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Antiangiogenic Human Monoclonal Antibody Ramucirumab Radiolabelling: *In Vitro* Evaluation on VEGFR2-positive Cell Lines

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Abstract. *Background/Aim: Radiolabelling of monoclonal antibodies (mAbs) could be beneficial in cancer diagnosis and therapy, however it may cause structural changes and consequently deteriorate their immunoreactivity. Materials and Methods: The therapeutic mAb ramucirumab (RAM) was technetium-99m labelled using either a direct or an indirect method with the use of two bifunctional chelating agents (HYNIC, DTPA). The radiochemical purity was assessed using instant thin-layer chromatography (ITLC) and high-performance liquid chromatography (HPLC) technique. The affinity of radiolabelled RAM was tested on human cancer cell lines. Results: The radiolabelling provided the following stable compounds: [^{99m}Tc]RAM, [^{99m}Tc]HYNIC-RAM and [^{99m}Tc]DTPA-RAM. Their radiochemical purity was over 95%. All prepared radiopharmaceuticals showed moderate affinity to the targeted receptor, in vitro. However, their affinity was one order lower compared to that of the natural mAb. Moreover, directly and DTPA-radiolabelled RAM demonstrated less favourable binding kinetics. Conclusion: Radiolabelling negatively affected the affinity of RAM to its targeted receptor.*

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Cancer incidence and mortality are rapidly increasing worldwide. There were about 18.1 million new cancer cases and 9.6 million cancer deaths in 2018 (1). Chemotherapy, radiation therapy and surgical treatment are still irreplaceable methods of cancer treatment, but they are usually damaging to the healthy cells and tissues. Over the past few decades, intense research was headed towards the development of potentially more effective and safer biologically-targeted therapeutics for the disease. A promising strategy for treating malignancies that exploits their biological phenotype is targeted *in situ* radiotherapy (2). In this approach, mAbs that specifically recognize and bind to tumour-growth factor receptors, are used as vehicles to selectively deliver radionuclides to cancer cells for *in situ* radiation therapy (3, 4). All currently clinically used therapeutic mAbs are immunoglobulin G monoclonal antibodies (5).

During the preparation of the mAb bearing the therapeutic radionuclide, chemical changes are usually made in the mAb and its structure is exposed to non-physiological conditions (*e.g.* pH alteration, redox reaction). This study focused on the susceptibility of a model antiangiogenic mAb to the conjugation conditions and the potential changes this caused to its immunoreactivity.

Angiogenesis is driven by a complex signalling system, which includes signalling molecules and their specific receptors. Vascular endothelial growth factors (VEGFs) are an important group of signalling molecules involved in angiogenesis. The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF). An isoform named VEGF165, has been found to be overexpressed in some human tumours, which are characterized by high invasiveness and metastatic activity (6).

VEGFs act *via* binding to transmembrane vascular endothelial growth factor receptors (VEGFRs) that are divided into the three subtypes, VEGFR1, VEGFR2, and VEGFR3 (7). VEGFR2 is the main receptor involved in VEGF-A, VEGF-C and VEGF-D induced signalling. VEGFR2 is expressed in almost all adult vascular endothelial cells (8). It plays a role in vasculogenesis, angiogenesis, endothelial cell migration, survival, proliferation and in increasing vascular permeability (9). VEGFR2 is considered as the primary VEGF family receptor driving angiogenesis (10). Increased expression of VEGFR2 has been found in various tumours - *e.g.* gastric and oesophageal cancer, non-small cell lung cancer, metastatic colorectal cancer, and metastatic breast cancer (11).

Ramucirumab (RAM) is a recombinant human IgG1 mAb (12), which binds VEGFR2 with an IC_{50} of 1-2 nM (13). Binding of RAM to VEGFR2 inhibits binding of VEGF-A possibly by both the steric blockade of the ligand, as well as the induction of a conformational change in the receptor (14). RAM was the first mAb targeting VEGFR2 to be used in phase III clinical trials, in which it rendered modest survival benefit (10, 15). The FDA has approved its use for metastatic gastric or gastro-esophageal junction carcinoma with paclitaxel in the second-line treatment and for metastatic colorectal cancer in combination with FOLFIRI (irinotecan, 5-FU and folinic acid) in 2014 (12).

In this study, RAM was radiolabelled with technetium-99m using three different conditions to assess the susceptibility of the mAb to the changes implemented during the radiolabelling process. RAM was radiolabelled either directly following reduction of the disulphide bonds of the mAb, or indirectly with the use of the bifunctional chelating agents HYNIC and DTPA. The prepared radiolabelled products were assessed on their affinity to the cell surface VEGFR2 using two *in vitro* techniques.

Materials and Methods

Cell cultures and reagents. The VEGFR2 expressing human Caucasian prostate adenocarcinoma PC-3 cell line (CLR 1435; ATCC, Rocksville, MA, USA) and human Caucasian ovarian adenocarcinoma SKOV3 (HTB-77; ATCC) cells were used in this study (16, 17). VEGFR2 expression was verified using western blot analysis (data-not-shown). F-12 Kaighn's modification (GE Healthcare Life Science, Chicago, IL, USA) for PC-3 cells, and McCoy's 5A cell culture media (Sigma-Aldrich, St. Louis, MI, USA) for SKOV3 cells were used, supplemented with 10% foetal calf serum (Sigma-Aldrich). The cells were grown at 37˚C in an incubator with a humidified atmosphere and 5% CO_2. Cells intended for the real-time automated affinity analysis were seeded in a local area (approximately 5 cm^2 large) near the edge of a 10 cm Petri dish (Nuclon, dish size 100*20; NUNC A/S, Roskilde, Denmark) as described previously (18) in the quantity of 1×10^6 cells per dish.

Monoclonal antibody ramucirumab. The anti-VEGFR2 mAb ramucirumab (Eli Lilly, IN, USA) was obtained in its commercially available form (Cyramza, 10 mg/ml) as an infusion solution. Before radiolabelling, the ramucirumab solution was purified on a NAP-5 column (GE Healthcare, Waukesha, WI, USA) equilibrated with PBS (pH 7.4, 140 mmol/l NaCl) for direct and indirect labelling using HYNIC or $NaHCO_3$ (coupling buffer; 0.2 M, pH 9, metal-free) for indirect labelling using DTPA. The mAb was then radiolabelled directly or indirectly (HYNIC and DTPA) as described below.

Direct radiolabelling of ramucirumab with technetium-99m. The human mAb ramucirumab was directly labelled with ^{99m}Tc using 2-mercaptoethanol (2-ME) as the reducing agent. Direct radiolabelling followed the previously published protocol (19) with some modifications. Briefly, the disulphide bridges in the RAM molecule were reduced with 2-ME (Sigma-Aldrich) at a molar ratio 1:2,000 (RAM and 2-ME respectively) for 30 min at room temperature with continuous stirring. The reduced mAb was purified on a NAP-5 column. The reduced and purified mAb ramucirumab (100 μg) was labelled with Tc 99m [20 MBq, [^{99m}Tc]TcO_4^- eluted from a Mo-99/Tc-99m generator, (GE Healthcare Life Science, IL, USA)] in the presence of $SnCl_2$ (4 μg) as the technetium reducing agent. No purification of the prepared radioconjugate [^{99m}Tc]ramucirumab was necessary.

The technetium 99m labelling of ramucirumab using the bifunctional chelating agent HYNIC. The mAb ramucirumab was conjugated with the bifunctional chelating agent HYNIC and then radiolabelled according to a previously published protocol (20). Ramucirumab was conjugated with Succinimidyl hydrazinium nicotinate hydrochloride (NHS-HYNIC; Synchem UG & Co. KG, Germany) in the molar ratio 1:5 at room temperature in the dark for 2.5 h while gently stirring. The unconjugated fraction of NHS-HYNIC was removed from the mAb by gravity elution on a NAP-5 column. One hundred μg of NHS-HYNIC conjugated ramucirumab (HYNIC-RAM) were labelled with Tc 99m (20 MBq) in the presence of SnCl2 (4 μg) and tricine (0.5 mg) as co-ligand at 37˚C in the dark for 30 min. Following incubation, $MgCl_2$ (4.5 μg) was added to stabilize the prepared radioimmunocomplex (20).

Technetium 99m labelling of ramucirumab using the bifunctional chelating agent DTPA. All procedures were carried out in metal-free conditions and according to a previously published protocol with some modifications (21). Ramucirumab was conjugated with DTPA in the molar ratio 1:100 in coupling buffer $NaHCO_3$ (0.05 M, pH 9.0) for 2.5 h at room temperature and then overnight at 4˚C. The reaction mixture was then purified on a VIVASPIN 6 (Sartorius) to remove unconjugated DTPA molecules with a NH_4OAc (0.15 M, pH 6.9) solution.

The prepared DTPA-ramucirumab (100 μg) was labelled with Tc-99m (20 MBq) in the presence of $SnCl_2$ (4 μg) at room temperature in the dark for 30 min. Following incubation, the radioimmuno-conjugate was purified on a NAP-5 column.

Quantification of the number of bifunctional chelating agents bound to the monoclonal antibody. The DTPA/mAb ratio was determined using a modified version of the commonly used Arsenazo(III) (A(III)) assay (22-24). A working solution of A(III) was prepared using 0.155 mg of A(III), 10 μl of 1 mg/ml standard copper atomic

absorption solution and 5 ml of metal-free 0.15 M ammonium acetate, pH 7.0 (all from Sigma-Aldrich); hereafter this solution will be referred as the "Cu reagent solution". The Cu reagent solution was stored at room temperature in the dark to avoid degradation over time. Standard concentration solutions of DTPA chelating agent were prepared in the conjugation buffer (1 M $NaHCO_3$, pH 9.0). Aliquots (10 μl) of standard chelating agent concentrations and modified antibody samples were mixed with 190 μl of Cu reagent solution, incubated for 30 min at 37˚C and the absorbance was measured at 630 nm. The assay was performed in 96-well plates. The final DTPA/antibody ratio was calculated as the ratio of DTPA concentration (μM) to the antibody concentration (μM), as determined by a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

A modified spectroscopic method (25) was used to determine the HYNIC/mAb ratio after its conjugation to the antibody molecule. Briefly, the hydrazine content in the molecule of the modified antibody was analysed after reaction with o-sulfonic benzaldehyde (Sigma-Aldrich). Standard concentrations of the HYNIC chelating agent were prepared in the conjugation buffer (PBS, pH 7.4). Aliquots (10 μl) of the modified antibody and standard chelating agent concentrations were incubated with 100 μl of the o-sulfonic benzaldehyde solution (1 mg/ml, 0.1 M sodium acetate, pH 4.7) at room temperature overnight in the dark. The absorption of the hydrazone adduct was measured at 343 nm. The antibody concentration after the conjugation was determined using the BCA protein assay kit (Pierce Biotechnology). The assay was performed in 96-well microtiter plates. The HYNIC/antibody ratio was calculated as the ratio of HYNIC concentration (μM) to the antibody concentration (μM).

Radiochemical purity control. The prepared radiopharmaceuticals [^{99m}Tc]ramucirumab, [^{99m}Tc]DTPA-ramucirumab and [^{99m}Tc]HYNIC-ramucirumab were analyzed for their radiochemical purity with the use of two standard methods. Instant thin-layer chromatography (ITLC) was made on silica gel coated glass fibre plates (Varian, USA), and a saline solution (sodium chloride 0.9 % solution, pH 7.4) for [^{99m}Tc]ramucirumab and citrate buffer (0.1 M, pH 5) for [^{99m}Tc]DTPA-ramucirumab and [^{99m}Tc]HYNIC ramucirumab were employed as mobile phases. The ITLC chromatographic strips were analysed by a Rita Star analyser (Raytest, Straubenhardt, Germany) shortly after radiolabelling (0 h) and after a 24 h incubation at 4˚C. In both systems the radiolabelled antibody stays at the origin whilst common impurities – free Tc-99m, [^{99m}Tc][tricine]$_2$ complex or technetium-99m chelating complexes move with the front of the mobile phase. [^{99m}Tc]TcO_2 colloid does not migrate with the mobile phase, thus cannot be distinguished from the antibody using ITLC.

High-performance liquid chromatography (HPLC) (HPLC system Agilent 1100 Series, Agilent Technologies Inc., Santa Clara, CA, USA) analysis was performed using PBS (pH 6.8) as the mobile phase (flow rate 1.0 ml/min) and the BioRad Gel column BIO-Sil Sec 250, 300×7.8 mm for the separation with concomitant radiometric detection. The analysis of the prepared radiopharmaceuticals was carried out after their radiolabelling (0 h) and following 24-h incubation at 4˚C. The radiochromatograms were compared to UV/VIS chromatograms of the non-conjugated mAb to verify the antibody position.

Real-time automated affinity analysis. The *in vitro* affinity analysis of the prepared radiopharmaceuticals was carried out with the employment of the LigandTracer Yellow instrument (Ridgeview Instruments AB, Vänge, Sweden) as previously described (18). Briefly, the affinity analysis was carried out in a Petri dish with cells seeded in a local area of approximately 5 cm^2. The dish was placed on a tilted rotating platform. Radioactive signal coming either from the area with cells or the reference area without cells (negative control) was detected with a scintillation detector placed above the highest point of the rotating platform in the instrument lid. The measurement was recorded by the software LigandTracer Control 2.2.2 (Ridgeview Instruments AB), which showed the binding curve for radioligand – receptor interaction in a real-time manner.

The measurements were obtained according to a previously published protocol (26), following targeting of the VEGFR2 expressed on the surface of the human cancer cell lines PC-3 and SKOV3 by the radiolabelled ligands. To prevent the potential nonspecific binding of the mAb, the cells were pre-incubated in Krebs Ringer bicarbonate buffer containing 1% BSA for 1 h at 37˚C. The association phase of the measurement consisted of two consecutive radioligand additions to the final concentrations of 3 and 9 nM in 3 ml of Krebs-Ringer bicarbonate buffer (Sigma-Aldrich). Following the association phase, the buffer was changed with a fresh one (3 ml) without the radiopharmaceutical and the dissociation phase measurements were performed. All measurements proceeded at 37˚C, in an incubator without a humidified atmosphere. The resulting data were evaluated using the TraceDrawer software (Ridgeview Instruments AB, Vänge, Sweden).

The manual saturation study. The manual saturation study was performed to test the affinity of the differently radiolabelled ramucirumab to VEGFR2 expressed on the surface of PC-3 and SKOV3 cells according to a previously described protocol with minor modifications (27). The cells were seeded in a 24 well plate in a 1×10^5 cells per well density and kept at 37˚C under a humidified atmosphere with 5% CO_2 for two days. On the third day, the cells were washed with PBS and Krebs-Ringer bicarbonate buffer was added (0.5 ml per well) with the tested radioligand at a specific concentration in order to assess specific binding (SB). In order to assess non-specific binding (NSB), unlabelled RAM (1 μM) was used. Overall, six concentrations of labelled RAM (3, 9, 15, 30, 45 and 90 nM) in triplicates for SB assessment were prepared. The incubation time of cells with radiopharmaceuticals was 2 h at 37˚C. Following incubation, cells were lysed, homogenized and analysed for receptor binding/internalization activity with the gamma counter (2480 Wizard2™, PerkinElmer, Waltham, MA, USA). Cell protein concentration in the lysed samples was analysed using BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) according to the previously described protocol (28). The cell bound activity was calculated per the amount of cellular protein for each concentration in triplicate, and the data were analyzed in GraphPad Prism 7 and statistically evaluated in MS Office Excel.

Results

The radiochemical purity analysis of the prepared radioimmunoconjugates. The radiochemical purity of the prepared radiopharmaceuticals was assessed with the employment of ITLC or HPLC. The analysis was performed at 0 and 24 h (data not shown) after radiolabelling to produce [^{99m}Tc]RAM, [^{99m}Tc]DTPA-RAM and [^{99m}Tc]HYNIC-RAM.

All radiopharmaceuticals were of high purity, which was over 95%, as it is demonstrated in the ITLC representative radiochromatograms in Figure 1. The main peak, on the left of the chromatograms, corresponded to technetium labelled RAM, and the small peak, on the right, corresponded to the negligible presence of free Tc-99m. The direct technetium 99m labelling was characterized by the highest presence of free Tc 99m compared to all other preparations (Figure 1a). The numerical values of the assessed radiochemical purity are summarized in Table I.

Table I. *Radiochemical purity (%) of technetium-99m labeled RAM as arithmetic mean value (n=3) with SD (in brackets) measured at 0 h and 24 h after radiolabelling.*

Time (h)	[^{99m}Tc]RAM	[^{99m}Tc]DTPA-RAM	[^{99m}Tc]HYNIC-RAM
0	96.27 (2.96)	99.51 (0.28)	99.27 (0.93)
24	95.92 (3.16)	99.12 (0.12)	98.78 (1.29)

Similar results on the radiochemical purity control and stability up to 24 h were obtained using HPLC, as shown in Figure 2. The peak of the radiolabelled RAM at the radiochromatogram had a retention time of about 7.5 min. Figure 3 depicts an HPLC UV/VIS representative chromatogram of unlabelled RAM with a 7.5 min retention time. The position of the native RAM in the UV/VIS chromatogram corresponded to the position of the prepared radiopharmaceuticals depicted in the radiochromatograms of Figure 2.

The quantification of the number of bifunctional chelating agents bound to the monoclonal antibody. To plot a standard curve relating DTPA concentration to Cu reagent solution absorbance, the range of DTPA concentrations (0-500 μM) were used and the linear range was found in the generated standard curve. Using the standard curve equation, the amount of DTPA chelating agent available for the metal binding in the conjugated antibody was directly calculated. The unconjugated antibody was used as a negative control. After the conjugation of ramucirumab using the DTPA-chelating agent in three independent experiments, the mean DTPA/mAb ratio was found to be 7.5±0.7.

The standard curve relating the hydrazine adduct absorption to the HYNIC concentration was constructed using standard HYNIC concentration solutions (0-1,000 μM) and in the tested concentration range the relationship was linear. Using the standard curve equation, the HYNIC concentration presented in the modified antibody solution was calculated. The unmodified antibody was used as a negative control. In the three independent experiments, the mean HYNIC/mAb ratio was 6.1±0.8.

Real-time automated affinity analysis. The affinity of all prepared radiopharmaceuticals to VEGFR2 expressed on the surface of the human cancer cell lines PC-3 and SKOV3 was tested with the employment of the real-time automated affinity analysis, which provided the binding curves of the radioligand to the receptor in a real-time measurement. The curves were further analyzed using the radioligand equilibrium dissociation constant (KD) to find out the affinity of the radioligand to its target receptor. All measurements provided binding curves and the corresponding KD values are summarized in Table II. The directly ([^{99m}Tc]RAM) and indirectly ([^{99m}Tc]DTPA-RAM) labelled mAb exhibited increased binding to the plastic surface. However, this NSB was eliminated following a 1-h pre-treatment with Krebs-Ringer bicarbonate buffer containing 1% BSA. The [^{99m}Tc]HYNIC-RAM did not exhibit increased NSB to the plastic surface before BSA pre-treatment and the KD values before and after the pre-treatment did not differ. Moreover, [^{99m}Tc]RAM and [^{99m}Tc]DTPA-RAM exhibited the less steep slope of the association curve which suggests a slower receptor/ligand interaction relatively to [^{99m}Tc]HYNIC-RAM. Similarly, the faster decrease in the dissociation curve of the [^{99m}Tc]RAM and [^{99m}Tc]DTPA-RAM indicates the weaker receptor/ligand binding. The representative runs of [^{99m}Tc]RAM, [^{99m}Tc]HYNIC-RAM and [^{99m}Tc]DTPA-RAM obtained using PC-3 and SKOV3 cells are depicted in Figure 4.

The manual saturation study. The manual saturation study, which was carried out using human cancer cells expressing VEGFR2 at their surface, rendered the information about the affinity of the prepared radiopharmaceuticals. The saturation study could not provide any information about the changes in binding kinetics as in the case of real-time analysis. However, the KD values obtained with this method corresponded to the ones obtained with the real-time automated affinity analysis. The KD values obtained using the manual saturation technique are presented in Table II.

Discussion

Although cancer therapy is the main goal of anti-cancer research, the development of diagnostic tools, which could enable imaging of cancer processes is equally important. To accomplish this goal, mAbs are chemically modified with the employment of non physiological conditions that could potentially alter their immunoreactivity. Therefore, we tested the immunoreactivity of the mAb RAM radiolabelled with the SPECT radionuclide Tc-99m.

Technetium-99m radiolabelling of RAM was performed using three methods. The first method included direct labelling following disulphide bond reduction while the other methods included radiolabelling *via* the bifunctional chelating agents DTPA or HYNIC. Although, all methods led

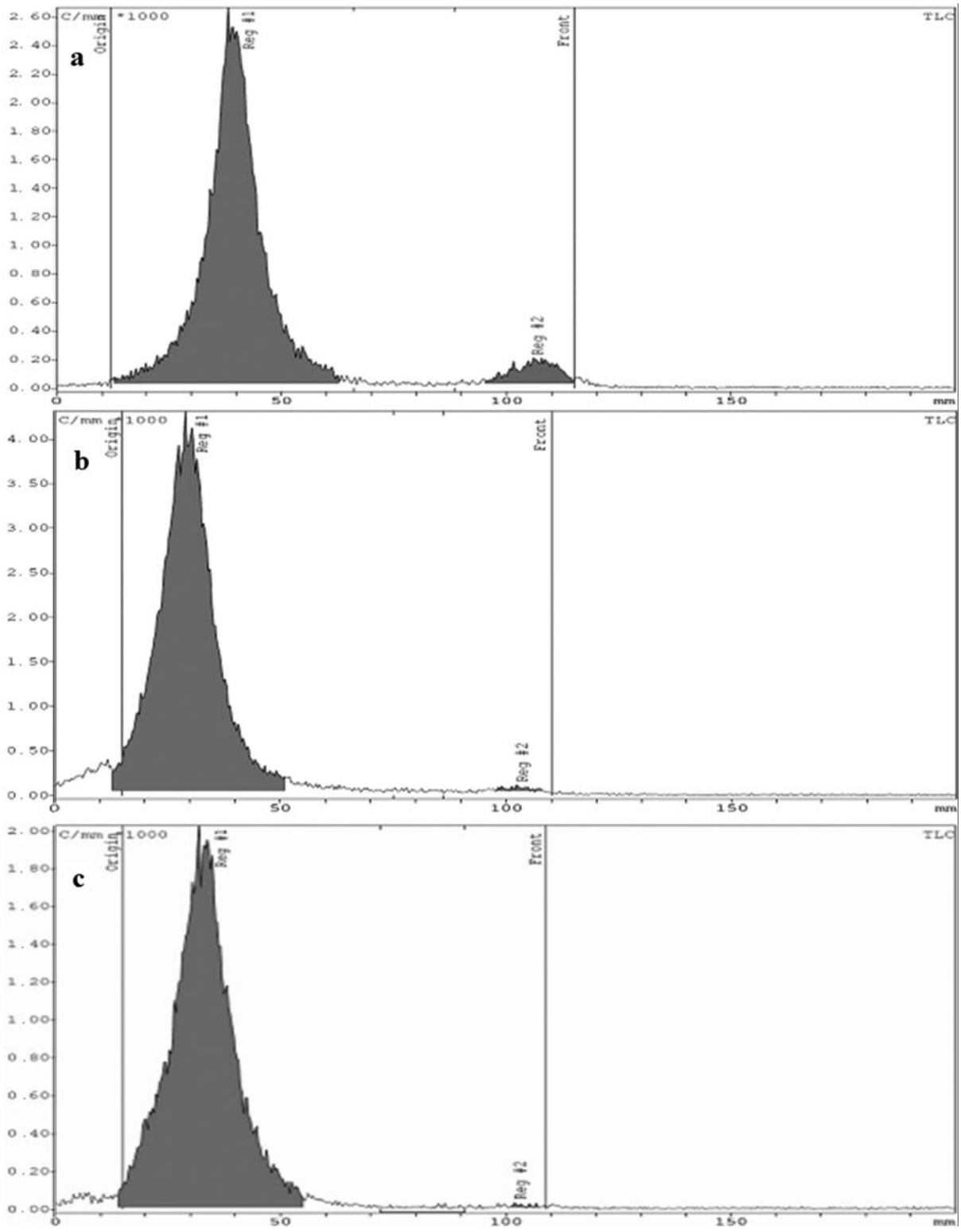

Figure 1. *Representative chromatograms of radiochemical purity analysis assessed with ITLC. The large peak at the start represents the radiolabelled mAb ramucirumab. The small peak at the end is radiochemical impurity (free Tc-99m). The purity analysis at 0 h after radiolabelling for [^{99m}Tc]RAM, [^{99m}Tc]DTPA-RAM and [^{99m}Tc]HYNIC-RAM is demonstrated in figures a, b and c respectively. The radiochemical purity for the all radiopreparations was over 95%.*

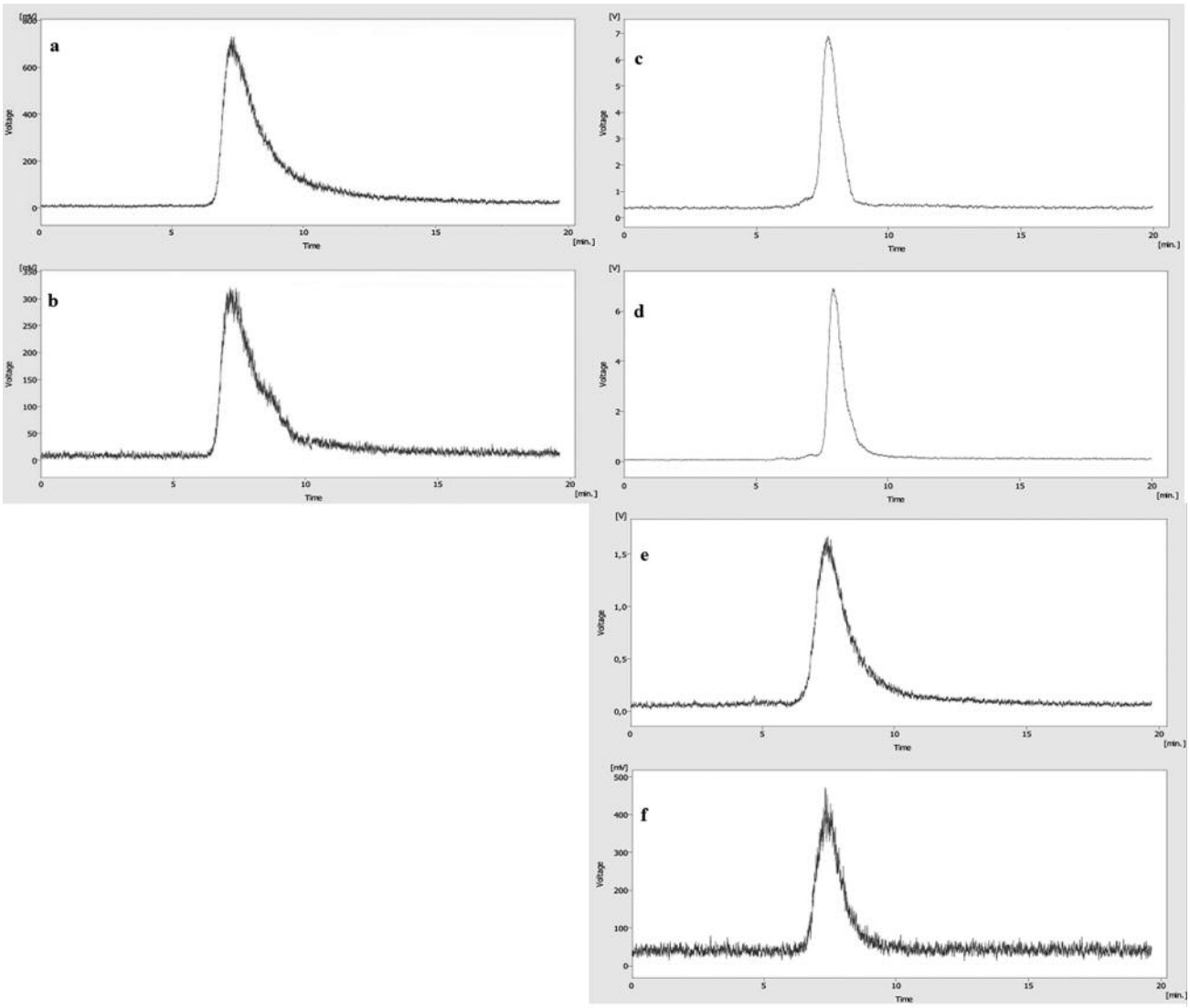

Figure 2. *Representative radiochromatograms of HPLC analysis of the radiolabelling. The peak corresponds to radiolabelled mAb ramucirumab, which was eluted at about 7.5 min. The analysis was carried out at 0 h (Figures 2a, 2c and 2e) and 24 h (2b, 2d and 2f) after radiolabelling for [^{99m}Tc]RAM, [^{99m}Tc]DTPA-RAM and [^{99m}Tc]HYNIC-RAM respectively.*

to the preparation of stable radiopharmaceuticals with over 95% radiochemical purity, the direct and DTPA-mediated labelling were accompanied by a slightly worse binding of the mAb to VEGFR2.

The affinities of the various radiopharmaceuticals to VEGFR2 did not differ from each other significantly, as summarized in Table II, but there were still some distinct small differences. [^{99m}Tc]HYNIC-RAM had higher affinity, as represented by the lower KD values compared to [^{99m}Tc]RAM and [^{99m}Tc]DTPA-RAM. The lower binding ability of [^{99m}Tc]RAM and [^{99m}Tc]DTPA-RAM is also seen in Figure 4. The slope of the association phase curve of these radiochemicals was less steep than that of [^{99m}Tc]HYNIC-RAM, which points to their slower interaction with VEGFR2, particularly seen in the association phase for the second radioligand addition (9 nM).

In addition, the pattern of the slope in the dissociation phase (no free radioactive ligand is included in the incubation media) provides information about the strength of the binding between a ligand and its receptor (Figure 4). In general, a very slow decrease in the dissociation phase curve, indicates strong binding between a ligand and a receptor, and *vice versa*. A faster decrease in the dissociation phase curve was regularly observed for

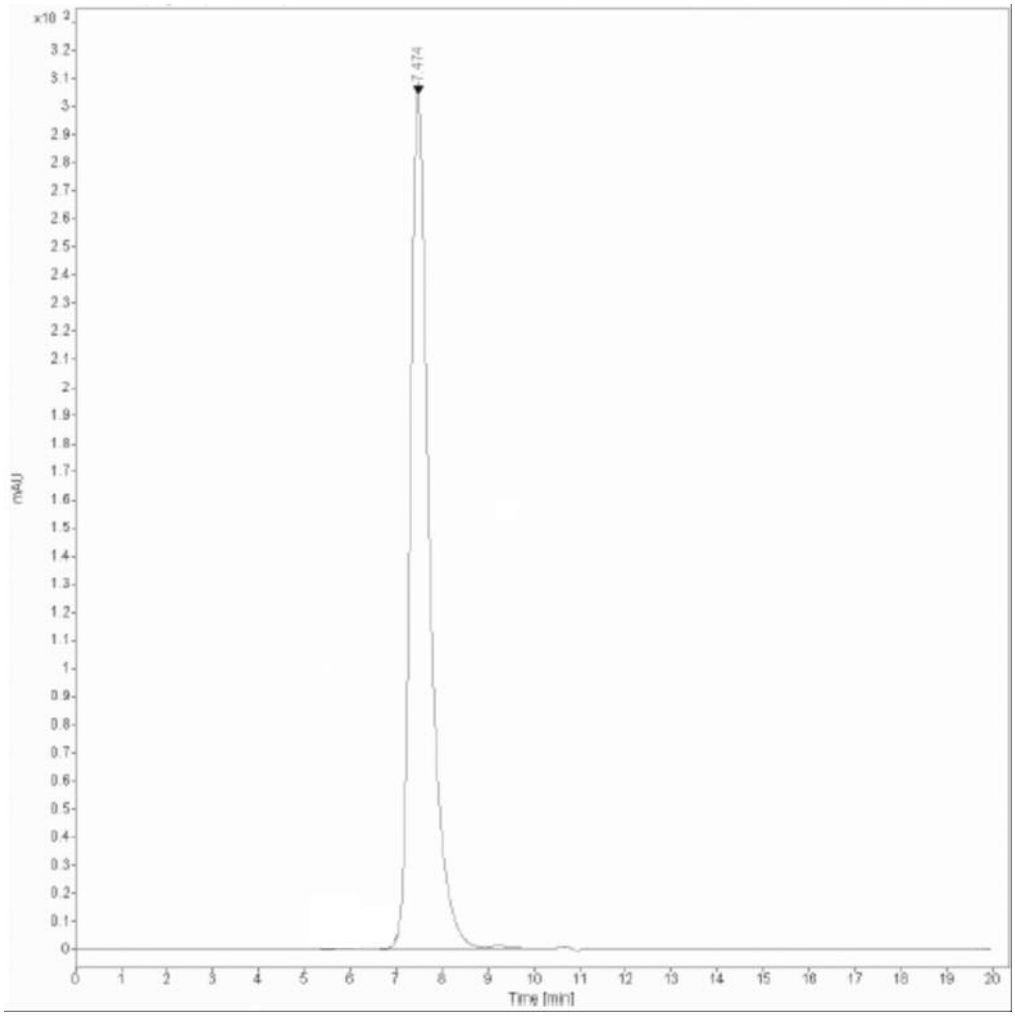

Figure 3. *The representative HPLC UV/VIS chromatogram of unlabeled mAb ramucirumab with elution time 7.5 min at mobile phase flow 1 ml/min.*

Table II. *The affinity values (nM) of [^{99m}Tc]ramucirumab, [^{99m}Tc]DTPA ramucirumab and [^{99m}Tc]HYNIC ramucirumab to VEGFR2 expressed on PC-3 and SKOV3 cells assessed by the real-time automated affinity analysis (RT) and the manual saturation technique (MT). The affinity of the radioligand is presented as the equilibrium dissociation constant (KD). The KD values were calculated in the TracerDrawer software (the automatic method) and GraphPad Prism (the manual technique) and are presented as the arithmetic mean value (n=3) with SD (in brackets).*

Cell line	[^{99m}Tc]RAM		[^{99m}Tc]DTPA-RAM		[^{99m}Tc]HYNIC-RAM	
	RT	MT	RT	MT	RT	MT
PC-3	31.5 (5.3)	30.6 (1.0)	19.5 (5.4)	16.8 (1.9)	14.0 (6.1)	19.6 (5.4)
SKOV3	24.3 (1.1)	28.2 (1.1)	19.8 (0.7)	25.2 (2.3)	17.8 (1.2)	17.0 (2.3)

[^{99m}Tc]RAM and also for [^{99m}Tc]DTPA-RAM (SKOV3 cells curve). In conclusion, the binding of these radiopharmaceuticals to VEGFR2 was weaker than that of [^{99m}Tc]HYNIC-RAM.

The results of the manual saturation technique for the analysis of the binding of the radiopharmaceuticals to VEGFR2 were in accordance with those obtained using the real time automated affinity assay. Taken together, the results

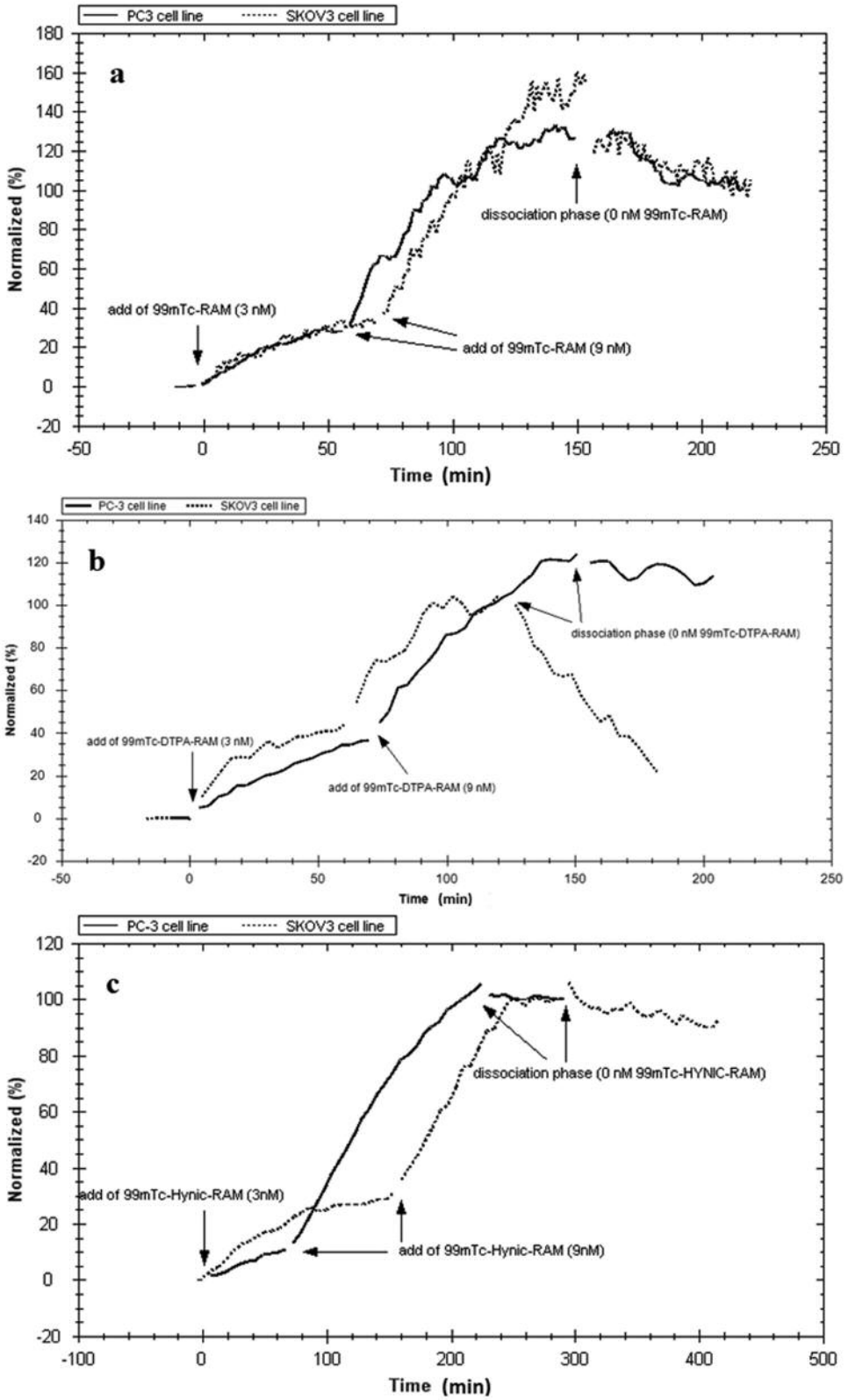

Figure 4. *The representative examples of real-time radioimmunoassay binding interaction runs between either [^{99m}Tc]RAM (4a) or [^{99m}Tc]DTPA-RAM (4b) or [^{99m}Tc]HYNIC-RAM (4c) and VEGFR2 expressed on the cell surface. The association phase consisted of the two additions (3 and 9 nM) of RAM. The dissociation phase proceeded with no added RAM. The full line is for PC-3 cells and the dashed line is for SKOV3 cells.*

from both affinity techniques proved the assumption that technetium-99m labelling reduces that binding affinity of the mAb. The natural RAM equilibrium dissociation constant value was one order lower than those of the tested radiopharmaceuticals. This has been probably caused by either the conjugation of the chelating agent or the radiolabelling processes (13). In the case of direct labelling, the conditions of disulphide bond reduction might have been responsible for the reduction in the affinity. Apparently, the harsh environment of mAb modification negatively influenced its structure and decreased its binding affinity. The change of pH to a more alkaline environment in the coupling buffer could be potentially responsible for the negative influence on the [^{99m}Tc]DTPA-RAM molecule, since loss of immunoreactivity after exposure to the alkaline pH has been observed (29). In addition, binding of the chelating agent to mAb could also have a negative effect on its binding affinity. Too intense derivatization of the mAb may lead to the loss of immunoreactivity (30). However, in our case both [^{99m}Tc]DTPA-RAM and [^{99m}Tc]HYNIC-RAM carried approximately seven chelator molecules per mAb. In another study, five molecules of the macrocyclic chelator were conjugated to the mAb with no apparent loss of immunoreactivity (31). Therefore, the difference in the binding kinetics of the indirectly labelled mAb is likely due to the different pH during the conjugation process.

As previously published (32), the affinity of a specific ligand for a receptor expressed on the surface of different cell lines might differ. In this *in vitro* study, two Caucasian cell lines of different origin (prostate and ovarian) carrying surface VEGFR2, were used. The culture conditions, the number of the seeded cells, the conditions of the real-time automated affinity analysis technique and the manual saturation technique protocol were identical for both cell lines. Therefore, the obtained small differences in KD values among these cells may derive from the levels of the expression of the receptor at the cell surface or the presence of other cell surface molecules that influence the interaction process between the tested ligand and its receptor as has previously been reported (32).

In vitro experiments can result in discrepancies due to the cells and the chosen conditions. In this study, *in vitro* binding was examined at 37˚C in order to assess binding at a physiological temperature. But the physiological processes at this temperature can influence the result of the affinity studies (33). The possible internalization of VEGFR2 bound radioligand and the following receptor degradation or recycling process at 37˚C could have influenced the calculated affinity constants. However, analysis of [^{99m}Tc]HYNIC-RAM and [^{99m}Tc]RAM binding to VEGFR2 expressed on either SKOV3 or PC-3 at 4˚C (data not shown), resulted in KD values similar to those obtained at 37˚C.

Conclusion

This study tested the influence of the radiolabelling process on the affinity of ramucirumab to VEGFR2. Direct ([^{99m}Tc]RAM) and indirect radiolabelling *via* chelating agents ([^{99m}Tc]DTPA-RAM and [^{99m}Tc]HYNIC-RAM) were successfully performed. The radiopharmaceuticals' affinity was one order lower than that of the natural mAb, while the specificity of the binding was preserved. The half-life of technetium-99m was 6 h and did not match the half-life of the mAb *in vivo*, but the *in vivo* application of these radiopreparations was not the purpose of this study.

Conflicts of Interest

The Authors have no conflict of interest to declare regarding this study.

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