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An automated synthesis method for ^{68}Ga-labelled ubiquicidin 29-41

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Abstract

Published methods for radiolabelling of 1,4,7-triazacyclononane-1,4,7-triacetic acid ubiquicidin (NOTA-UBI) 29-41 to date describe manual or kit-based procedures. The purpose of this study was to develop an automated synthesis method for the synthesis of [^{68}Ga]Ga-NOTA-UBI. NOTA-UBI was successfully labelled with gallium-68 using the three different automated procedures. The use of radical scavengers to improve radiochemical purity is also discussed. The automated procedures showed a high degree of robustness and repeatability. The validated automated synthesis protocols using a Scintomics GRP Module will contribute to provide GMP-compliant [^{68}Ga]Ga-NOTA-UBI for clinical infection imaging.

Keywords: automated synthesis, ubiquicidine, NOTA-UBI, ^{68}Ga NOTA-UBI, Good Manufacturing Practice, radiolabelling

1. Introduction

Bacterial infections are a major contributor to the increasing costs of health care. Early, accurate detection of such infections may improve outcome and therefore reduce the costs associated with bacterial infection. Early detection and localisation also plays an important role in patient management as the process of identifying the site of infection is often difficult and time consuming which contributes to health care costs [1].

In order to make an accurate diagnosis, a number of steps are followed which include taking a detailed patient history and physical examinations, followed by a variety of laboratory tests such as erythrocyte sedimentation rate and C-reactive protein measurements. Various imaging modalities are utilised to localise the site of infection. These modalities include X-rays, ultrasonography, magnetic resonance imaging and computed tomography.

Nuclear imaging techniques to localise infections date back several decades. The use of gallium-67 citrate has been extensively described for imaging of infections [2–4]. Indium-111 or technetium-99m (^{99m}Tc) labelled leucocytes and ^{99m}Tc-labelled ciprofloxacin) with better imaging qualities than gallium-67 made them more attractive as infection imaging agents [2, 5, 6]. To date, imaging in vitro labelling of white blood cells with technetium-99m or indium-111 is still considered the gold standard for detection of peripheral infection [5].

The dawn of positron emission tomography (PET) has seen a rise in the need for tracers that can be labelled with positron emitters such as fluorine-18 (^{18}F) or gallium-68 (^{68}Ga). Labelling tracers with positron emitters offers the advantage of imaging with a higher spatial resolution than conventional single photon emission tomography (SPECT).

2-Deoxy-2-[^{18}F]fluoroglucose, ([^{18}F]FDG) has been widely used in the imaging of bacterial infections [7–9]. The low specificity of [^{18}F]FDG has been described as a major limitation. It

further cannot distinguish between infections and sterile inflammatory processes, malignancies and the normal wound healing process [10, 11]. These limitations of [^{18}F]FDG as the current ideal infection imaging agent have led to a continued quest for a PET imaging agent that will not only allow for specific detection of bacterial infections, but also be able to distinguish between sterile inflammation and bacterial infections.

2.   Theory

Ubiquicidin (UBI) is a human antimicrobial peptide and synthetic derivatives of this peptide have been suggested as a possible agent for imaging infections [12, 13]. The UBI fragment 29-41 (TGRAKRRMQYNRR) has been successfully labelled with technetium-99m [14, 15]. As mentioned previously, better spatial resolution can be obtained using PET radionuclides such as gallium-68, as opposed to technetium-99m, a conventional SPECT radionuclide. Besides the favourable imaging qualities of gallium-68, a physical half-life of 67.71 min coincides well with the biokinetics of low molecular weight peptide radiopharmaceuticals [16].

Since the introduction of cost-efficient ^{68}Ge/^{68}Ga generators, labelling of various peptides such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraaceticacid–1-NaI^3-octreotide (DOTA-NOC), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraaceticacid- 1-Tyr^3-octreotate (DOTA-TATE), and the peptidomimetic inhibitor of prostate specific membrane antigen (PSMA) with gallium-68 has been well described [7, 17–19]. ^{68}Ge/^{68}Ga generators have the advantage of a PET radionuclide being readily available as opposed to cyclotron produced radionuclides such as fluorine-18 and carbon-11. Radiolabelling of UBI 29-41 fragments with gallium-68 has been performed utilising bi-functional chelators like 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA). This ^{68}Ga-labelled radiopharmaceutical is currently being investigated

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as a potential infection imaging agent [20]. Ebenhan and co-workers also indicate that selective binding to bacterial cells is not compromised by the labelling procedure.

Published methods for radiolabelling of UBI initially only d manual processes [14, 15, 21, 22]. More recently, kit-based labelling methods have also been published [23, 24]. Kit based methods do not require expensive synthesis modules but do not address the possible risk of a higher radiation exposure to operators. Automation of labelling procedures has the benefit of reducing radiation exposure to personnel and due to standardization, makes these procedures more reliably compliant with good manufacturing practices (GMP) [25].

The automated labelling methods introduced by this study do not require HPLC purification of the final product, making these labelling methods especially suitable for radiopharmacies in a clinical setting.

3.   Experimental

3.1   Labelling methods

All steps in the automated labelling procedure were performed using a Scintomics GRP automated synthesis module (Scintomics, Germany). Freeze-dried NOTA-UBI (Shanghai, China or ABX, Germany) was dissolved in Millipore water (18.5Ω) and subdivided in 50 µl aliquots (1 µg/µl) and frozen at - 20°C. 50 µg of frozen NOTA-UBI was used for each of the labelling methods, except in our study investigating scavengers, where 100µg NOTA-UBI was used to improve radioyield of the cationic purification method. ^{68}Ga was obtained by eluting a ^{68}Ge/^{68}Ga generator (iThemba Labs, South Africa) using 0.6 M HCl (ABX, Germany). C-18 SEP-PAK (tC-short) cartridges (Waters, USA) were pre-conditioned on the synthesis module at the start of the synthesis using 5 ml HPLC-grade ethanol (Merck, USA).

1.5 M 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer (pH = 5.0) and PS-H$^+$ cartridges as well as GMP-prepared kits for the Scintomics synthesis module were obtained from ABX, Germany and sodium acetate trihydrate from Honeywell Riedel-de-Haën, Germany. Sodium chloride 0.9% (B.Braun, South Africa) and pharmaceutical grade ethanol (Merck, USA) were used to prepare the ethanol/saline (50% v/v) solution. Ultrapure water was freshly prepared with a Milli-Q water purification system (Millipore, USA). Ascorbic acid (North East Pharmaceutical Group, South Africa), genticic acid (Merck, USA) and pharmaceutical grade ethanol (Merck, USA) were tested as radical scavengers. Ammonium formate (Kimix, South Africa) was used to prepare a 1.0 M ammonium formate solution. HPLC analyses were carried out using HPLC-grade acetonitrile (Merck, USA) and trifluoroacetic acid (Sigma Aldrich, Germany).

Reference manual labelling

Certified 10 ml glass vials, prepared with a solution containing 25 µg NOTA-UBI per vial, were freeze-dried overnight and stored at - 20 °C u ntil the day of radiosynthesis. Manual radiosyntheses (n = 14) were performed according to a previously published method to create a reference dataset [21]. Briefly, the manual method consisted of fractional eluting a ^{68}Ge/^{68}Ga generator with 0.6 M HCl. Sodium acetate – buffered ^{68}Ga was added to 50 nM NOTA-UBI 29-41and incubated at 90°C for 15 minutes. The radiolabelled product was purified using a C-18 SEP PAK cartridge and filter through a 0.22µm sterile filter.

Automated Method 1) Fractional generator elution and pH adjustment with HEPES

The generator was eluted in 2.0 ml fractions. The first 2.0 ml fraction of the elution was discarded to the waste container. The second fraction of 2.0 ml was used for the labelling procedure. The ^{68}Ga was slowly added to a volume 1.5 ml of 1.5 M HEPES buffer (pH = 5.0) to render a buffered eluate mixture with a pH of between 3.5 and 4.0. The buffered ^{68}Ga

eluate mixture was then slowly added to the reaction vessel containing 50 µg NOTA-UBI. The concentration of NOTA-UBI in this mixture was 14.3 µg/ml. This mixture was heated for 10 minutes at 90°C, cooled for one minute and purified using a C-18 SEP-PAK cartridge. ^{68}Ga-labelled NOTA-UBI was desorbed from the C-18 cartridge using 2 ml ethanol/saline (50% v/v), and passed through a 0.2 µm sterile filter into a sterile vial. The final product was further diluted to 15 ml with phosphate buffered saline (PBS).

Automated Method 2) Fractional generator elution and pH adjustment with sodium acetate

^{68}Ga chloride was fractionally eluted with 0.6 M HCl as described earlier, followed by the slow addition of the ^{68}Ga eluate to a volume of 2.8 ml of 1.0 M sodium acetate solution (pH = 8.5) to render a buffered eluate mixture to a pH level between 3.5 and 4.0. The buffered ^{68}Ga was mixed with 50 µg NOTA-UBI providing a concentration of 10.4 µg NOTA-UBI per ml in the reaction mixture. The synthesis continued further as described in method 1 above.

Automated Method 3) Eluate processing by cationic purification and radiolabelling

The cationic eluate processing method was based on a method published by Martin et al. [26]. Briefly, the ^{68}Ge/^{68}Ga generator was eluted with 10 ml 0.6 M HCl and diluted to 18 ml with Milli-Q water. The diluted HCl solution was then slowly passed over a PS-H$^+$ cartridge (ABX, Germany) to retain most of the ^{68}Ga. Purified ^{68}Ga was recovered from the PS-H$^+$ cartridge using 1.5 ml 5.0 M NaCl solution which was then transferred into the reaction vessel which contained 50 µg NOTA-UBI in 1.3 ml of 1.0 M acetate solution. The concentration of NOTA-UBI in this reaction mixture was 17.9 µg/ml. The synthesis further continued as described above.

3.2   Analytical methods

Instant thin layer chromatography (ITLC)

Instant thin layer chromatography was performed as described by Breeman et al. [27] using a glass microfiber chromatography medium impregnated with silica gel (ITLC-SG, Varian, USA). The mobile phase consisted of 0.1 M sodium citrate (pH = 5.0). Measurement of radioactivity was performed using a Curiementor PTW dose calibrator (PTW, Germany) by way of cutting and measuring the ITLC strip into distinct pieces or running the entire ITLC strip on a radio-chromatographic scanner (Lablogic, United Kingdom).

HPLC analyses

HPLC analysis was initially performed using Waters HPLC system (Waters, USA). A sample of the labelled product was analysed by HPLC using a variable wavelength PDA UV-detector (Waters, USA) and a Raytest gamma detector (Raytest, Germany). The mobile phase (v/v) for the isocratic HPLC analysis was 15% acetonitrile, 85% ultrapure water, supplemented with 0.1% of trifluoroacetic acid (TFA). The flow rate was set at 1 ml/min. A Waters C-18 Symmetry analytical column (4.6 x 250mm x 4.6 mm x 5μm, Waters, USA) was used as the stationary phase for all analyses.

Subsequent analyses of the labelled product were done using a Schimadzu, Nexera XR HPLC system (Shimadzu, Japan) with a variable wavelength PDA UV-detector and a Raytest gamma detector (Raytest, Germany). The mobile phase (v/v) for the gradient HPLC analyses was 0.1% TFA in ultrapure water and 0.1% TFA in acetonitrile. The flow rate was set at 1 ml/min. A Waters C-18 column (Waters, USA) was used as the stationary phase for all analyses. [68Ga]Ga-NOTA-UBI eluted at 10.0 - 11.0 min on radio-HPLC, while free gallium-68 eluted at 3.0 – 4.0 minutes. The retention time of free gallium-68 was confirmed by HPLC analysis using a buffered 68Ga eluate solution with a pH of 3.5 – 4.0. Using a flow rate of

2 ml/min and adjusting the gradient time of the analysis to shorten the analysis was also investigated.

Radionuclidic identity

A sample of [^{68}Ga]Ga-NOTA-UBI was measured in a dose calibrator and the radioactivity recorded every 2 minutes for a period of 10 minutes. The half-life was calculated using a standard decay formula.

Germanium-68 breakthrough

Germanium-68 (^{68}Ge) breakthrough was routinely measured in each of the labelled products using a Curiementor PTW dose calibrator (PTW, Germany). This was performed 48 hours post synthesis when the ^{68}Ga decay was >10 half-lives. ^{68}Ge was measured indirectly by way of detection of ^{68}Ga produced only by leaked ^{68}Ge in the sample [28].

Determination of HEPES content

The European Pharmacopoeia (Ph. Eur.) describes an ITLC method to be used for the determination of HEPES content following syntheses with ^{68}Ga where HEPES is used as a buffering agent [29]. The limit for HEPES is 200 µg/V where V is the maximum injected dose in milliliters. In our institution this limit was calculated to be 13.3 µg/ml. This method uses silica-gel ITLC strips as the stationary phase and a mobile phase of acetonitrile (Merck, USA) and Millipore water (80:20 v/v). Fifteen microliters (15 µl) of an in-house prepared HEPES reference solution (13.3 µg/ml) and [^{68}Ga]GaNOTA-UBI (test solution) were applied at the origin of the TLC plate. The mobile phase was allowed to migrate to two thirds of the height of the strip and the strip then removed and allowed to dry. The dried strip was developed in a chamber containing iodine crystals. The intensity of the spot obtained with the test solution was compared to the intensity of the spot obtained with the reference solution.

3.3 Further quality control measures

In addition, the following tests were performed after each radiosynthesis to justify the product validity for human administration and to comply with specifications for batch release of radiopharmaceuticals:

Residual radioactivity on PS-H$^+$ cartridges was measured in a Curiementor PTW dose calibrator (PTW, Germany). Residual radioactivity on the C-18 cartridge at end of synthesis (EOS) was also measured in all instances irrespective of the labelling method used.

The pH of ^{68}Ga-labelled NOTA-UBI was tested with pH indicator strips with a range of 5.0 – 10.0. (Merck, USA). The pH value was read in increments of 0.5. Integrity of the sterilisation filter was tested using a Millipore pressure gauge (Millipore, USA).

During synthesis, labelled [^{68}Ga]Ga-NOTA-UBI was desorbed from the SEP-PAK C-18 cartridge using a mixture of 1 ml ethanol and 1 ml 0.9 % sodium chloride (50% v/v). The final volume of the labelled compound was programmatically set to 15 ml using the Scintomics software. The ethanol content therefore never exceeded 10% v/v.

3.4 Microbiological studies

Sterility testing

Sterility testing was performed by the National Health Laboratory Services at Tygerberg Hospital. A peptone nutrient broth, *Brucella* agar plates and Sabouraud dextrose agar plates were utilized to test for aerobic, anaerobic organisms and fungi respectively. Briefly, a sample from test the solution was withdrawn and each culture media inoculated using aseptic technique. The peptone nutrient broth and Brucella agar plates were incubated at 35 °C for a minimum of 5 days while Sabouraud dextrose agar plates were incubated at 30 °C for the same period.

Bacterial endotoxin spectrophotometry

Rapid endotoxin unit (EU) spectrophotometry (SPM) was performed using the Endosafe portable testing system (PTS) (Charles Rivers, USA) [30]. The analysis of the radiopharmaceutical samples was deemed acceptable if the recorded values were within the following specification: sample reading: < 10 EU/ml, sample coefficient of variance: < 25%, spike coefficient of variance: < 25%, and recovery: 50 – 200%

Methods 1- 3 were subjected to three full scale validation studies (see methods above) using our in-house release criteria (Table 1) for releasing radiopharmaceuticals for human use. Validation studies were performed using sterile cassettes (ABX, Germany) and reagents which included a phosphate buffered saline solution, ethanol, water for injection and 5.0 M NaCl solution (ABX, Germany). The 50:50 (v/v) ethanol/saline mixture was prepared fresh in-house and sterilised using a 0.22 µm sterile membrane filter.

Table 1 Summary of quality control procedures and release criteria.

Quality control procedure	Release criteria	Method
Visual appearance	Clear, colourless, particle free	Visual inspection
Radiochemical purity	≥ 95% [^{68}Ga]Ga-NOTA-UBI	ITLC / HPLC
Radionuclidic identity (half-life)	63 – 73 minutes	Dose calibrator
pH of final product	4.0 – 8.0	pH strips
Bacterial endotoxin	< 10 EU/ml	Endosafe PTS SPM
Residual ethanol content	< 10% v/v	Direct calculation
Sterile product filtration	≥ 3.45 bar	Filter integrity test
Bacterial growth testing	sterile (pass)	Broth testing
Germanium breakthrough	<0.001 %	Dose calibrator
Chemical purity	[^{68}Ga]Ga-NOTA UBI peak at retention time = 10-11 min	HPLC

Notes: EU = Endotoxin units, PTS = portable endotoxin system, SPM = spectrophotometer

4.   Results and Discussion

The manual labelling method described by Ebenhan et al. formed the basis for our in-house automated labelling procedure [20]. This method was considered too rigid to make a seamless translation to an automated module. The main challenges were the small total labelling volume and the concentrations of reagents, the peptide molarity and type of buffer. In addition, a suitable cation-exchange based generator eluate pre-processing method has not been investigated. This study set out to develop a robust radiosynthesis solution applicable to automated modules. A full scale generator elution requires 10 ml 0.6 M HCl to yield all the elutable ^{68}Ga activity; however, based on the nature of the generator elution profile, 84 - 92 % of that activity can be collected in 2 - 3 ml which also leads to a concentrated ^{68}Ga eluate [29].

The relatively small volume (± 550 µl) of 2.5 M sodium acetate used during the manual procedure for pH adjustment was too small to be effectively incorporated into an automated synthesis method (see Table 2 for a comparison of manual and automated methods). Sodium acetate (1.0 M) and HEPES (1.5 M) were considered to be applicable buffering agents to adjust the pH of the labelling mixture. Sodium acetate has been widely described as a buffering agent in the synthesis of ^{68}Ga-labelled peptides [31–34] and is considered the buffer of choice because of its safe biological profile [35]. Alternately, we opted to investigate HEPES buffer for the reasons described above. HEPES is a zwitterionic buffer with a pKa_1 and pKa_2 of 3.0 and 7.55 respectively and belongs the Good's group of buffers used in biological research [36]. The pKa value of sodium acetate on the other hand is 4.76 [37], thus, HEPES is expected to have a better buffering capacity than sodium acetate at a pH range of 3.0 – 4.0. Table 2 presents a comparison of the manual method with the three automated labelling methods.

Table 2 Comparison of the manual with the automated radiolabelling methods.

	Manual (Reference) (n≥9)	Automated (Scintomics module)		
		Method 1 (n=6)	Method 2 (10)	Method 3 (n=7)
Generator age during study (d)	120 – 244	145 - 181	112 – 180	145 - 153
Type of generator elution	FE	FE	FE	Full scale
Volume: ^{68}Ga activity (ml)	1.0	2.0	2.0	10
Buffering solution	2.5 M NaOAc	1.5 M HEPES	1.0 M NaOAc	1.0 M NaOAc
Volume of buffer used (µl)	278	1500	2800	1300
pH of labelling mixture	3.5 - 4.0	3.5 - 4.0	3.5 – 4.0	3.5 – 4.0
NOTA-UBI concentration / labelling (µg/ml)	19.6	14.3	10.4	17.9
Heating time (min)	10-15	10	10	10
Heating temperature (°C)	90	90	90	90
Radiochemical yield	65.5 ± 22.6	83.4 ± 6.7	71.8 ± 3.5	78.9 ± 3.6
Radioynthesis time (min)	31 ± 7	38 ± 2	38 ± 2	44 ± 2
% Average radiochemical purity	97.1 ± 1.9	99.6 ± 0.2	99.6 ± 0.5	99.0 ± 1.7
Activity yield (MBq)	473 ± 234	616 ± 21	537 ± 52	514 ± 24
Molar activity (MBq/nmol)	20.4 ± 11.4	26.5 ± 0.8	21.3 ± 2.0	20.6 ± 0.9
Activity retained on C-18 at EOS (MBq)	65.9 ± 55.9	6.5 ± 3.1	9.8 ± 3.8	31.5 ± 8.3
Residual activity on P-SH$^+$ cartridge at EOS (MBq)	-	-	-	79.3 ± 10.4
Retained C-18 activity (%)	10.0 ± 8.9	0.59 ± 0.3	1.3 ± 0.4	6.2 ± 1.6

Notes: EOS = End of synthesis, FE = fractional elution

Fractional elution method using HEPES buffer

Six successful automated syntheses using 1.5 M HEPES buffer were performed to prove that a satisfactory labelling can also be obtained using HEPES buffer. During the

development of the automated procedure various volumes of eluate and buffer solution were tested to determine optimum labelling conditions. Eluate and buffer volumes of 1000 – 2000 µl and 1200 – 1600 µl were respectively used. Routine syntheses were carried out using an eluate volume of 2000 µl buffered with 1500 µl 1.5 M HEPES.

Radiolabelling was carried out using a Scintomics automated synthesis module. The average radiochemical yield was $83.4 \pm 6.7\%$ (n = 6). The average radiochemical purity was 99.6 ± 0.2 % (n = 6) using a thin layer chromatographic method. Total synthesis time was 39 - 41 minutes which included pre-conditioning of the C-18 SEP-PAK cartridge, fractional elution and a final rinsing of the cassette tubing with water to flush away residual radioactivity from the cassette.

With HPLC analysis using the gradient method as described above, we were able to successfully distinguish between free gallium-68 (3.0 minutes) and ^{68}Ga labelled NOTA-UBI (10.0 – 11.0 minutes). The presence of a radiochemical impurity due to radiolysis was observed at retention time $\pm$ 9 minutes. (See figure Fig 1a).
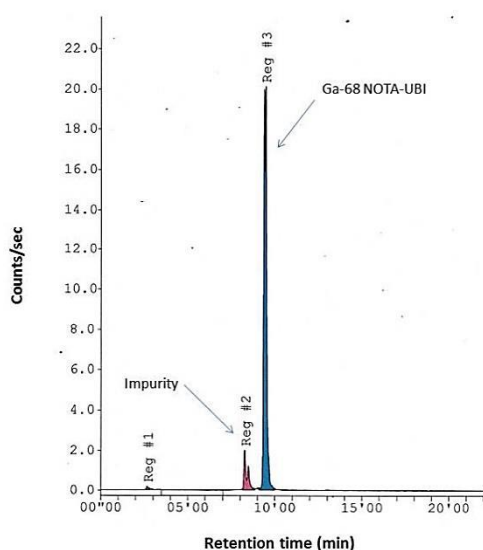

Fig.1a Radio-HPLC of [^{68}Ga]Ga-NOTA-UBI using fractional elution and HEPES buffer.

Results for the determination of the HEPES content in the final radiolabelled product indicated a HEPES content higher than the Ph.Eur/BP limit. The reason for this observation is not fully understood as HEPES is routinely used as a buffer in our institution in the labelling of [^{68}Ga]Ga- DOTA-NOC and PSMA (data not shown). The HEPES content in the latter two radiolabelled products does not exceed our in-house specification of 13.3 µg/ml. The unexpected high HEPES content in [^{68}Ga]Ga-NOTA-UBI necessitated the investigation of an alternative buffer such as sodium acetate. An automated labelling method using HEPES was therefore not further developed and validated.

Fractional elution method using 1.0 M sodium acetate solution

The same method used in the fractional elution method with HEPES was used but HEPES was replaced with sodium acetate solution. The volume of sodium acetate buffer tested during the developmental phase ranged from 2000 – 3000 µl. Development of this method was based on a fractional elution method where the second eluate fraction of 2000 µl was buffered with   2800 µl 1.0 M sodium acetate solution.

Radiolabelling was carried out as described above. The average radiochemical yield was 71.8 ± 3.5 % (n = 10). The average radiochemical purity was 99.6 ± 0.5 %   (n = 9) using a thin layer chromatographic method. Total synthesis time was 39 - 41 minutes which included pre-conditioning of the C-18 SEP-PAK cartridge, fractional elution steps and final rinsing of cassette tubing.

A sample from the labelled [^{68}Ga]Ga-NOTA-UBI was taken for HPLC analysis. Using the gradient method as described above, ^{68}Ga labelled NOTA-UBI eluted at 10 – 11 minutes as shown in the figure 1b. The presence of a radiochemical impurity due to radiolysis was again observed at retention time ± 9 minutes.
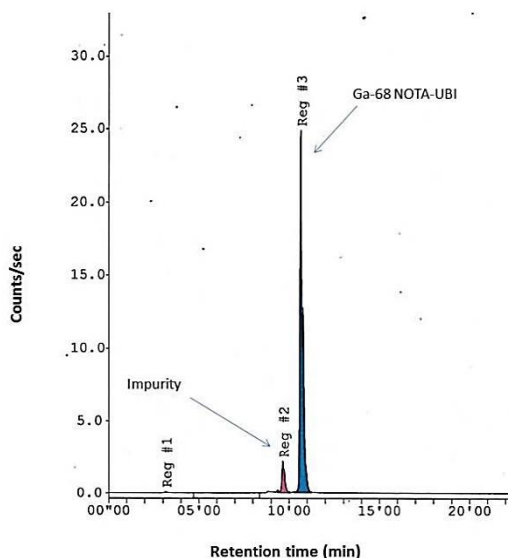
Fig.1b Radio-HPLC of [^{68}Ga]Ga-NOTA-UBI using fractional elution and sodium acetate buffer.

Fractional elution of the ^{68}Ge-68/^{68}Ga generator however does not guarantee that all metal impurities are removed from the eluate during the first elution. Cationic or anionic pre-purification of generator eluates are therefore suggested, especially in ^{68}Ge/^{68}Ga generators that have shown a greater tendency of higher levels of metal contaminants eluting from the generator column. This is considered an important factor especially when ^{68}Ge-68/^{68}Ga generators are eluted in a large volume and at a low pH [30]. The presence of metal impurities has a detrimental effect on the labelling process.

Cationic pre-purification method

A cationic pre-purification method using a PS-H$^+$ cartridge and 1.0 M sodium acetate solution as a buffering agent was further developed. The advantages of cationic pre-purification of the ^{68}Ga eluate using a PS-H$^+$ cartridge eluted with a solution of 5.0 M sodium chloride have been well described by Martin et al. [38].

The development phase consisted of testing sodium acetate volumes of 1200 – 2100 µl to adjust the pH of the labelling solution to a required pH of 3.5 – 4.0. A volume of 1300 µl sodium acetate solution was used during routine syntheses.

Radiolabelling was carried out as described above. The average radiochemical yield was 78.9% (n = 7). The average radiochemical purity was 99.0% (n = 7) using a thin layer chromatographic method. Total synthesis time was 43 - 45 minutes which included pre-conditioning of the C-18 SEP-PAK cartridge, generator elution, as well as final rinsing of the cassette tubing.

Using HPLC analysis, ^{68}Ga labelled NOTA-UBI eluted at 10 – 11 minutes as shown in figure 1c. The radiochemical impurity at retention time ± 9 minutes is again present.
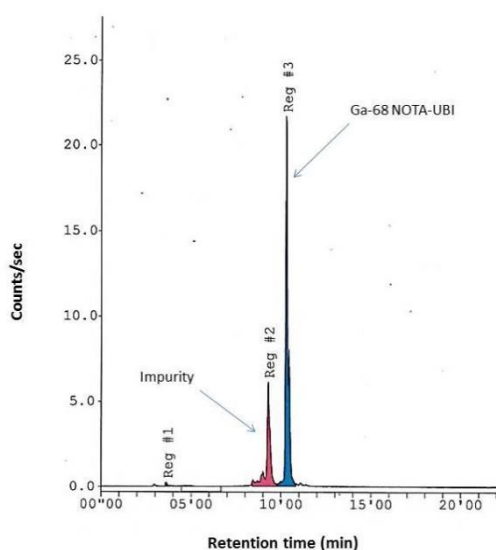

Fig. 1c Radio-HPLC of [^{68}Ga]Ga- NOTA-UBI using sodium acetate buffer and cationic pre-purified eluate.

Total HPLC analysis time was 40 minutes. Increasing the flow rate to 2 ml/min and adjusting the gradient time of the analysis resulted in a shorter analyses time of 25 minutes. This shorter method could be successfully used for routine analyses. Using the shorter

method, non-chelated gallium-68 eluted at 1-2 minutes while ^{68}Ga labelled NOTA-UBI eluted at 6 – 7 minutes.

Radical scavengers use to reduce radiochemical impurities during synthesis

Our labelling methods described above, resulted in a significant formation of a radiolysis impurity which was observed on radio-HPLC (see figures 1a - c). For this reason, it was deemed necessary to investigate the use of radical scavengers to improve radiochemical purity. The use of scavengers has been well described [39, 40].

Scavengers investigated for labelling methods 2 and 3 included ascorbic acid, genticic acid and ethanol. For reasons described above, the used of a scavenger in method 1 was not investigated.

Results from our study using scavengers listed above, concluded that for the fractional elution method (method 2), ascorbic acid 1.4% was effective in reducing the impurity. Our results further improved when sodium acetate buffer was replaced with 1.0 M ammonium formate solution. Using this combination of buffer and scavenger, a radiochemical purity of ≥ 95% was achieved on radio-HPLC (n = 3). Formic acid is currently used as part of a buffering solution in the preparation of FDA approved NETSPOT® [41].

For method 3, 1.0 M sodium acetate buffer (supplemented with concentrated hydrochloric acid to a pH of 4.5) was used. This buffer together with a combination of 350 µl of a 1.4% ascorbic acid solution and 170 µl ethanol (5% of total labelling mixture volume), reduced the radiolysis impurity significantly, increasing the radiochemical purity to ≥ 95% on radio-HPLC (n = 3).

Table 3 provides a summary the important labelling parameters using scavengers and alternative buffers.

Table 3 Automated method using radical scavengers

	Method 2 n = 3	Method 3 n = 3
Type of generator elution	FE	Full scale
Buffering solution	1.0 M ammonium formate	1.0 M NaOAc (pH = 4.5)
Volume of buffer used (µl)	2000	1500
Scavenger volume used (µl)	350 µl 1.4% ascorbic acid	350 µl 1.4% ascorbic acid
	170 µl ethanol	
NOTA-UBI concentration / labelling (µg/ml)	11.2	27.4
Radiochemical yield	63.2 ± 1.5	57.3 ± 3.8
% average radiochemical purity (TLC)	98.9 ± 0.3	99.31 ±0.1
% average radiochemical purity (radio-HPLC)	96.4 ± 0.9	97.3 ± 0.5
Activity yield (MBq)	690 ± 22	580 ± 99
Molar activity (MBQ/nmol)	27.6 ± 0.9	11.4 ± 1.9
Activity retained on C-18 at EOS (MBQ)	11.4 ± 10.0	14.2 ± 7.4
Residual activity on P-SH$^+$ cartridge at EOS (MBq)	-	129 ± 30
Retained C-18 activity	1.6 ± 1.4	2.5 ± 1.5

Notes: EOS = End of synthesis, FE = fractional elution

Radio-HPLC chromatograms of labelling methods 2 and 3 which include the addition of one or more scavenger, are presented in figure 2 a – b below. These chromatograms clearly show the improvement in radiochemical purity when scavengers are incorporated into the labelling methods. Stability studies (n = 3) confirmed that the final product was stable for up to three hours post synthesis.
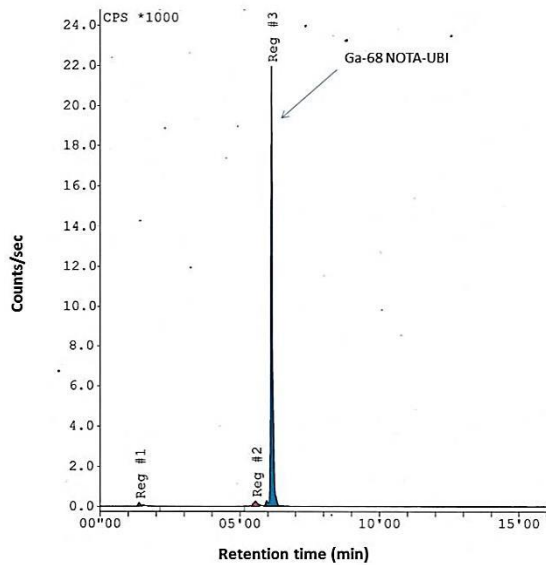
Fig. 2a Radio-HPLC of [^{68}Ga]Ga-NOTA-UBI using fractional elution and ammonium formate buffer with the addition of ascorbic acid.

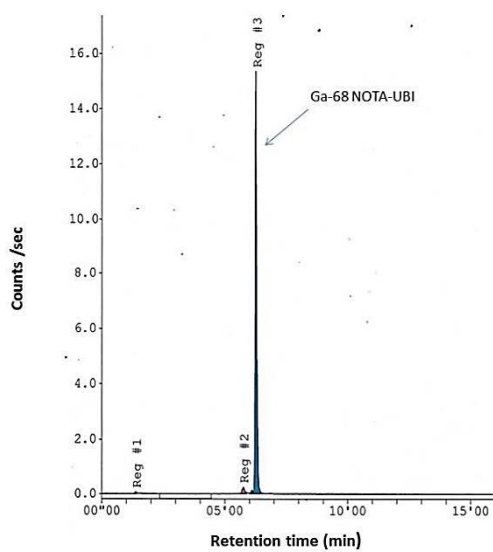Fig. 2b Radio-HPLC of [^{68}Ga]Ga-NOTA-UBI using cationic purification fractional elution and sodium acetate buffer with the addition of ascorbic acid and ethanol.

Instant thin layer chromatography

Labelled [^{68}Ga]Ga-NOTA-UBI was retained at the origin (Rf = 0 – 0.1) on the ITLC strip, while any free ^{68}Ga migrated with the solvent front (Rf = 0.8 - 1.0). No significant differences were found when the radiochemical purity was determined using the dose calibrator or the radio-chromatographic scanner. Figure 3 illustrates an example of a typical readout from a [^{68}Ga]Ga-NOTA-UBI ITLC analysis:
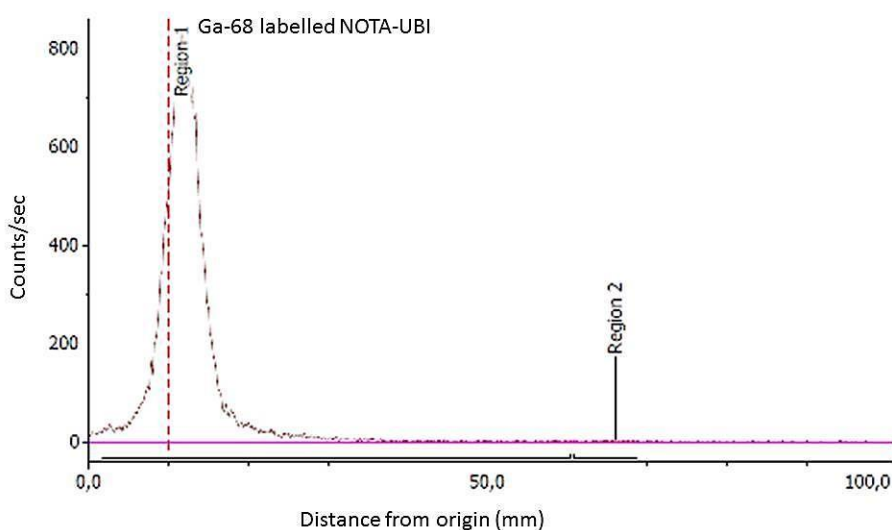

Fig. 3 ITLC analyses of [^{68}Ga]Ga-NOTA-UBI

Region 1: ^{68}Ga-labelled NOTA-UBI; Region 2: free ^{68}Ga

Note: a wider than usual region of interest was chosen for region 1 to compare this method with a "cut and measure" method using a dose calibrator.

Germanium breakthrough

Our institution uses an iThemba Labs ^{68}Ge/^{68}Ga generator which is routinely eluted with 0.6 M HCl. This generator has previously shown significant ^{68}Ge breakthrough [42]. Three different generators with varying ages were used during the development of these automated

synthesis methods. Germanium breakthrough was observed in the eluates of one of the generators used in the development of the automated labelling methods. It was therefore deemed necessary to determine germanium breakthrough in the final product for each of the automated labelling methods.

No germanium breakthrough was observed in any of the final products synthesised with each of the different labelling methods. These results further confirmed that, should germanium be present in eluates, it is sufficiently removed during the synthesis process.

NOTA-UBI concentration of labelling mixture (µl/ml)

The concentration of NOTA-UBI in the final labelling mixture of all three labelling methods varied from 14.3 – 17.9 µg/ml. In the study using radical scavengers, the concentration NOTA - UBI varied from 11.2 – 27 µg/ml. Results indicated that a higher concentration of NOTA-UBI in the final labelling mixture did not necessarily result in a better yield, irrespective of the labelling method and buffer used.

Molar activity of labelled product

The molar activity of the [^{68}Ga]Ga-NOTA-UBI was calculated for each of the three labelling methods. The fractional elution method using HEPES as buffer resulted in the highest molar activity (26.5 ± 0.8 MBq/nmol) compared to the molar activity obtained with the fractional elution (21.3 ± 2.0 MBq/nmol) and cationic pre-purification (20.6 ± 0.9 MBq/nmol) methods using sodium acetate as buffer.

In our study that investigated the use of scavengers, the highest molar activity (27.6 ± 0.9 MBq/nmol) was achieved with the fractional elution method using 1.0 M ammonium formate as buffer and ascorbic acid as a scavenger. The molar activity of the cationic purification method using a combination of ascorbic acid and ethanol as scavengers and buffered sodium

acetate was 11.4 MBq/nmol. This was expected as 100 µg of NOTA-UBI peptide was used for the synthesis. This higher amount of peptide is not expected to produce any toxicity in humans. The study by Akhtar et el. intravenously injected a dose of 400 mg of ubiquicidine 29-41 labelled with ^{99m}Tc, without any side effects. [12].

Validation of automated synthesis method

Three validation studies were performed on each of the automated labelling methods. Labelling of NOTA-UBI with ^{68}Ga using a fractional elution method has been successfully developed, tested and validated. The final product fulfilled all our in-house criteria for GMP release.

Labelling of NOTA-UBI with ^{68}Ga using a cationic pre-purification method has also been successfully developed, tested and validated. The final product fulfilled all our in-house criteria for GMP release.

Choice of automated labelling method

^{68}Ga labelling of NOTA-UBI can be successfully performed using both fractional elution and cationic purification automated methods. The fractional elution method using HEPES as buffering agent method gave the best radiochemical yield. The fractional elution method using HEPES as buffer resulted in the highest molar activity (26.5 MBq/nmol). Velikyan also reported a higher molar activity obtained when using HEPES buffer in the labelling of DOTA-NOC with ^{68}Ga [33]. Unfortunately, this study also indicated that HEPES is not a suitable buffer for the labelling of NOTA-UBI as the HEPES content in the final product exceeded the limits published in the Ph. Eur. and BP. All three automated labelling methods resulted in radiochemical yields in excess of 71%. Radiochemical yields for the study investigating the use of scavengers for method 2 and 3, resulted in an average radiochemical

yield of 60%. The radiochemical purity on TLC for all three automated labelling methods, including the study which investigated radical scavengers, was consistently more than 98%. As previously mentioned, fractional elution does not guarantee that all metal impurities will be removed in the first fraction and these metal impurities may result in sub-optimal labelling conditions. Pre-purification with cationic exchange cartridges such as a PS-H$^+$ cartridge have been widely used to purify generator eluates from metal impurities. The choice of an automated labelling method will therefore heavily depend on the characteristics of the generator used during the labelling. To reduce the presence of impurities due to radiolysis, radical scavengers such as ascorbic acid and ethanol were successfully incorporated in labelling method 2 and 3, resulting in an increase in radiochemical purity using radio-HPLC.

More recently, kit-based labelling of [^{68}Ga]Ga-NOTA-UBI 29-41 has also been described. Kit-based labelling of radiopharmaceuticals has the advantage that it does not require an expensive synthesis module for the labelling process. However, the need for pre-purification of generator eluates and the post-purification of the radiolabelled product will play an important role in the choice of a labelling method. The use of ^{68}Ge/^{68}Ga generators known with high levels of germanium breakthrough and metal impurities may limit a kit-based approach.

Limitations

The ITLC method utilized during quality control analyses could not distinguish between colloidal impurity and the labelled compound. Although final purification was performed using a C-18 SEP-PAK cartridge, ITLC could not confirm if all colloidal impurities are indeed removed during this step. It has however been reported for ^{68}Ga peptide synthesis that C-18 purification will lead to the removal of colloidal impurities [43].

Further investigation of the labelled product to clearly characterise the origin of the double peak seen on the HPLC chromatogram, to exclude the possible formation of impurities would also be valuable.

We did not investigate and develop an automated method using pre-purification with a PS-H$^+$ cartridge and HEPES as buffering agent. It is therefore not known if the HEPES content would also exceed pharmacopoeial accepted limits as observed with the fractional elution method.

In this study, a standard peptide quantity of 50µg NOTA-UBI (100 µg in the study investigating scavengers using method 2) and in varying buffer volumes was used for all labellings. The effect of standardising peptide concentration but using different volumes of buffer on labelling parameters was not investigated.

The use of L-methionine as a radical scavenger to improve radiochemical purity was not investigated.

Total synthesis time was 39 - 41 minutes and 43 - 45 minutes for the fractional elution and pre-purification methods respectively. The additional pre-purification step increased total synthesis time by 4 - 5 min. Flow rates used after the heating step were based on the published manual method. Faster flow rates were not investigated during this study.

The flow rate in the following post-heating steps can influence total synthesis time:

• Adsorption of labelled [^{68}Ga]Ga-NOTA-UBI on the C-18 cartridge.

• Washing of the C-18 cartridge with PBS.

• Desorption of labelled [^{68}Ga]Ga-NOTA-UBI from the C-18 cartridge.

It is therefore possible that total synthesis time can be reduced by increasing the flow rates in the post-heating steps. Shorter synthesis time will also lead to an increase in the amount of radiolabelled product.

Despite a longer total synthesis time, the pre-purification method using a cationic exchange cartridge still has the benefit of ensuring that metallic impurities are removed prior to the labelling step. Furthermore, an automated synthesis method has the added benefit of producing [^{68}Ga]Ga-NOTA-UBI with a high degree of consistency and robustness, satisfying GMP requirements for the production of radiopharmaceuticals.

Conclusions

Three different fully automated methods for the labelling of [^{68}Ga]Ga-NOTA-UBI have been successfully developed and tested. The use of radical scavengers to reduce radiochemical impurities was successfully introduced in method 2 and 3. Both the fractional elution and pre-purification methods can be used for the synthesis of [^{68}Ga]Ga-NOTA-UBI under GMP compliant conditions. This study confirms that automated synthesis methods have the benefit that radiopharmaceuticals are produced in a robust and reproducible manner.

The availability of a PET radiopharmaceutical such as [^{68}Ga]Ga-NOTA-UBI, that can be synthesised using an automated synthesis module, is expected to positively contribute to the medical management of patients with infections.

Notes

Compliance with Ethical Standards

Conflict of interest:

The authors declare that they have no conflict of interest.

Acknowledgements:

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