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Separation and purification of no-carrier-added arsenic from bulk amounts of germanium for use in radiopharmaceutical labelling

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Summary. Radioarsenic labelled radiopharmaceuticals could add special features to molecular imaging with positron emission tomography (PET). For example the long physical half-lives of ^{72}As ($T_{1/2} = 26$ h) and ^{74}As ($T_{1/2} = 17.8$ d) in conjunction with their high positron branching rates of 88% and 29%, respectively, allow the investigation of slow physiological or metabolical processes, like the enrichment and biodistribution of monoclonal antibodies in tumour tissue or the characterization of stem cell trafficking. A method for separation and purification of no-carrier-added (nca) arsenic from irradiated metallic germanium targets based on distillation and anion exchange is developed. It finally converts the arsenic into an $^{+}$As(III) synthon in PBS buffer and pH 7 suitable for labelling of proteins *via* As−S bond formations. The method delivers radioarsenic in high purity with separation factors of 10^6 from germanium and an overall yield from target to labelling synthon of $> 40\%$. In a proof-of-principle experiment, the monoclonal antibody Bevacizumab, directed against the human VEGF receptor, was labelled with a radiochemical yield $> 90\%$ within 1 h at room temperature with nca $^{72/74/77}$As.

1. Introduction

1.1 Arsenic

The element arsenic is well known as the favourite poison of the Savellis, the Borgias and Agatha Christie [1]. There were also speculations that Napoleon Bonaparte died because of arsenic poisoning [2]. Because arsenic is viewed as synonymous with toxicity it is still used today for homicides or suicides [3]. A worldwide problem is the local high pollution of natural water with arsenic under natural conditions or as a result of additional human pollution [4]. Therefore the removal of arsenic from drinking water is an important challenge. Despite its toxic properties arsenic was used 2000 years ago by Greek and Chinese healers as therapeutic agent to treat everything from syphilis to cancer [1]. More recently, the use of arsenic trioxide (ATO) in European medicine increased after the invention of Fowler's solution which was used for a number of systemic illnesses from 18th to 20th century. Over the last 100 years, the concerns about toxicity and potential carcinogenicity of arsenic administration have declined its use for medical application [5]. At the end of the last century it was found that ATO has very good therapeutic characteristics for treatment of acute promyelocytic leukaemia (APL) and clinical trials have been confirmed all around the world [6, 7]. Some years ago ATO was approved by the U.S. Food and Drug Administration (FDA) as Trisenox® for this indication [8].

Another application of arsenic radionuclides might be its use as radioactive probe in sub-toxic trace amounts for biological or medical purposes. The tracer concept of radiopharmaceutical chemistry allows the application of no-carrier-added (nca) amounts of radioactive isotopes (*e.g.* of arsenic) that are used for labelling of interesting biological carriers like monoclonal antibodies (mab) and the imaging of their biological behaviour *in vivo*. The element arsenic provides a range of isotopes for non-invasive PET imaging like ^{72}As ($T_{1/2} = 26$ h; 88% β^+), and ^{74}As ($T_{1/2} = 17.8$ d; 29% β^+). These isotopes are produced by (p, n)-reactions on natural or isotopically enriched targets of elemental germanium or germanium(IV) oxide and are therefore available in nca state. Additionally, the β^- emitter ^{77}As ($T_{1/2} = 38.8$ h; 100% β^-) is produced by bombardment of the same target materials with neutrons, followed by β^- decay of ^{77}Ge ($T_{1/2} = 11.3$ h; 100% β^-).

1.2 Separations

Various methods for the separation of germanium and arsenic have been reported so far. A collection of separating procedures for germanium and arsenic from fission products is given elsewhere [9–11]. The majority of these methods is based on distillation of germanium as $GeCl_4$ followed by distillation of arsenic as $AsCl_3$. Other methods were based on solvent extraction or precipitation [12] of one of the two elements. Most of these methods fail for radiopharmaceutical application because arsenic carrier was used, which is not in common with the tracer concept. A review on the chemical behaviour of radio germanium is given by Mirzadeh and Lambrecht [13],

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involving separation techniques for radioarsenic. Separation of As(III) from antimony and bismuth was achieved in 8 M HCl and extraction into benzene [14]. A systematic study on separation of As(III) and Ge(IV) in HCl and HI systems by liquid–liquid extraction into several solvents was carried out by Brink *et al.* [15]. An easy separation of arsenic from germanium was achieved by solvent extraction of arsenic from sulphuric acid solutions after addition of KI into toluene [16, 17]. Another strategy for the separation of As from GeO_2 in HCl is the oxidation of As to As(V) and extraction of the Ge into organic solvents [18–20]. After the separation the As(V) is reduced again to As(III) and extracted into organic solvents. Tolmachev and Lundqvist [21] processed a germanium(IV)dioxide target after proton bombardment by dry distillation followed by wet chemistry workup for additional purification. The low target loss of less than 1% was mentioned as far as a further target recovery is not needed. Some methods based on distillation of $GeCl_4$ are reported whereas arsenic was kept in the non-volatile form of As(V) [22] followed by solvent extraction [23, 24], cation exchange [25] or anion exchange [26]. Schindewolf and Irvine [27] absorbed Ge and Ga from diluted HF solutions on anion exchange resin AG1X8 while As(III) was not absorbed. Some more data about the behaviour of As(V) in HF media [28] and mixtures of HF/HNO_3 media [29] on AG1X8 are available although data for Ga differ in [27] and [28]. The behaviour of Ge, As(III) and As(V) in HCl media on AG1X8 is very well known from literature [30–32] and some data about the absorption in HCl/acetone media are available [33]. Ge and As(III) are strongly absorbed at high HCl concentrations and can easily be separated from As(V) which shows only slight absorption. Korkisch and Feik [34] used the same resin, but HCl/acetic acid medium for separation of Ge and As(III)/As(V). The behaviour of arsenic on cation exchange resin is determined by very low absorption coefficients in HCl and $HClO_4$ media [35]. A slightly higher absorption was found in HCl/acetone media [36] and in HBr media [37]. Jennewein *et al.* [38] developed a separation method for GeO_2 targets based on solid phase extraction after dissolving the target in HF_{conc}. The radioarsenic was fixed on a BOND ELUT ENV cartridge after treatment with NaI while the GeF_6^{2-} formed in HF passed through the column without interaction. After drying the cartridge with nitrogen gas the radioarsenic was eluted with ethanol. This method so far is the only one that leads to a radioarsenic labelled mab [39] that was used for tumour imaging with PET *in vivo* [40].

There are also some interesting approaches that are far-out the normal standard techniques. Maki and Murakami [41] developed a separation technique for ^{77}Ge, ^{77}As(III) and ^{77}As(V) by TLC with and without the addition of arsenic carrier. Caletka and Kotas [42] developed a separation technique based on the absorption of germanium in 8 M HCl or other mineral acids on silica gel columns. Under these conditions elements like gallium, arsenic and zinc are eluted from the column with 8 M HCl whereas germanium stayed on the column.

1.3 Targetry

Preliminary tests on the production of arsenic radioisotopes using GeO_2-targets at high neutron flux density reactors and charged particle beams at cyclotrons showed for long term irradiations of 1 week (reactors) or high proton beam currents (more than 2 μA) that, following dissolution in HF_{conc}, appreciable amounts of the target material was transformed in insoluble compounds as a consequence of thermal stress and radiolysis. This insoluble compound was identified as elemental germanium. However, aiming for medical application of arsenic radioisotopes, high neutron fluxes or high proton beam currents need to be applied on the targets for high production yields. Therefore, instead of GeO_2, elemental germanium of natural composition was chosen as target material.

1.4 Approach

The aim of this work thus was to set up a procedure for separation of nca amounts of arsenic radioisotopes from macroscopic amounts (100–200 mg) of irradiated metallic germanium. The desired high purity of the final radioarsenic fraction was intended by crude separation of germanium by distillation of $GeCl_4$ followed by anion exchange chromatography to remove the remaining trace amounts of germanium. Additionally, a separation of radioactive contaminants (gallium and zinc radionuclides formed during neutron bombardment [43], namely $^{72/73}$Ga and ^{69m}Zn) was considered. In cyclotron irradiations, the major contaminant was found to be ^{67}Ga. The separation of those contaminants is very important as far as a radioarsenic based tracer for possible human is intended. Thus radioarsenic use must be available in highest radiochemical purity. The combination of a distillation technique with an anion exchange column has been used in a similar setup before [26]. This method led to a high purity *As(V) fraction in 10 M HCl not feasible for labelling of biomolecules. The challenge for the labelling of mab is thus first to reduce the *As(V) to *As(III) and then to remove the 10 M HCl since mab labelling requires a pH of around 7. For aspired *in vivo* experiments, the final product needs to be concentrated to low volumes of about 500 μl or less.

2. Materials and methods

2.1 Materials

If the radioarsenic is used for labelling chemistry of proteins in nanomolar concentrations, care must be taken that the addition of cold arsenic carrier in used chemicals is minimized. This is also the when the radiolabeled product is used in a clinical environment. Therefore all chemicals were purchased in the highest purity available, *e.g.* suprapure acids. Elemental germanium (99.999%) was purchased from Goodfellow as a plate of $50 \times 50 \times 0.5$ mm^3 and laser-cut into discs of 9.9 mm diameter (204 mg each) and used for cyclotron irradiations. For reactor irradiations, elemental germanium (99.9999%) was purchased from Chempur in small pieces ranging from 100–200 mg. Concentrated nitric acid (65%, suprapur), hydrochloric acid (30%, suprapur) and EDTA (disodium salt, biological grade) were purchased from VWR. CuCl (99.995+%) and chloroform (analytical grade) were purchased from Sigma Aldrich. Anion exchange resin AG1X8 (200–400 mesh) was purchased from

BioRad. PBS buffer, TCEP (tris(2-carboxyethyl)phosphine) and hydroxylamine hydrochloride were purchased from Pierce.

2.2 Proteins

The monoclonal antibody Bevacizumab was chosen as a model protein for the demonstration of the ability of this method to provide radioarsenic in a form suitable for labelling of SH group bearing molecules. The Bevacizumab was purchased from LaRoche at a concentration of 25 mg/ml. Bevacizumab is directed against the VEGF receptor and used as anti cancer agent in human therapy [44, 45]. Each labelling experiment was performed by using 50 µl (corresponding to 1.25 mg) of the original antibody solution. The solution was filled up with 450 µl of PBS buffer and added to the radioarsenic solution.

2.3 Irradiations

The positron emitters ^{72}As and ^{74}As were produced simultaneously *via* (p, n)-reactions on ^{nat}Ge targets at the German Cancer Research Center (DKFZ) cyclotron MC32NI in Heidelberg. Cross sections for the production of those positron emitting arsenic isotopes were measured by Spahn *et al.* [46]. Detailed decay properties of arsenic isotopes can be found elsewhere [38, 47, 48]. For the irradiations, germanium discs were placed into an aluminium container and covered with a 50 µm Havar foil. The beam current was up to 30 µA with 15 MeV protons and irradiation time ranged between 0.5 to 5 h. Integrated loading was up to 100 µA h. This setup showed no destruction of target material. Ge targets of natural composition led to the formation of ^{70,71,72,73,74,76}As *via* (p, xn) reactions. The major isotope after 1 d cooling time was ^{72}As. If the target was allowed to cool for 1 week, the major isotope was ^{74}As with small contaminations of $^{71/73}$As. In a cyclotron irradiated target any Ge isotopes formed are not useful for γ-ray spectroscopy due to undesirable low cross sections. The system ^{77}Ge/^{77}As provides a unique option for detection of both elements simultaneously. The nca isotope ^{77}As ($T_{1/2} = 38.8$ h; 100% β^-) was produced by (n, γ) reaction on ^{nat}Ge target in a nuclear reactor. It was used for optimization of the chemical separation procedure, determination of yields and for the optimization of labelling chemistry. Detailed cross sections for nuclear reactions of neutrons on germanium can be found elsewhere [43]. The production of ^{77}As was carried out by irradiation of 100–200 mg elemental germanium pieces for 6 h at a neutron flux density of 4.2×10^{12} n cm^{-2} s^{-1} at the TRIGA Mark II reactor of the Institute of Nuclear Chemistry, Mainz. The target was allowed to cool for 18 h to form ^{77}As from decay of ^{77}Ge and used directly for separation. Therefore the separation factors have been measured in this system and the same procedure was then applied to the positron emitting, cyclotron produced arsenic isotopes.

2.4 Radiochemical separation

2.4.1 Target dissolution

The germanium target (100–200 mg) was placed in a quartz distillation apparatus and 4 ml of *aqua regia* were added. The apparatus was heated to 120 °C and during this time the irradiated elemental germanium dissolved.

2.4.2 Distillation of $GeCl_4$

After complete dissolution of the target the temperature was maintained at 120 °C for distillation of $GeCl_4$. For acceleration of this process a stream of argon was bubbled through the solution. Over a period of 1.5 h additional 6 ml of 10 M HCl were added. The $GeCl_4$ was trapped in an ice cooled vessel containing 20% H_2SO_4. After complete clearance the solution was condensed to less than 500 µl.

2.4.3 Anion exchange

The distillation solution was filled to 500 µl with 10 M HCl. The solution was transferred onto an anion exchange column (3×100 mm, AG1X8) in the chloride form and eluted with 500 µl fractions of 10 M HCl. Arsenic *As(V) was eluted in the fractions 2 and 3. After 10 fractions the eluent was switched to 0.1 M HCl for removal of gallium, germanium and zinc radionuclides.

2.4.4 Reduction of As(V) to As(III), extraction into CCl_4 and back extraction into PBS-buffer

Fractions 2 and 3 were combined (1 ml solution) and mixed with 50 mg CuCl. The mixture was heated at 60 °C for different periods ranging from 5 to 120 min, with 60 min finally applied for the batch experiments. The As(III) was extracted twice with 500 µl CCl_4. Combined CCl_4 fractions were extracted with 500 µl PBS-buffer containing 25 mM EDTA and 0.5 M hydroxylamine.

2.4.5 Speciation of As(III) and As(V)

The oxidation state of the radioarsenic in the CCl_4 and the PBS fraction was determined by radio TLC. 0.5 µl of each fraction was spotted on a Si-60 silca plate (Merck, Germany) and developed with 0.01 M sodium tartrate/methanol 3 : 1. TLC plates were analyzed using an Instant Imager from Packard.

2.4.6 Determination of radiochemical purity and radiochemical separation yield

The radiochemical purity and separation yield were determined by γ-ray spectroscopy. The activity of the undissolved Ge target and of 500 µl aliquots of the solution after each step were measured in the same geometry and compared quantitatively. The γ-ray spectroscopy was performed using an Ortec HPGe detector system and Canberra Genie 2000 software. For the ^{77}Ge/^{77}As-system, the two nuclides were determined by their most intense γ-lines of 239 keV (1.6%) for ^{77}As and 264 keV (53.9%) for ^{77}Ge. Since ^{77}Ge has a much higher gamma emission rate compared to ^{77}As it is clearly visible in the target spectrum but could not be detected in the purified fraction. Care was taken that the dead time of the detector remained always below 10%. The irradiated target was measured in 1 m distance to the detector for 15 min, whereas the purified fraction was measured for 12 h directly on the surface of the detector. All data were

normalized to the time point of the first acquisition by the acquisition software. The detector was calibrated for efficiency at all positions with the certified standard solution QCY48, R6/50/38 from Amersham.

2.4.7 Labelling of Bevacizumab

The 500 µl of the purified radioarsenic solution in the PBS fraction was combined with 500 µl of Bevacizumab solution (1.25 mg, 8 nmol). 10 µl of TCEP (10 mg/ml, 420 nmol) were added and the mixture was allowed to stand at room temperature for 1 h. At various time points aliquots were taken for analysis and detection of labelling yields *via* gel filtration HPLC (Waters 1525 HPLC-system equipped with 2489 UV-detector and Berthold LB 509 radio flow detector, HiTrap desalting column from GE Healthcare, eluent: 0.9% NaCl solution) or radio TLC (Si-60, Merck, eluent: 0.01 M sodium tartrate/methanol 3 : 1).

3. Results and discussion

3.1 Irradiations

Under the chosen conditions, the neutron irradiations at the TRIGA Mark II reactor Mainz resulted in about 4 MBq ^{77}As and about 2 MBq ^{77}Ge at beginning of target workup (18 h EOB) for a 150 mg Ge target. The ^{72}Ga ($T_{1/2} = 14.1$ h) and ^{69m}Zn ($T_{1/2} = 13.9$ h) were produced in low yields of about 4 kBq and 1 kBq, respectively, due to low cross sections for fast the neutron reactions ^{72}Ge$(n, p)^{72}$Ga and ^{72}Ge$(n, \alpha)^{69m}$Zn. The production yield for the positron emitting isotopes of arsenic under the chosen conditions (15 MeV protons) was about 1 MBq/µA h for ^{74}As and 6 MBq/µA h for ^{72}As. The ^{67}Ga ($T_{1/2} = 78.3$ h) was produced by the ^{70}Ge$(p, \alpha)^{67}$Ga reaction (about 35 kBq/µA h).

3.2 Target dissolution

Although elemental germanium is slowly attacked by *aqua regia* [13] it was chosen as solvent. The nitric acid oxidizes the Ge(0) to Ge(IV) which is precipitated as GeO_2 in the HCl. The dilution of the target accelerates with increasing temperature. Under these oxidative conditions the nca radioarsenic is also oxidized to *As(V). The subsequent distillation requires about 120 °C, the germanium is dissolved within 30 min which are required for heating the solution. Dissolution of elemental germanium target results in a milky solution of germanium(IV) oxide.

3.3 Distillation of $GeCl_4$

After dilution of the target the temperature is maintained at 120 °C to distil the $GeCl_4$ (b.p. = 84 °C). Additionally a slight stream of argon was bubbled through the solution to accelerate the distillation. Under these conditions the radioarsenic stays in oxidation state *As(V) which is not volatile. In contrast to other publications [9], no other oxidizing reagent (except the initial amount of nitric acid) was added to keep the arsenic in the pentavalent oxidation state. During 1.5 h distillation period a total volume of 6 ml 10 M HCl was added periodically for distillation of $GeCl_4$ and destruction of the remaining nitric acid. Larger amounts of nitric acid will interfere in later reduction of *As(V) to *As(III) and lead to low extraction yields. Finally the solution is condensed to less than 500 µl and taken out of the distillation apparatus. An average loss of arsenic of 10% was observed in this step. It is basically attributed to absorption on the walls of the glassware. In this step an average separation factor of germanium of 2×10^4 was achieved. Care should be taken to avoid condensation of $GeCl_4$ on the surface of the vessel which might give some additional contamination of the solution with germanium. The non-arsenic radionuclides produced during irradiation at cyclotron and reactor (namely $^{67/72}$Ga, ^{69m}Zn) cannot be removed by distillation and remain inside the arsenic solution.

3.4 Anion exchange purification

According to the behaviour of arsenic, germanium, zinc and gallium on anion exchange resin AG1X8 in HCl [30–32], *As(V) is only slightly retained compared to Ge, Zn and Ga at high HCl concentrations. Consequently, 10 M HCl was chosen to elute *As(V) from the column. The *As(V) is eluted in fraction two and three, containing > 90% of arsenic activity in 1 ml of 10 M HCl. Ge, Zn and Ga are only eluted after changing the eluent to 0.1 M HCl. A typical elution profile for *As(V), *Zn(II) and *Ga(III) is given in Fig. 1. However, according to [30] Ge is eluted at HCl concentrations < 5 M and is strongly retained at 10 M HCl with a distribution coefficient of about 200 compared to 4 of As(V). For determination of the final radionuclidic purity an aliquot of the combined fractions two and three was measured for 12 h at the closest position of the detector. No ^{77}Ge activity could be observed. As the detection limit for this setup was determined to be 0.25 Bq ^{77}Ge and the experiment started from 2 MBq of ^{77}Ge, an overall separation factor for the anion exchange column of ^{77}Ge/^{77}As of > 10^6 is deduced.

3.5 Reduction of As(V) to As(III), extraction into CCl_4 and back extraction into PBS-buffer

Batch experiments showed that both NaI and CuCl are suitable reducing agents for *As(V) to *As(III). In the case of NaI with nca activities of *As always re-oxidation to *As(V)

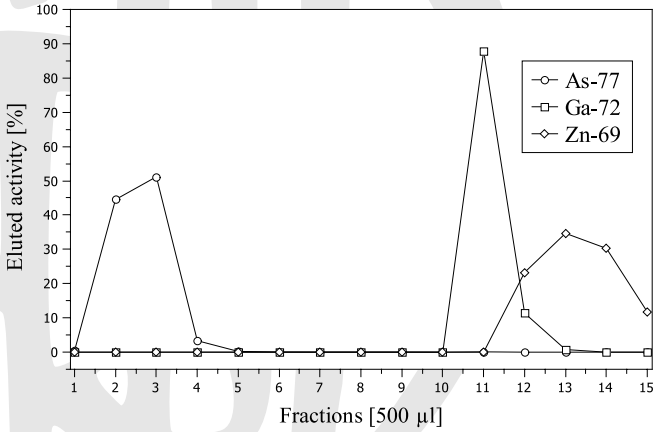

Fig. 1. Elution profile of ^{77}As(V) distillate from reactor-irradiated Ge target. ^{77}As(V) is eluted in fractions 2 and 3 in 10 M HCl. Eluent was changed to 0.1 M HCl in fraction 11. Under these conditions Ga and Zn radionuclides are eluted from the column.

was observed in CCl_4 by co-extraction of I_2 formed. Therefore CuCl was used which did not show comparable effects and delivered *As(III) in CCl_4. Reduction of *As(V) was achieved in high yields by heating the solution for 1 h at 60 °C. Extraction of As(III) twice in 500 µl CCl_4 portions gave an average yield of > 70%. This offers the ability to label molecules in non aqueous medium. Back extraction was performed by extraction from the combined organic phases with 500 µl PBS buffer an average yield of > 60%. The overall yield was 40%. Extraction yields might be upgraded by repeated extraction. However, the aim was also to minimize the final volume to about 500 µl PBS buffer to achieve high concentrated solutions of arsenic activity.

3.6 Speciation of As(III) and As(V)

One of the key steps for labelling of mab with arsenic radioisotopes is the availability of *As(III) in the labelling solution. This was monitored by a radio TLC method. While mixtures of HCl/acetone [13, 41] lead to destruction of the TLC plate, a mixture of sodium tartrate and methanol was chosen. The R_f values of 0.9 for *As(V), 0.6 for *As(III) and 0 for *As-labelled mab (see Fig. 2) are comparable to those found in HCl/acetone mixtures [41]. Care should be taken that the separated samples are used for labelling immediately or stored frozen in an atmosphere of argon to prevent the *As(III) from re-oxidation. The final fraction in 500 µl PBS contained > 95% *As(III).

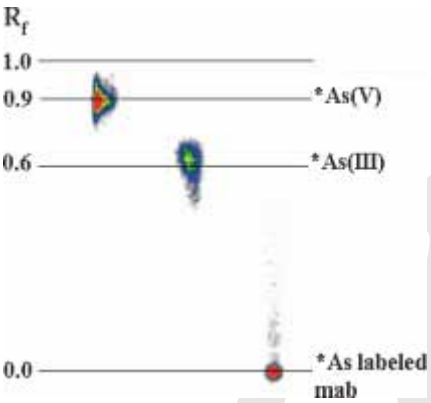

Fig. 2. Radio TLC of *As(V), *As(III) and *As labelled mab.

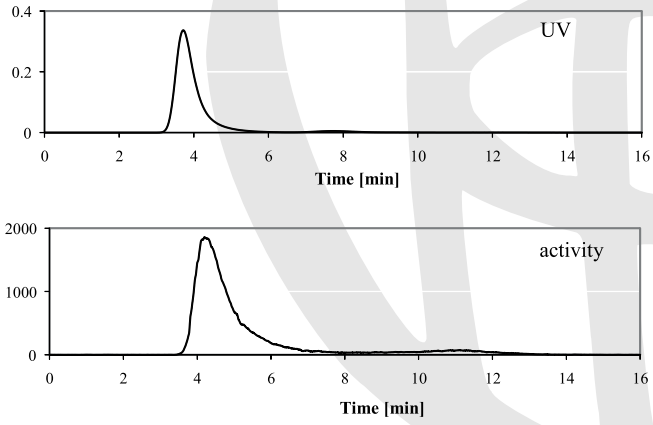

Fig. 3. Gel-filtration chromatogram of ^{77}As-mab after 1 h (UV- and corresponding radio signal).

3.7 Labelling of Bevacizumab

The principle of labelling strategy for radioarsenic is based on its high affinity to free SH-groups. The thiol-free reducing agent TCEP reduces some disulfide bonds inside the mab that will subsequently react with the *As(III), obtained from the separation method. To prevent a loss of immunoreactivity of the mab, a low concentration of TCEP (420 nmol) was used as mentioned in the instruction manual [49]. TCEP alone shows no interaction with arsenic and thus can stay inside the solution during the reaction. After 1 h at room temperature the labelling yield was > 90%, determined by TLC and > 99% by gel filtration HPLC (Fig. 1). The antibody can be purified by gel filtration from the excess of free TCEP.

4. Conclusion

A highly selective method for the separation of nca arsenic radioisotopes from bulk amounts of germanium targets and trace contaminants of Zn and Ga radionuclides was developed. The method can be applied to natural germanium targets or isotopically enriched target material irradiated at a nuclear reactors or a cyclotron. It is also possible to use GeO_2 as target material. Nca radioarsenic and macroscopic germanium are separated in a two step procedure. Elemental germanium and As(V) are first separated by distillation with an average separation factor of 2×10^4. This is followed by purification with anion exchange chromatography for separation of the remaining germanium and radioactive trace amounts of Zn and Ga formed during irradiation. The overall decontamination factor for germanium/arsenic was $> 1 \times 10^6$. In order to apply the separated radioarsenic for labelling of proteins, the *As(V) was reduced to *As(III) with CuCl at elevated temperature within 1 h. As far as the labelling of monoclonal antibodies requires neutral pH conditions, the *As(III) was first extracted into CCl_4 and then back extracted into PBS buffer. The overall yield of *As(III) from the target to the final 500 µl PBS fraction is > 40%.

Labelling of antibodies was successfully exemplified with the monoclonal antibody Bevacizumab providing labelling yields of > 99% after 1 h incubation at room temperature. This demonstrates that the radiochemical separation procedure is not only effective in terms of radiochemical parameters, but also adequate for the application of radioarsenic for syntheses of relevant protein based radiopharmaceuticals.

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