average value of 71.3 % ± 1.4 (n = 3), 71.1 % ± 0.9 (n = 3) and 75.8 % ± 3.3 (n = 9) respectively, conjugate bound to HER2 versus free conjugate.

4.8 Kit stability over time

Labelling performed with kit 75ZG5 of various storage time showed no decrease in yield. The kit storage time stretches from 1 day to 28 days (see Figure 8).

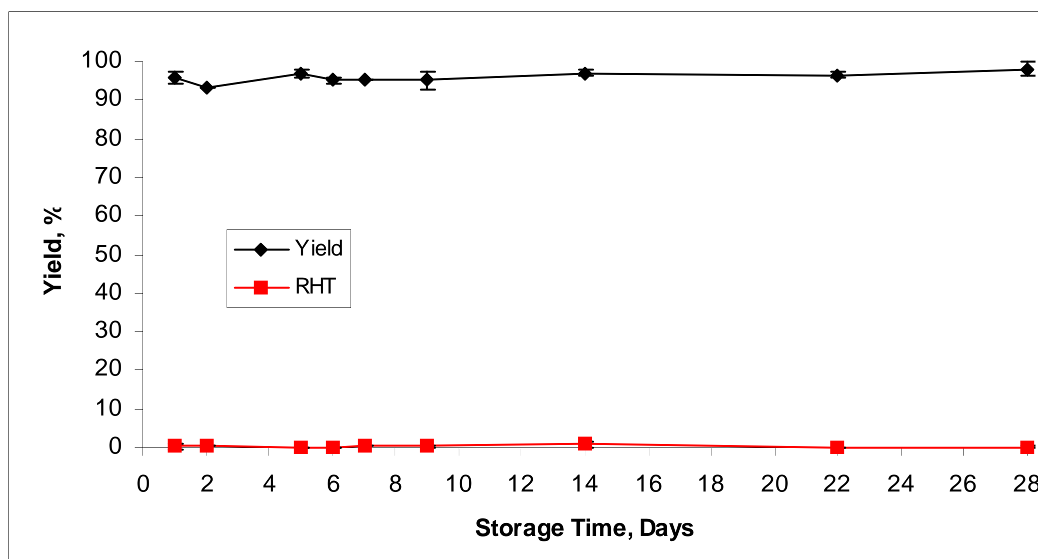

Figure 8a. Labelling yield and RHT formation as a function of time passed since kit 75ZG5 was prepared. The different time points, as days, are: 1 (n = 6), 2 (n = 1), 5 (n = 3), 6 (n = 2), 7 (n = 1), 9 (n = 4), 14 (n = 2), 22 (n = 2) and 28 (n = 2).

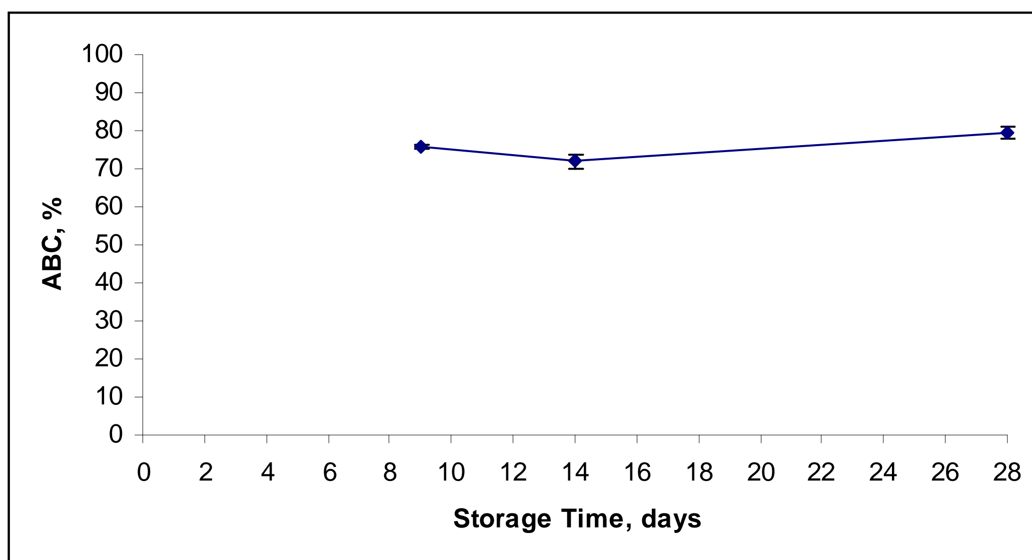

Figure 8b. ABC-test results as a function of time passed since kit 75ZG5 was prepared. All data points are triplicates. The error bars represent one standard deviation.

4.9 Biodistribution in NMRI mice

Summarization of the biodistribution 4 h p.i. of ^{99m}Tc-$Z_{HER2:2395}$-C conjugate, labelled using kit 75ZG5 can be seen in Figure 9. The biodistribution showed low uptake of radioactivity in

all normal tissues except the kidneys (114.7 ± 8.7 % IA/g). All other samples were below 1 % IA/g except the liver (1.2 ± 0.1 % IA/g). Intestine, carcass and tail were measured as whole sample. These data were in good agreement with data obtained earlier using two-vial kit (Ahlgren, unpublished).

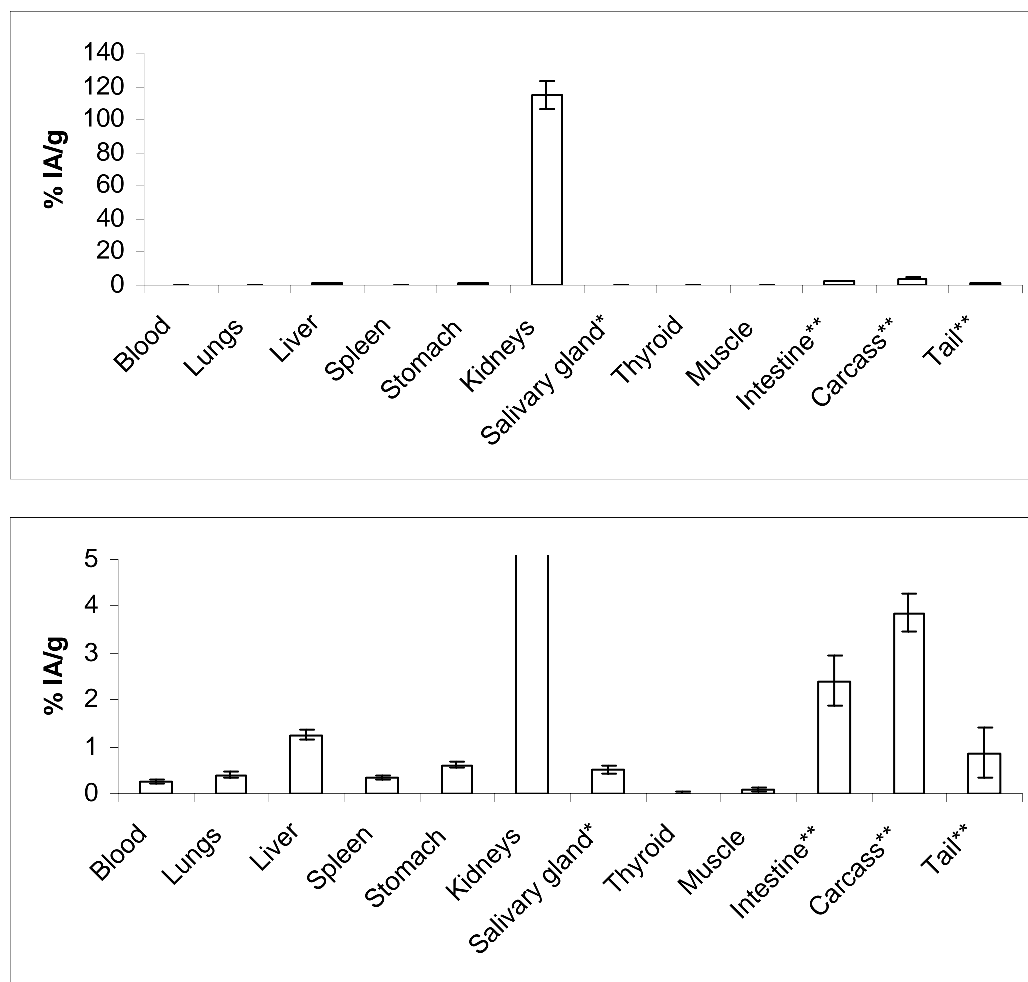

Figure 9. Biodistribution of ^{99m}Tc-$Z_{HER2:2395}$-C, labelled using kit 75ZG5, 4 h p.i. The bars represent an average of % IA/g ± one standard deviation (n = 4). *Data for salivary gland is from three animals. **For thyroid, carcass, tail and intestine the data represent % IA per whole sample. For intestine content is included.

5. Discussion

The results from the experiments to find an adequate concentration of $SnCl_2$, glucoheptonate and EDTA were relatively straightforward to interpret. Variation in the concentration of $SnCl_2$ showed a maximum yield at 75 μg/kit and relative robustness to variation. Therefore this concentration of $SnCl_2$ was chosen for variation of glucoheptonate.

Labelling efficiency due to concentration of glucoheptonate showed that there is a need to include it in the kit formulation, to avoid colloid formation. No definite point of best concentration could be seen in the graphs. The best concentration should however be close to 3.75 mg, so this concentration was chosen for further optimization of the kit. For this concentration the graph indicated a very robust kit formulation, concerning variation in glucoheptonate concentration. When calculating mean values for this concentration (kit G5) three values, from labelling done the same day, have been excluded due to abnormal variation both within themselves as well as with the other six remaining values. This might be due to an accumulation of ground-state technetium in the generator.

A similar situation, as with glucoheptonate, was seen when the concentration of EDTA was investigated. No clear point of best concentration could be deducted. However, the labelling yield should be very stable for concentrations around 100 μg/kit and this concentration was therefore chosen.

Variation in concentration of $Z_{HER2:2395}$-C (performed on kit S10) showed the expected increase in labelling yield with increased concentration $Z_{HER2:2395}$-C. For 100 μg protein per kit a plateau have more or less been reached, indicating that there is little to be gained from an increased concentration of $Z_{HER2:2395}$-C.

Increasing the volume of technetium pertechnetate when labelling, using kit 75ZG5, showed a decrease in yield. However, no major conclusion can be drawn since this test was only performed once.

For one-vial kits no PBS was used in the labelling procedure, simply because it was not necessary. The reason for the loss in labelling yield that is seen in kit ZG5 compared to kit G5

is unknown. Pre-reduction of protein before kit formulation did not increase the yield further. Therefore the following kits were done using non pre-reduced protein.

Decreasing the concentration of glucoheptonate, EDTA and $SnCl_2$ to 75 % compared to kit ZG5, while keeping the concentration of $Z_{HER2:2395}$-C constant, increased the labelling yield by a few percent units. For calculations of average values for this kit (75ZG5) six abnormal values have been excluded. These values all came from labelling performed with pertechnetate from one specific generator, while no other parameter had been changed. This lead to the conclusion that something generator associated affected the labelling, hence these values were excluded.

The in vitro specificity test only works as an indication whether the Affibody® molecule binds specifically to its receptor or not, no quantitative values can be obtained. Tests of ^{99m}Tc labelled $Z_{HER2:2395}$-C showed a binding percentage above 30% while pre-blocking of the receptor lead to a significant decrease of conjugate associated to the cells. From these values it can be seen that the $Z_{HER2:2395}$-C still binds specifically after labelling with ^{99m}Tc using kit S10, G5 and 75ZG5 respectively. The more quantitative ABC-test has shown good correlations with test previously described test the literature (Lindmo *et al.*, 1984). In the ABC-test, a ratio 100:1 of receptor to binder is used as an approximation of infinite excess of receptors. With the ABC-test it was shown that, 70 - 80 % of $Z_{HER2:2395}$-C binds to its receptor when labelled using either of the kits G5, E1 or 75ZG5. From these values it can be seen that $Z_{HER2:2395}$-C affinity towards HER2 is preserved at a high level using said kit formulations.

No tendencies towards a decreased labelling yield or loss of affinity were seen, as the kit was stored longer. However, 28 days is not a long time and measurements with kits stored for a longer period should be performed.

The biodistribution study showed good correlation with previously published data, where Affibody® molecules have been labelled with radioactivity (Ahlgren *et al.*, 2008; Orlova *et al.*, 2007). Low levels of radioactivity were seen in blood, in agreement with in vitro tests. This suggests that the conjugate is stable with respect to transchelation i.e. the exchange of technetium from its cheleate to transferrin. An accumulation in stomach, salivary gland and thyroid would suggest that free pertechnetate had been released, however the level of

radioactivity in these organs are low. No indication of colloid formation was evident considering the low level of radioactivity seen in the liver, spleen and lungs.

The high kidney uptake is due to the small size of the Affibody® molecule, leading to renal excretion followed by tubular reabsorption (Behr *et al*., 1998). From an imaging perspective this accumulation should be acceptable since breast cancer metastases do not allocate to the kidneys. The activity needed for imaging should allow the renal uptake to be within an acceptable level for the patient.

Low levels of activity were seen in all samples but the kidneys. This should allow for a high contrast image of HER2 positive tumours, since the kidneys have a well-defined form. This is under the assumption that $Z_{HER2:2395}$-C high affinity for HER2, seen in the ABC-tests, is preserved in vivo after labelling.

6. Conclusions

A single vial kit for labelling of $Z_{HER2:2395}$-C with ^{99m}Tc was optimised. A composition of 2.8 mg glucoheptonate, 75 µg EDTA, 56 µg $SnCl_2$ and 100 µg $Z_{HER2:2395}$-C gave a yield of 96.4 % ± 1.7 and RHT content of 0.35 ± 0.45. The conjugate, ^{99m}Tc-$Z_{HER2:2395}$-C, labelled using said kit formulation, was shown to conserve the $Z_{HER2:2395}$-C specificity (p value = 9.6×10^{-4} and 8.2×10^{-4} respectively) and affinity (75.8 % ± 3.3) to the HER2. The conjugate was stable against tranchelations and no decrease in labelling yield or affinity could be seen over 28 days.

7. Further Studies

Verifications that the kit formulation produce stable labelling yields over a longer time span are needed. The batch-to-batch variation must also be investigated for this kit formulation to be of interest in hospital pharmacy. Furthermore, conformations that the affinity seen on SKOV-3 cells is conserved in vivo are necessary.

If this one-vial kit formulation passes these tests it should be of interest for diagnosis of HER2-status in hospital pharmacy. A one-vial formulation promises ease of use and minimize human errors, allowing it to be used by untrained personnel.

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