# Optimization of production methods for gallium-68 PET radiopharmaceuticals in a hospital radiopharmacy

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# Declaration

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This dissertation includes one original paper published in a peer-reviewed journal, one accepted for publication and two unpublished papers. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

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# Abstract

Production of radiopharmaceuticals intended for human use and research purposes is generally performed in well-equipped commercial or research facilities that usually have access to advanced equipment for the synthesis and quality control of radiopharmaceuticals. Nuclear Medicine departments are in most cases situated in hospitals. Radiopharmacies in these departments usually have limited space and equipment which necessitates careful consideration of suitable production methods. Optimization may include methods to simplify quality control procedures through the use of less sophisticated equipment and procedures.

The purpose of this study was to demonstrate how to optimize production methods in an environment with limited resources using ubiquicidin labelled with gallium-68 as an example.

The peptide ubiquicidin is currently investigated for localization of infections in patients using positron emission tomography (PET). Until recently, labelling ubiquicidin with gallium-68 was limited to a manual labelling method. Manual labelling methods are not recommended for the routine production of radiopharmaceuticals because of difficulty to comply with Good Manufacturing Practice (GMP). Manual labelling methods can also result in high radiation exposure to personnel. These disadvantages can be addressed by automation of production methods.

The research conducted in this study shortly entails the following aspects:

- Automation of a manual labelling method of ubiquicidin with gallium-68
- Optimization of the synthesis methods using radical scavengers
- In-depth comparison of the labelling characteristics of the manual method to that of the automated methods
- Conducting a literature search into the toxicity of HEPES in humans and animals in order to clarify its use as a buffering agent in the labelling of radiopharmaceuticals

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- Investigating thin-layer chromatography as method to determine the radiochemical purity of gallium-68 ubiquicidin

Two different automated synthesis methods were developed in this study. Optimization of the labelling methods was achieved by adding free-radical scavengers to reduce the formation of impurities. A comparison of the labelling characteristics of the manual labelling method with the automated methods showed that the results obtained with automated methods were more robust and repeatable.

The literature search into the toxicity of HEPES showed that its toxicity in humans and animals may be overestimated by pharmacopoeias. The current limits applied by pharmacopoeias may be too strict.

An evaluation of several thin-layer chromatography methods indicated that the method currently described in the literature may underestimate the presence of colloidal impurities in the final product. None of the other methods investigated in this study could distinguish the colloidal impurity from the labelled product. This aspect highlights the need for a final purification step to reduce the presence of colloidal impurities in the final product.

The work presented in this study creates an important basis for optimization of production methods in a clinical environment. The study can further serve as a guideline to other nuclear medicine departments for optimization of radiopharmaceutical production methods.

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# Opsomming

Produksie van radiofarmaseutika vir menslike gebruik en navorsing geskied oor die algemeen in goed toegeruste kommersiële- of navorsingsfasiliteite wat meestal oor gevorderde toerusting vir die sintese en gehaltebeheer van radiofarmaseutika beskik. Kerngeneeskunde-afdelings is oor die algemeen geleë in hospitale en hul radiofarmasie fasiliteite beskik dikwels slegs oor beperkte ruimte en toerusting wat deeglike oorwegingvan produksiemetodes noodsaak. Die optimiseringsproses kan vereenvoudiging van ingwikkelde gehaltebeheerprosedures insluit deur gebruik te maak van minder komplekse toerusting en prosedures.

Die doel van hierdie studie was om met behulp van 'n produkvoorbeeld, nl ubiquicidin wat met gallium-68 gemerk word, ondersoek na die belangrikste aspekte van radiofarmaseutiese sinteses in 'n betreklik eenvoudige opset te doen.

Die peptied ubiquicidin word tans ondersoek om met die hulp van positron emissie tomografie (PET) infeksies in pasiënte op te spoor. Tot onlangs was die merking van ubiquicidin met gallium-68 hoofsaaklik gebaseer op ’n handmerkingsmetode. Handmerkingsmetodes word nie aanbeveel vir roetine produksie van radiofarmaseutika nie; enersyds weens probleme om aan vereistes vir goeie vervaardigingspraktyk te kan voldoen en andersyds weens ‘n hoër stralingsdosis wat werknemers ontvang. Hierdie nadele kan grootliks oorbrug word deur die gebruik van modules wat die merkingsmetdoes outomatiseer.

Die navorsing in hierdie studie behels kortliks die volgende aspekte:

- Outomatisering van ‘n handmerkingsmetode om ubiquicidin 29-41 met gallium-68 te merk
- Optimisering van die sintese prosedure deur die gebruik van vry-radikaalinhibeerders
- In-diepte vergelyking van die merkinsgeienskappe van die handmetodes teenoor dié van outomatiese metodes

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- Literatuurstudie na die toksisteit van HEPES in mense en diere om die gebruik daarvan as ’n buffer in die vervaardging van radiofarmaseutika in perspektief te stel
- Ondersoek van dunlaagchromatografiemetodes vir die bepaling van radiochemiese suiwerheid van gallium-68 ubiquicidin

Twee verskillende tipes outomatiese sintesemetodes is vir die doeleindes van hierdie studie ontwikkel. Optimisering van die merkinsgsproses is bewerkstellig deur die gebruik van ten minste twee vry-radikaalinhibeerders om die vorming van ongewenste onsuiwerhede te beperk. ′n Vergelyking van die merkinsgeienskappe van die handmerkingsmetode teenoor die outomatiese metodes dui daarop dat die outomatiese merkingsmetodes meer robuust en herhaalbaar is.

Die literatuurstudie na die gebruik van HEPES in mense en diere dui daarop dat die toksisiteit van HEPES in mense moontlik deur die farmakopieë oorskat word. Die grense wat tans deur die farmakopieë voorgestel word mag dalk te streng wees.

Die evaluering van verskeie dunlaagchromatografiemetodes dui daarop dat die huidige metode wat in die literatuur beskryf word die teenwoordigheid van ’n kolloïdale onsuiwerheid in die finale produk onderskat. Al die ander metodes wat in hierdie studie ondersoek is kon nie hierdie onsuiwerheid van die gemerkte produk onderskei nie. Hierdie aspek beklemtoon die belangrikheid van van ’n suiweringstap in die merkingsprosedure om die teenwoordigheid kolloïdale onsuiwerheid in die finale produk te voorkom.

Die werk wat in hierdie studie vervat word skep ’n belangrike grondslag vir toekomstige optimisering van produksiemetodes in ’n kliniese omgewing. Die studie kan verder ook deur ander kerngeneeskunde afdelings as riglyn gebruik word in die optimisering van produksiemetodes van radiofarmseutika.

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**Addenda**

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# List of abbreviations

| | |
|---|---|
| **$^{64}Cu$** | Copper-64 |
| **$^{18}F$** | Fluorine-18 |
| **$^{68}Ga$** | Gallium-68 |
| **$^{68}Ge$** | Germanium-68 |
| **$^{99m}Tc$** | Technetium-99m |
| **BP** | British Pharmacopoeia |
| **DFO** | Desferrioxamine |
| **DOTA** | 1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetra-acetic acid |
| **DOTA-NOC** | 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraaceticacid–1-NaI3-octreotide |
| **DOTA-TATE** | 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraaceticacid- 1-$Tyr^3$-octreotate |
| **EU** | Endotoxin units |
| **FDA** | Food and Drug Administration |
| **GMP** | Good Manufacturing Practice |
| **HBED** | N,N-bis(2-Hydroxybenzyl)ethylenediamine-N,N-diacetic acid |
| **HCl** | Hydrochloric acid |
| **HEPA** | High-efficient particulate air |
| **HEPES** | 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid |
| **HPLC** | High performance liquid chromatography |
| **ITLC** | Instant thin-layer chromatography |
| **LC-MS** | Liquid chromatography – mass spectrometry |
| **MBq** | Megabecquerel |

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| | |
|---|---|
| **NaOAC** | Sodium acetate |
| **$NH_4HCO_2$** | Ammonium formate |
| **NODAGA** | 1,4,7-triazacyclononane,1-glutaric acid-4,7-acetic acid |
| **NOTA** | 1,4,7-tri-azacyclononane-1,4,7-triacetic acid |
| **PBS** | Phosphate buffered saline |
| **PET** | Positron emission tomography |
| **Ph. Eur** | European Pharmacopoeia |
| **PSMA** | Peptidomimetic inhibitor of prostate specific membrane antigen |
| **PVD** | Pyoverdine |
| **RCP** | Radiochemical purity |
| **RCY** | Radiochemical yield |
| **RGD** | Arginine-glycine-aspartate |
| **Rf** | Retardation factor |
| **RT** | Retention time |
| **SPE** | Solid phase extraction |
| **SPECT** | Single photon emission computed tomography |
| **TAFC** | Triacetylfusarine C |
| **TFA** | Tri-fluoro acetic acid |
| **TLC** | Thin-layer chromatography |
| **TRAP** | 1,4,7-triazacyclononane phosphinic acid |
| **UBI** | Ubiquicidin 29-41 |
| **UPLC** | Ultra-performance liquid chromatography |
| **$^{89}Zr$** | Zirconium-89 |
| **[$^{68}$Ga]Ga-NOTA-UBI** | [$^{68}$Ga]Ga-ubiquicidin (29-41) |

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# Overview of Authors' Contributions

Declaration by candidate

With regards to the articles presented in this dissertation the scope of my contribution was as follows:

| Publication | Nature of contribution | Extent of contribution (%) |
|---|---|---|
| **Chapter 2:** An automated synthesis method for $^{68}$Ga labelled ubiquicidin 29–41 | Literature review, study design, data collection, processing and analysis, manuscript writing and preparation | 70 |
| **Chapter 3:** Impact of radical scavengers on the radiolabelling characteristics and purity of Gallium-68-ubiquicidin | Literature review, study design, data collection, processing and analysis, manuscript writing and preparation | 75 |
| **Chapter 4:** A comparison of labelling characteristics of a manual and automated synthesis methods for gallium-68 labelled ubiquicidin | Literature review, data collection, processing and analysis, manuscript writing and preparation | 70 |
| **Chapter 5:** The use of HEPES-buffer in the production of gallium-68 radiopharmaceuticals – time to reconsider strict pharmacopoeial limits? (Technical Note) | Literature review, data collection, processing and analysis, manuscript writing and preparation | 72 |

*(Declaration with signature in possession of candidate and supervisor.)*

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Date: August 2020

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The following co-authors contributed to the articles in this dissertation:

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| | **Chapter 3 Manuscript**<br>Study design, manuscript review | 15 |
| | **Chapter 4 Manuscript**<br>Manuscript review, processing and analysis | 15 |
| | **Chapter 5 Manuscript**<br>Manuscript review | 8 |
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| | **Chapter 3 Manuscript**<br>Manuscript review | 10 |
| | **Chapter 4 Manuscript**<br>Study design, data collection, manuscript review | 15 |
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**Declaration by co-authors**

The undersigned hereby confirm that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors in the specified chapters/articles.
2. No other authors contributed to the specified chapters/articles beside those specified above.

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3. Potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in the specified chapters/ articles of this dissertation.

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# Chapter 1

# Introduction

## Background information

Recent years have seen a drastic increase in the development of radiopharmaceuticals used for positron emission tomography (PET) [1, 2]. Despite this development of new radiopharmaceuticals for clinical use, not many are available in South Africa on a routine basis. Of all PET radiopharmaceuticals, 2-[$^{18}$F]fluoro-2-deoxy-D-glucose ([$^{18}$F]FDG) is probably the best known [3]. Fluorine-18's half-life of 109 minutes makes this radionuclide attractive for regular use and it has been widely incorporated into various ligands for PET imaging [4, 5]. Other PET radionuclides such as carbon-11, nitrogen-13 and oxygen-15 are also used in the labelling of tracers for PET studies, but with half-lives of 20 minutes, 10 minutes and 2 minutes respectively, regular use in a clinical setting is limited. Use of these radionuclides further also requires an on-site cyclotron.

Hospital radiopharmacies in South Africa are usually limited to the use of radiopharmaceuticals that are commercially available. This limits researchers in a clinical setting to perform research with only one or two commercially available radiopharmaceuticals. [$^{18}$F]FDG is a typical example of such a radiopharmaceutical. It has been commercially available in South Africa since 2006 and has for almost a decade been the only commercially available radiopharmaceutical for PET imaging. [$^{18}$F]sodium fluoride and [$^{18}$F]fluoroethylcholine were only available on special request.

The introduction of gallium-68 from germanium-68/gallium-68 ($^{68}$Ge/$^{68}$Ga) generators has launched a wider range of PET radiopharmaceuticals [6]. $^{68}$Ge/$^{68}$Ga-generators provide a readily available supply of a positron emitting radionuclide that can be used in a hospital radiopharmacy without the need of an on-site cyclotron. The past few years have seen a dramatic increase in the labelling of tracers with gallium-68 [6–9]. Advances in several aspects in the design of $^{68}$Ge/$^{68}$Ga-generators have made its application in a hospital radiopharmacy possible. These include development of

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generator eluates that are suitable for labelling a number of compounds and the rapid development of peptides that can be labelled with gallium-68 using mono- and bifunctional chelators [10].

Over the past few years, a number chelators have been developed which include non-macrocyclic and macrocyclic chelators [19-21]. Non-macrocyclic chelators include diethylenetriaminepentaacetic acid (DTPA), N'N-bis(2-hyroxybenzyl)ethylendiamin-N,N'-diacetic acid (HBED) and desferrioxamine (DFO). Macro-cyclic chelators include 1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetra-acetic acid (DOTA), 1,4,7-tri-azacyclononane-1,4,7-triacetic acid (NOTA), 1,4,7-triazacyclononane,1-glutaric acid-4,7-acetic acid (NODAGA) and 1,4,7-triazacyclononane phosphinic acid (TRAP). Both DTPA and DFO have the advantage that labelling can be performed at room temperature which is important when labelling heat sensitive molecules such as antibodies. DTPA labelling also requires less acidic labelling environments (pH = 5.0 – 7.4) [20]. DTPA has been extensively used as the chelator of choice in the labelling of indium-111 pentetreotide while DFO derivatives can complex gallium-68 and zirconium-89 [20,24].

DOTA is probably the best known macrocyclic bifunctional chelator and has been extensively used in the labelling of PET radiopharmaceuticals. More recently NOTA has gained more popularity in gallium-68 labelling of radiopharmaceuticals. Gallium-68 fits better into the smaller ring structure of NOTA. NOTA also has the advantage that labelling can be performed at room temperature. Some compounds may however require higher temperature to increase radiochemical yield (RCY). NODAGA is another important bifunctional chelator in gallium-68 labelling [20]. NODAGA has a glutaric arm instead of an acetic arm. The advantages of the hexadentate $N_3O_3$ structure is that it does not get destroyed as opposed to conjugated NOTA because it creates a space between the target molecule and chelator. Ubiquicidin 29-41 has also been successfully labelled with gallium-68 using a NODAGA chelator. [22]. The use of TRAP has also gained interest. TRAP can be labelled at room temperature and in a relatively short time (10 min.). It has also shown to be less affected by impurities present in gallium generators.

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The development of automated synthesis modules that can be connected with $^{68}Ge/^{68}Ga$-generators make it possible for hospital radiopharmacies to produce a PET radiopharmaceutical for clinical use [11–15]. Some modules also allow preparation of several different gallium-68 radiopharmaceuticals using the same synthesis unit. The Nuclear Medicine Division at Tygerberg Hospital currently produces [$^{68}$Ga]Ga-DOTA-NOC and [$^{68}$Ga]Ga-PSMA-11 using the same module. Automated modules can be pre-programmed to synthesise PET radiopharmaceuticals according to a standard method. The generator and synthesis module are placed in a hot cell, reducing radiation exposure to the operator. Hot cells with high-efficient particulate air (HEPA) filters also make it possible to produce radiopharmaceuticals under Good Manufacturing Practice (GMP) compliant conditions. Particular advantages of using automated synthesis modules are as follows: [16]

- They help limiting radiation exposure to operators.
- They ensure that radiopharmaceuticals can be prepared in a reproducible manner.
- The modules are compact enough to be used in a hot cell.
- The methods can be certified according to Good Manufacturing Practice to comply with local or international regulations for the production of radiopharmaceuticals.
- Cassette-based systems have the further advantage that these can be supplied as sterile, pyrogen-free units, making compliance with GMP regulations easier.

Automated synthesis modules on the other hand have the following disadvantages:

- These modules and cassette systems are generally very expensive.
- They require regular and planned preventative maintenance.
- They require trained personnel for regular maintenance and repairs.

If synthesis modules are equipped with software that allows the user to further customize the step-by-step procedures of the module, new labelling or synthesis methods can be developed in-house. A typical module set-up for the synthesis of ubiquicidin 29-41 using a $^{68}Ge/^{68}Ga$-generator is shown in addendum A. Synthesis modules are either equipped with fixed tubing systems, or they can make

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use of cassette-based systems for the transfer of reagents during synthesis [21]. The use of fixed tubing has become less attractive due to difficulty to comply with GMP principles. Strict cleaning procedures, which have been extensively validated, are important to prove that the no residual contaminants from the previous synthesis are present at the start of the next synthesis. Fixed tubing can also not be sterilized prior to a synthesis to minimize the risk of bacterial and endotoxin contamination of the final product. Cassette-based systems on the other hand can be supplied as a sterile unit which has been manufactured according to GMP principles. These cassettes are typically manufactured as a single use item and are discarded prior to the start of the next synthesis. Nowadays it is possible to purchase fully GMP compliant synthesis kits that contain not only a sterile and pyrogen-free cassette, but also all the reagents and consumables required for the synthesis, manufactured under GMP conditions.

Establishing an automated labelling method is usually based on a manual labelling method used in the initial development of the radiopharmaceutical. Conversion of a manual synthesis method to an automated method requires a thorough optimization process. Small quantities of radioactivity and precursors are normally used in the developmental phase to evaluate initial labelling characteristics such as radiochemical yield, chemical purity and stability. Also, the effects of adapting labelling parameters like pH, labelling temperature and heating times of the labelled product yield and purity may need to be assessed. Starting with low concentrations and gradually increasing the concentration of the precursor, the optimal precursor concentration for successful labelling is then identified.

Radiolysis can cause formation of free radicals during the labelling process, which can damage the labelled compound. Optimizing a labelling method for a specific radiopharmaceutical may require the addition of radical scavengers to reduce the formation unwanted radiolytic impurities. PET radiopharmaceuticals such as fluorine-18 and gallium-68 are especially prone to radiolysis because of high radioactive quantities and specific activity [17, 18].

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Novel radiopharmaceuticals, when intended for clinical investigations, should ideally be produced using an automated synthesis module to ensure compliance to GMP. Translation of a manual synthesis to an automated one may pose certain challenges. It may be difficult to transfer small volumes of eluate and buffer accurately with an automated synthesis unit. Pre-purification of a generator eluate may be preferred when generators with known high levels of metal impurities are used for the radiosynthesis [10]. Some automated synthesis protocols offer this as a pre-set step. Manual methods often do not include a generator eluate pre-purification step. Schematic layouts of the manual and proposed automated labelling methods are illustrated in addenda B to D.

Hospital radiopharmacies situated in Nuclear Medicine departments do not all have an extensive clean room suite and well-equipped analytical laboratories for the production and testing of radiopharmaceuticals. These facilities may be limited to basic equipment for relatively uncomplicated syntheses and quality control of the final product. Equipment usually includes standard instruments such a synthesis module, an HPLC instrument and a thin-layer chromatogram scanner to name a few. Access to more complex and expensive equipment like liquid chromatography - mass spectrometry (LC-MS) and Ultra Performance Liquid Chromatography (UPLC)may be less common.

**Purpose of study**

The purpose of this study was to investigate the most important factors that determine the successful optimization of production methods in a hospital radiopharmacy situated in a clinical setting with relatively limited facilities and equipment.

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**Research objectives**

The objectives of this study were as follows:

- To identify which aspects influence the process of converting a manual radiosynthesis method to an automated module based preparation of radiopharmaceuticals;
- To identify the general aspects and radiolabelling parameters that may affect the quality of the radiopharmaceuticals;
- To demonstrate how to optimize a production method in a hospital radiopharmacy with limited equipment;
- To compare the labelling characteristics of radiopharmaceuticals prepared by an automated method with those prepared by a manual method; and
- To evaluate the quality control procedures required for a newly synthesised product.

**Significance and motivation**

Labelling [$^{68}$Ga]Ga-NOTA-UBI (29-41) with an automated synthesis module has not been described in the literature. The results obtained from this study can contribute significantly to the existing knowledge in the following way:

- This study can serve as an example for the development of other automated methods for future radiopharmaceuticals.
- It highlights the critical aspects involved in the quality control of products prepared by a new method.
- It demonstrates how optimization of production can be achieved using limited facilities and equipment.
- It can serve as a guideline for other Nuclear Medicine departments to successfully optimize production methods in their departments.

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- It can lead to the expansion of current available radiopharmaceuticals in a routine clinical setting.

This study focused on the development and optimization of two automated synthesis methods for labelling NOTA-UBI (29–41) (further referred to as NOTA-UBI) with gallium-68, one using fractional generator elution and the second method using a generator eluate pre-purification step. Optimizing the automated methods also took into consideration the choice of a buffer used for radiolabelling as well as an investigation into radical scavengers to reduce radiolysis. The use of thin-layer chromatography (TLC) and the need to optimize TLC methods are also discussed.

**Aim**

The aim of this study was to optimize the production method of a radiopharmaceutical in a hospital radiopharmacy using gallium-68 ubiquicidin as an example. It also included an investigation into thin-layer chromatography methods used to determine the radiochemical purity of gallium-68 ubiquicidin.

**Delineation of the study**

The research presented in this study was limited to:

- Using only gallium-68 from tin-oxide based iThemba LABS generators for the labelling procedure;
- Optimization of a single radiopharmaceutical; and
- Using existing facilities and equipment available in the radiopharmacy of a South African teaching hospital.

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**Research Questions**

The research was designed around the following research questions:

1. Can the development of in-house methods for an automated synthesis unit provide the foundation to expanding the current range of radiopharmaceuticals in a clinical setting?
2. Which clinically acceptable radical scavengers can specifically improve the radiochemical purity of [$^{68}$Ga]Ga-NOTA-UBI?
3. Which labelling parameters are affected in the conversion of a manual radiosynthesis method to an automated one?
4. What has been reported on the acute and chronic toxicity of HEPES to animals and humans and are the strict limits imposed on HEPES as a buffering agent in radiopharmaceutical manufacturing by the European and British Pharmacopoeias necessary?
5. Can a suitable thin-layer chromatography method be identified that can be used to detect and quantify the presence of gallium-68 colloidal impurities in [$^{68}$Ga]Ga-NOTA-UBI?

**Brief overview of the dissertation**

This dissertation has been compiled in a hybrid format. It comprises of published and unpublished articles and one chapter. Following this introductory chapter, the first research article describes the development and optimization of two automated synthesis methods (Chapter 2). The second research article compares the labelling characteristics of the automated synthesis methods to that of the manual method on which the automated method was based (Chapter 3). The third research article describes the effect of radical scavengers on the radiochemical purity of the in-house developed synthesis method (Chapter 4). A technical note presents the currently reported evidence on the acute and chronic toxicity of 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) in animals and humans and includes a critical debate on the very strict pharmacopoeial limits for this compound in radiopharmaceutical production (Chapter 5). Chapter 6 presents a series of experiments using

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thin-layer chromatography techniques as quality control procedures to determine the presence of colloidal gallium-68 impurities that may be present in [$^{68}$Ga]Ga-NOTA-UBI 29-41 preparations. Chapter 7 provides an overall summary of the achieved results, the study limitations and recommendations as well as the study conclusion.

Note that the articles and references are presented in the format prescribed by the respective journals' guidelines.

All studies were approved by the Stellenbosch University Health Research Ethics Committee (S15/10/235). This research is purely based on experimental laboratory work that does not involve administration to animals or human beings.

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# Chapter 2

# Development of an automated synthesis method for $^{68}$Ga-labelled ubiquicidin 29-41

### Rationale for research covered in this chapter

The use of automated synthesis modules has in recent years become an important aspect of the production of radiopharmaceuticals. Such modules make it easier for production facilities to comply with GMP guidelines. The automated labelling method is usually based on an optimized manual method used during the development phase of the radiopharmaceutical. The article below describes the development of three automated synthesis methods for the labelling of gallium-68 ubiquicidin using a Scintomics GRP synthesis module.

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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.

**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

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vitro labelled 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 specific detection of bacterial infections, but also be able to distinguish between sterile inflammation and bacterial infections.

**Theory**

Ubiquicidin (UBI) is a human antimicrobial peptide and synthetic derivatives of this peptide have been suggested as a possible agents 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

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well described [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 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 described 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.

## Experimental

### 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 100 µg NOTA-UBI was used to improve radioyield of

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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). C18 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 until 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 C18 SEP PAK cartridge and filtered through a 0.22µm sterile filter.

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

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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 of 1.5 ml of 1.5 M HEPES buffer (pH = 5.0) to render a buffered eluate mixture with a pH 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 C18 SEP-PAK cartridge. $^{68}$Ga-labelled NOTA-UBI was desorbed from the C18 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.

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## 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 measuring the ITLC strip cut 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 C18 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 C18 column (Waters, USA) was used as the stationary phase for all analyses. [$^{68}$Ga]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 $^{68}$Ga-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.

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*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]Ga-NOTA-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.

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**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 C18 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 C18 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.

**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.

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*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 2.1**) 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.

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**Table 2.1** 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

## 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.

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 2.2** for a comparison of manual and automated methods). Sodium acetate (1.0 M) and

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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 2.2** presents a comparison of the manual method with the three automated labelling methods.

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**Table 2.2** Comparison of the manual with the automated radiolabelling methods

| | Manual (Reference) (n≥9) | Automated (Scintomics module) | | |
|---|---|---|---|---|
| | | Method 1 (n=6) | Method 2 (n=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 C18 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 C18 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

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*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 ± 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 C18 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 ± 9 minutes. (See **Figure 2.1a**).

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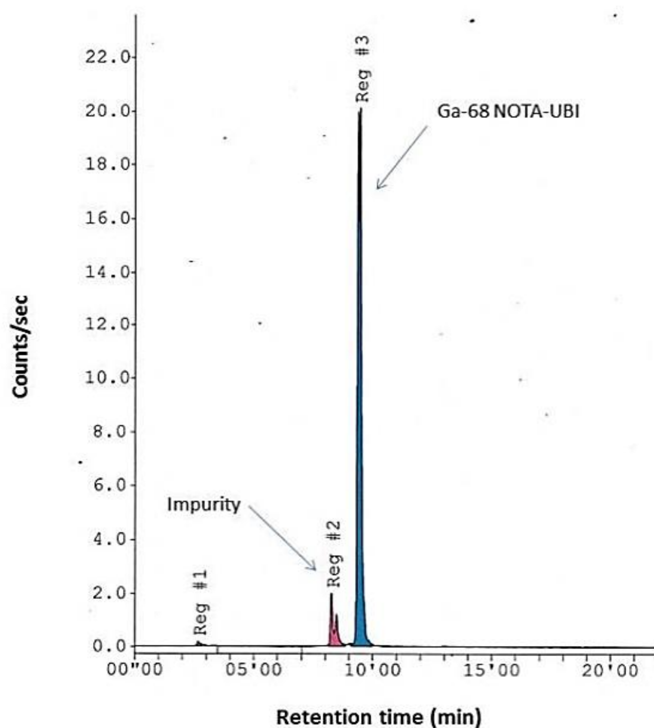


**Figure 2.1a** Radio HLPC of [$^{68}$Ga]Ga-NOTA-UBI using fractional elution and HEPES buffer

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 a 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

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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 C18 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 2.1b**. The presence of a radiochemical impurity due to radiolysis was again observed at retention time ± 9 minutes.

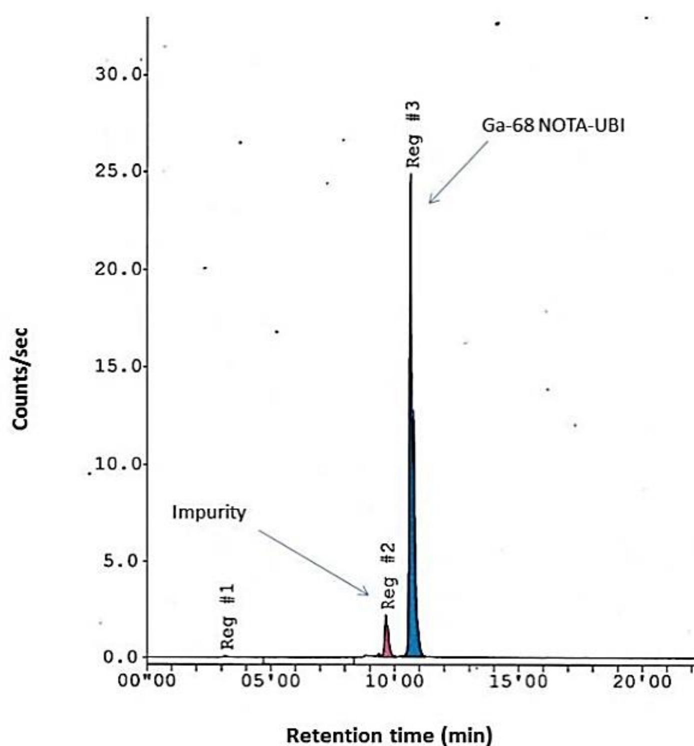


**Figure 2.1b** Radio-HPLC of [$^{68}$Ga]Ga-NOTA-UBI using fractional elution and sodium acetate buffer

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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 for higher levels of metal contaminants to elute from the generator column. This is considered an important factor, especially when $^{68}Ge/^{68}Ga$-generators are eluted in a large volume and at a low pH [38]. 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* [26].

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 C18 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 2c**. The radiochemical impurity at retention time ± 9 minutes was again present.

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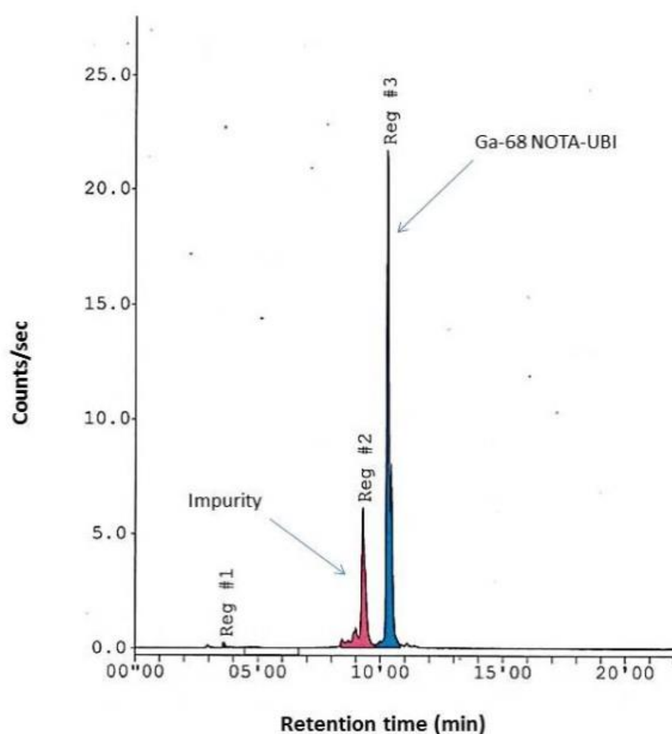


**Figure 2.1c** Radio-HPLC of [$^{68}$Ga]Ga-NOTA-UBI using sodium acetate buffer and cationic pre-purified eluate

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 2.2 a - 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.

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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 2.3** provides a summary of the important labelling parameters using scavengers and alternative buffers.

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**Table 2.3** 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<br>170 µl ethanol | 350 µl 1.4% ascorbic acid |
| 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 C18 at EOS (MBQ) | 11.4 ± 10.0 | 14.2 ± 7.4 |
| Residual activity on P-$SH^+$ cartridge at EOS (MBq) | - | 129 ± 30 |
| Retained C18 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 2.2a – 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.

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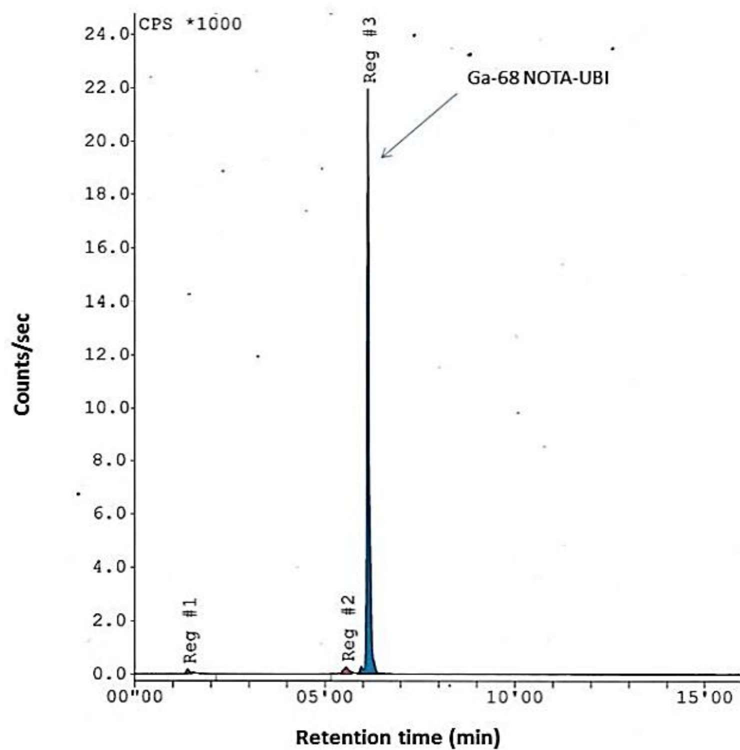


**Figure 2.2a** Radio-HPLC of [$^{68}$Ga]Ga-NOTA-UBI using fractional elution and ammonium formate buffer with the addition of ascorbic acid

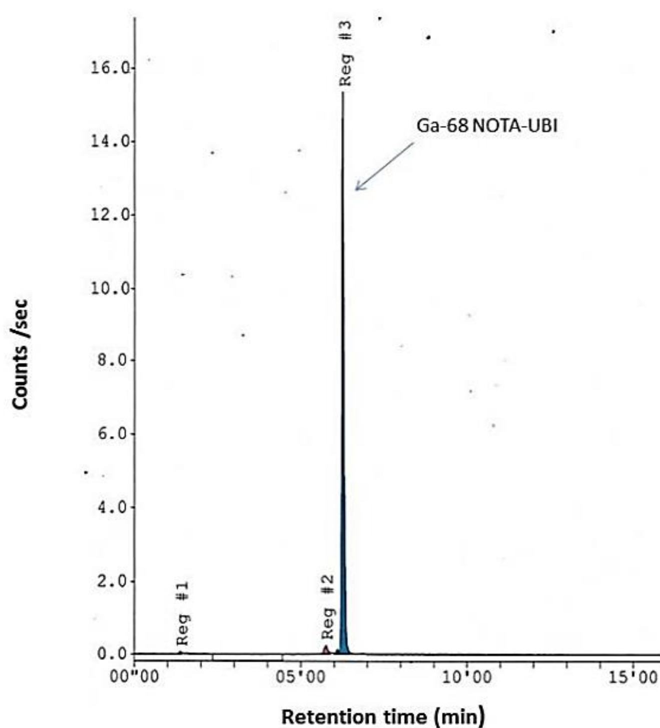


**Figure 2.2b** Radio-HPLC of [$^{68}$Ga]Ga-NOTA-UBI using cationic purification fractional elution and sodium acetate buffer with the addition of ascorbic acid and ethanol

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*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 2.3** illustrates an example of a typical readout from a [$^{68}$Ga]Ga-NOTA-UBI ITLC analysis. Note a wider region of interest was chosen for region 1 to compare this method with a "cut and measure" method using a dose calibrator.

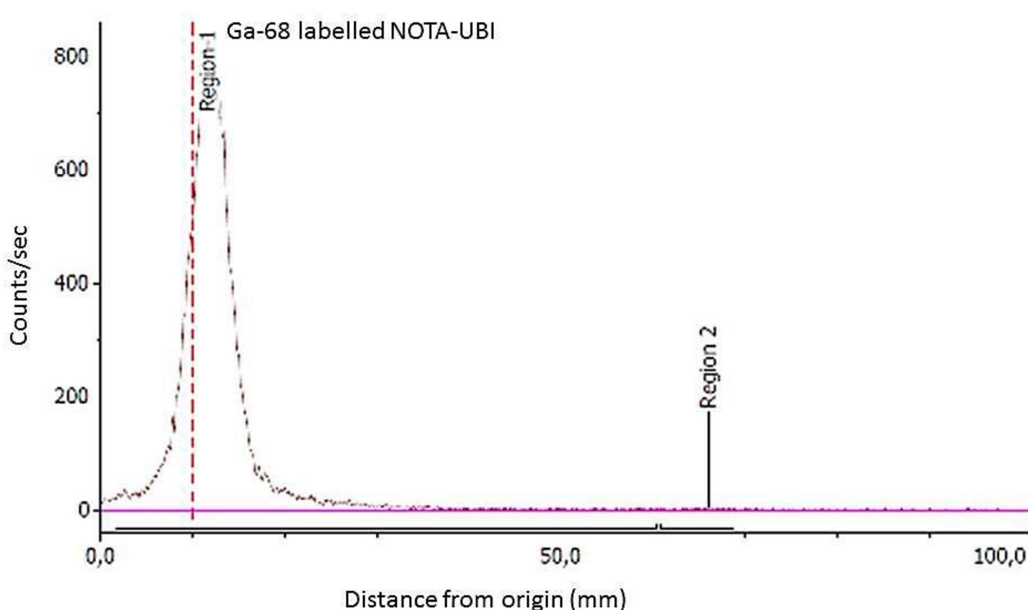


**Figure 2.3** ITLC analysis of $^{68}$Ga-NOTA UBI. Region 1 is [$^{68}$Ga[Ga-NOTA-UBI and region 2 is free gallium-68.

*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

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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

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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 al. intravenously injected a dose of 400 mg of ubiquicidin 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

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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 C18 SEP-PAK cartridge, ITLC could not confirm if all colloidal impurities are indeed removed during this step. It has been reported, for $^{68}$Ga-peptide synthesis, that C18 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.

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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 50 µg NOTA-UBI (100 µg in the study investigating scavengers using method 2) and in varying buffer volumes was used for all labelling experiments. 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 C18 cartridge.
- Washing of the C18 cartridge with PBS.
- Desorption of labelled [$^{68}$Ga]Ga-NOTA-UBI from the C18 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-

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UBI with a high degree of consistency and robustness, satisfying GMP requirements for the production of radiopharmaceuticals.

## Conclusion

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.

**Conflict of interest:** The authors declare that they have no conflict of interest.

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## Summary

Three automated labelling methods for gallium-68 ubiquicidin were succesfully developed. Two methods were further optimized using radical scavengers to reduce the fomation of radiolytic impurities. These methods were proven to be suitable for the routine production of this radiopharmaceutical in a GMP compliant manner.

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# Chapter 3

# The effect of radical scavengers on the radiolabelling characteristics of gallium-68 ubiquicidin

### Rationale for research covered in this chapter

Radical scavengers are often used to increase the radiochemical purity of radiopharmaceuticals. These radical scavengers can reduce the formation of radiolytic impurities caused by radiolysis and oxidation of the methionine group of methionine containing peptides.

Gallium-68 ubiquicidin (29-41) is an example of a methionine containing peptide. It is evident from the experimental work conducted in the previous chapter that there was a clear indication for the use radical scavengers to reduce radiolytic impurities. In this chapter the impact of several commonly used radical scavengers on the labelling characteristics of gallium-68 ubiquicidin is evaluated and discussed.

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## Impact of radical scavengers on the radiolabelling characteristics and purity of gallium-68-ubiquicidin

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**Abstract**

Radical scavengers have been widely used to reduce radiolysis in the labelling of radiopharmaceuticals. The purpose of this study was to evaluate the effectiveness of several commonly used scavengers in the labelling of [$^{68}$Ga]Ga-NOTA-UBI. We also compare our results with recent reports that have shown an increased interest in the use of ethanol as a scavenger and its beneficial effect on several labelling characteristics for some gallium-68 compounds. Results from our study indicate that, for [$^{68}$Ga]Ga-NOTA-UBI, a combination of at least two scavengers is required to reduce radiolysis to an acceptable level and render a final labelled product with a radiochemical purity ≥ 95%.

**Introduction**

Recent years have seen an unprecedented growth in the use of gallium-68 ($^{68}$Ga) for the labelling of radiopharmaceuticals for research and clinical application. The introduction of reliable $^{68}$Ge/$^{68}$Ga-generators significantly contributed to this growth because a major advantage of using a generator lies in the on-demand availability of a positron emitting radionuclide such as $^{68}$Ga without the need of an on-site cyclotron.[1,2]

Since $^{68}$Ge/$^{68}$Ga-generators became commercially available, many $^{68}$Ga-labelled radiopharmaceuticals have been successfully introduced into clinical practice.[2–7] $^{68}$Ga-labelled DOTA-TATE, DOTA-NOC and PSMA are but a few that have had a major effect in the clinical handling of cancers such as neuro-endocrine tumours and prostate cancer.[8–11]

Ubiquicidin (29-41) is a fragment of an antimicrobial peptide that can be labelled with $^{68}$Ga and is investigated as an agent for imaging infections using PET.[12–16] Recently we reported on development of an automated synthesis method for the labelling of $^{68}$Ga-ubiquicidin (29-41) (**Figure 3.1**).[17] Initial

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development and testing of an automated synthesis method revealed the formation of an impurity most likely caused by radiolysis.

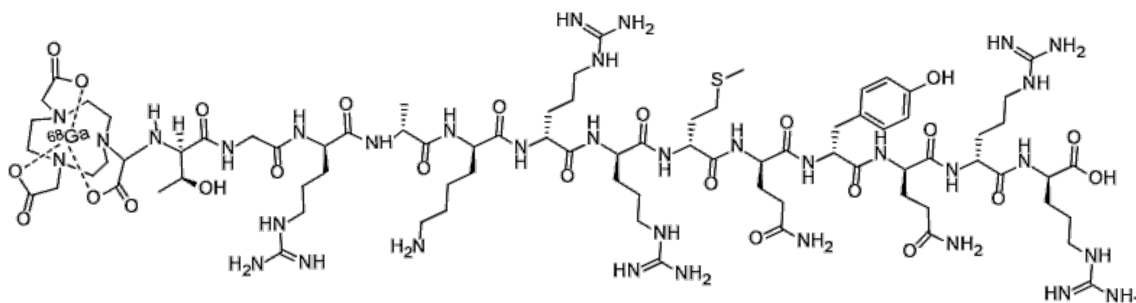

**Figure 3.1** The chemical structure of [$^{68}$Ga]Ga-NOTA-UBI (29-41)

Radiolysis is dependent on factors like total radioactivity concentration and the chemical structure of the radiopharmaceutical.[18] The high positron emitting fraction (maximum energy: 1.92 MeV and mean energy: 0.89 MeV) of $^{68}$Ga may be responsible for an increased risk of radiolysis.[10] The presence of the hydroperoxyl and hydroxyl free radicals (•OOH and •OH) is caused by high energy β-particles emitted by $^{68}$Ga.[10] When high levels of radioactivity are used for radiolabelling, radiolysis cannot always be prevented without the use of free radical scavengers. This may be especially important when $^{68}$Ga is produced using a cyclotron.[19–22] Peptides containing methionine, such as ubiquicidin (TGRAKRRMQYNRR) and gastrin, seem to be especially prone to radiolysis. During synthesis, auto-oxidation to sulphoxides is a common occurrence.[23]

The use of radical scavengers has been well described. [10,24–30] Ascorbic acid, ethanol, gentisic acid and L-methionine have all been suggested as scavengers to improve radiochemical purity.[30,31] Other less commonly used scavengers include sodium thiosulphate, sodium nitrate and sodium iodide.[28] The use of formic acid combined with a scavenger such as ascorbic acid has also been described.[32]

Improved radiolabelling of [$^{68}$Ga]Ga-DOTA-TOC by the addition of ethanol to the labelling mixture has recently been reported.[33] Besides an improvement in radiochemical purity by reducing radiolysis, it also significantly improved radiochemical yield. These studies have shown that increasing the ethanol content of the labelling mixture from 0 - 30% (v/v) when using only 10 µg

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DOTA-TOC precursor as opposed to 50 µg recommended by the manufacturer, resulted in an increase in radiochemical yield from 24% to 96% at 70 °C. The use of ethanol to improve radiolabelling with automated synthesis modules has also been demonstrated by Meisenheimer *et al*. They report using ethanol in increased concentrations of 10 - 40% or 0 - 30% (v/v), which led to an increase in RCP, yield and specific activity of [$^{68}$Ga]Ga-DOTA-TOC.[24] In contrast we found that using ethanol (10% v/v) in the radiolabelling of [$^{68}$Ga]Ga-NOTA-UBI, the radiochemical purity (RCP) was well below our acceptance criteria of 95%.

Previously we reported on the introduction of a cationic pre-purification method for the labelling of [$^{68}$Ga]Ga-NOTA-UBI using an automated synthesis module.[17] This method has proven to yield metal free $^{68}$Ga eluates using a 5 M NaCl solution; however, the average RCP for [$^{68}$Ga]Ga-NOTA-UBI was only 65 ± 3% (n = 3). Whilst there were negligible amounts of unchelated $^{68}$Ga detected with radio-HPLC, unfortunately 33 ± 3% radiolytic impurities were detected in the final product solution. The current work demonstrates the effectiveness of well-known scavengers to reduce radiolytic impurities during the automated synthesis of [$^{68}$Ga]Ga-NOTA-UBI.

## Experimental

### *Materials*

The automated labelling procedures were performed using a Scintomics GRP synthesis module (Scintomics, Germany). Freeze-dried 1,4,7-triazacyclononane-1,4,7-triacetic acid ubiquicidin (NOTA-UBI) obtained from GL Biochem (China) or ABX (Germany) was dissolved in ultrapure water (18.5 Ω) and subdivided in 100 µl aliquots (1 µg/µl) and frozen at −20 °C. 100 µg of frozen NOTA-UBI was used for each of the labelling methods. $^{68}$Ga was obtained by eluting a $^{68}$Ge/$^{68}$Ga-generator (iThemba LABS, South Africa) with 0.6 M HCl (ABX, Germany). A cationic pre-purification method with PS-$H^+$ cartridges (ABX, Germany) was used to purify the $^{68}$Ga-eluate for all labelling procedures. C18 Sep-Pak cartridges (Waters, USA) were pre-conditioned on the

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synthesis module at the start of the synthesis using 5 ml HPLC-grade ethanol (Merck, USA). Sodium acetate buffer (1.0 M) was prepared using sodium acetate trihydrate (Sigma Aldrich, 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), gentisic 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 as an alternative to sodium acetate. Sodium chloride (5 M) was prepared using pharmaceutical grade sodium chloride (Merck, USA). HPLC analyses were carried out using HPLC-grade acetonitrile (Merck, USA) and trifluoroacetic acid (Sigma Aldrich, Germany) as mobile phases.

*Radiolabelling*

The $^{68}Ge/^{68}Ga$-generator eluate pre-processing method was adopted from a method published by Martin *et al.* [34] Briefly, the $^{68}Ge/^{68}Ga$-generator was eluted with 10 ml of a 0.6 M HCl solution and the eluate was diluted with 8 ml ultrapure water. The entire volume was then slowly passed over a PS-$H^+$ cartridge (ABX, Germany) to retain the $^{68}Ga$-activity and remove possible metal contaminants. Purified $^{68}Ga$ was recovered from the PS-$H^+$ cartridge using 1.5 ml of a 5.0 M NaCl solution. This solution was then transferred into the reaction vessel which contained 100 µg NOTA-UBI in 1.5 ml of 1.0 M acetate solution along with a radical scavenger or combination of scavengers. We used a standard scavenger mass of 5 mg for both ascorbic- and gentisic acid for all labelling procedures as suggested by Mueller *et al.* [31] **Table 3.1** presents a summary of the different scavengers or combination of scavengers resulting in 5 different radiolabelling methods. All mixtures were heated for 10 minutes at 90 °C, cooled for one minute and purified using a C18 Sep-Pak cartridge as previously described by Ebenhan et al.[13] [$^{68}$Ga]Ga-NOTA-UBI was desorbed from

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the C18 Sep-Pak cartridge using 2 ml ethanol/saline (50% v/v) and transferred into a sterile vial. The final product was further diluted to 15 ml with phosphate buffered saline (PBS) to provide a product that would be suitable for intravenous injection.

**Table 3.1** Summary of scavengers used for each labelling method

| M | Buffer volume | Radical scavenger volume | Total volume # during radiosynthesis | NOTA-UBI concentration |
|---|---|---|---|---|
| **1** | 1 M sodium acetate 1.5 ml | 1.4% ascorbic acid 0.350 ml | 3.350 ml | 30 µg/ml |
| **2** | 1 M sodium acetate 1.5 ml | 1.6% gentisic acid 0.310 ml | 3.310 ml | 30 µg/ml |
| **3** | 1 M sodium acetate 1.5 ml | ethanol 10% v/v 0.310 ml | 3.310 ml | 30 µg/ml |
| **4** | 1 M sodium acetate 1.5 ml | 1.4% ascorbic acid, ethanol 10% v/v 0.350 ml; 0.170 ml | 3.520 ml | 28 µg/ml |
| **5** | 1 M ammonium formate 2.0 ml | 1.4% ascorbic acid 0.350 ml | 3.850 ml | 26 µg/ml |

# total volume includes 1.5 ml 5 M NaCl eluant required to desorb $^{68}$Ga-activity from PS-$H^+$ cartridge; M = method

*Radioanalysis*

*Instant thin-layer chromatography (ITLC)*

Instant thin-layer chromatography was performed using the same method as previously described by Breeman *et al*. using a glass microfiber chromatography medium impregnated with silica gel (ITLC-SG, Varian, USA).[35] The mobile phase consisted of 0.1 M sodium citrate (pH = 5.0). Distribution of radioactivity on the strip was determined with a radio-chromatographic scanner (Lablogic, United Kingdom).

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*HPLC analysis*

Radio-HPLC analysis was performed using a Shimadzu Nexera XR HPLC system (Shimadzu, Japan) with a variable wavelength PDA UV-detector and a Raytest gamma detector (Raytest, Germany). A Waters C18 Symmetry analytical column (4.6 × 250 mm × 4.6 mm × 5 µm, Waters, USA) was used as the stationary phase for all analyses. 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 2 ml/min. The retention time (RT) for free gallium-68 and [$^{68}$Ga]Ga-NOTA-UBI was 2.0 – 3.0 minutes and 6.0 – 7.0 minutes respectively. Radiolytic impurities eluted either just before or after the main peak.

## Results and Discussion

Previous research performed on [$^{68}$Ga]Ga-NOTA-UBI highlighted the need for the use of a radical scavenger to reduce the effect of radiolysis.[17] The aim of this study was to evaluate the effect of several commonly used scavengers on the radiochemical purity of [$^{68}$Ga]Ga-NOTA-UBI and their effect on radiolysis. **Table 3.2** gives a summary of the labelling characteristics for [$^{68}$Ga]Ga-NOTA-UBI comparing method 1 to 5 using three commonly used radical scavengers.

**Table 3.2** Comparison of labelling characteristics for [$^{68}$Ga]Ga-NOTA-UBI using different combinations of radical scavengers and buffering solution (n=3)

| | **Method 1** | **Method 2** | **Method 3** | **Method 4$^{\#}$** | **Method 5** |
|---|---|---|---|---|---|
| Buffer | NaOAc$^{\dagger}$ | NaOAc$^{\dagger}$ | NaOAc$^{\dagger}$ | NaOAc$^{\dagger}$ | $NH_4HCO_2$$^{\ddagger}$ |
| Scavenger | 1.4% AA | 1.6% GA | 10% EtOH | 1.4% AA/ 10% EtOH | 1.4% AA |
| Generator age (days) | 9 ± 1 | 14 ± 1 | 7 ± 1 | 72 ± 6 | 12 ± 1 |
| Activity (MBq) | 1539 ± 119 | 1710 ±42 | 1687 ± 117 | 1467 ± 85 | 1740 ± 42 |
| Radiochemical purity (ITLC) (%) | 99.0 ± 0.7 | 99.4 ± 0.2 | 99.3 ± 0.2 | 99.3 ± 0.1 | 99.0 ± 0.3 |
| Radiochemical purity (HPLC) (%) | 90.7 ± 2.7 | 85.0 ± 1.5 | 89.1 ± 1.4 | 97.3 ± 0.4 | 90.0 ± 4.8 |
| Activity yield (MBq)* | 600 ± 38.4 | 830 ± 71.4 | 141 ± 17.6 | 580 ± 99.3 | 799 ± 109.0 |
| Radiochemical yield (%) | 61 ± 5.4 | 76 ± 7.5 | 13 ± 1.8 | 57.3 ± 3.8 | 72 ± 10.3 |
| Residual activity on PS-H$^{+}$ (MBq)* | 112 ± 20.5 | 87.1 ± 3.5 | 95.9 ± 8.8 | 129 ± 29.6 | 110.7 ± 16.6 |
| C18-SPE retained activity (MBq)* | 32.3 ± 12.7 | 22.7 ± 4.4 | 11.9 ± 7.0 | 14.2 ± 7.3 | 12.6 ± 5.9 |
| C18-SPE retained activity (%) | 3.3 ± 1.4 | 2.1 ± 0.2 | 1.1 ± 0.4 | 2.5 ± 0.6 | 1.2 ± 0.6 |

Notes: AA) ascorbic acid, GA) gentisic acid, EtOH) ethanol

* measured at the end of the synthesis; C18 SPE – disposable C18 based solid-phase extraction cartridge; PS-H$^{+}$ - disposable cartridge with a strong cation exchange resin

$^{\#}$ data from previous study, time from previous elution/synthesis ≥ 4 h.

$^{\dagger}$Sodium acetate; $^{\ddagger}$Ammonium formate

It is important to note that radio-ITLC results showed a RCP of > 98% for all labelling methods as opposed to a RCP range of 85.0 ± 1.5 - 97.3 ± 3.0 calculated with radio-HPLC. **Figure 3.2** represents a typical example of a radio-ITLC chromatogram.

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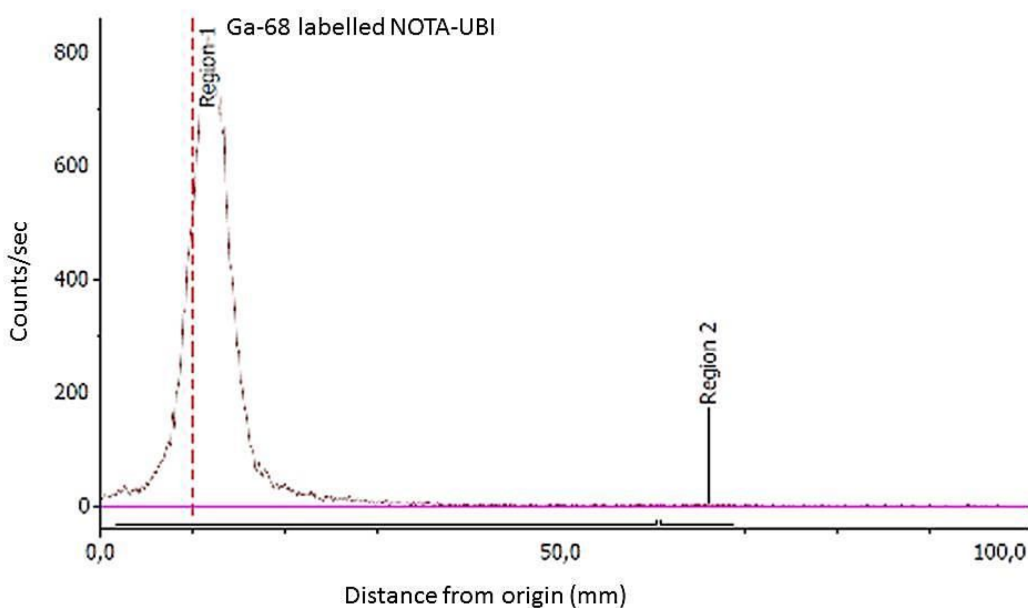


**Figure 3.2** Representative radio-ITLC chromatogram for quantitative analysis of [$^{68}$Ga]Ga-NOTA-UBI following C18-based purification. Radio-ITLC stationary phase ITLC-SG paper; mobile phase: 0.1M sodium citrate (pH 5); Chromatogram region 1: [$^{68}$Ga]Ga-NOTA-UBI; Region 2: detection of possible unchelated $^{68}$Ga.

The discrepancy in RCP between radio-HPLC and radio-ITLC occurred because the thin-layer paper-based chromatography technique lacked the capability to resolve peaks of [$^{68}$Ga]Ga-NOTA-UBI and its closely retained radiolytic impurities. Furthermore, this method[35] could not be used to detect the presence of any colloidal impurities. These results suggest that radio-ITLC analysis may be limited and unreliable to accurately determine the RCP of [$^{68}$Ga]Ga-NOTA-UBI following our radiosynthesis method.

Radio-HPLC chromatograms from each of the scavenger-buffer combinations are shown in **Figure 3.3**.

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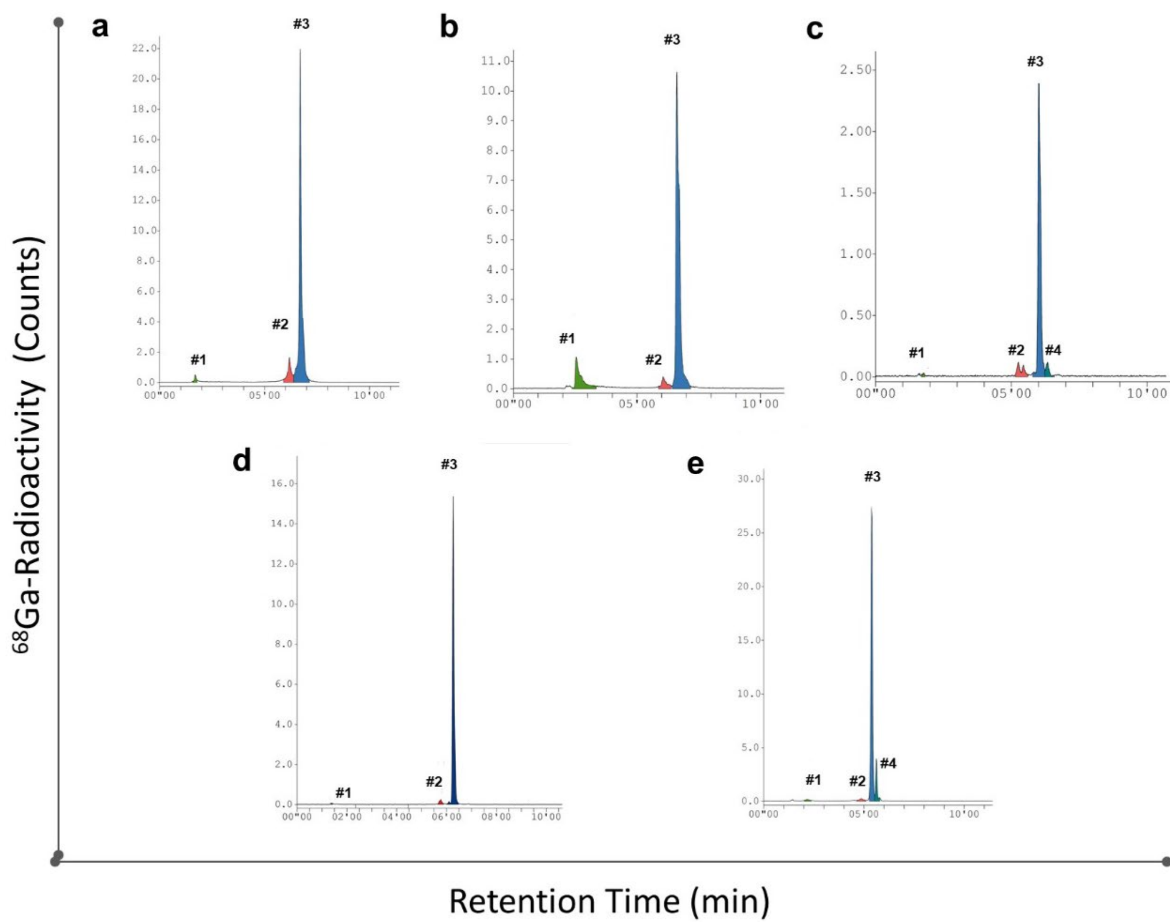


**Figure 3.3** Typical radio-HPLC chromatograms of [$^{68}$Ga]Ga-NOTA-UBI demonstrating the impact of different scavengers. #1= free $^{68}$Ga; #2 & #4 = radiolytic impurities; #3 = [$^{68}$Ga]Ga-NOTA-UBI, a) ascorbic acid scavenger, b) gentisic acid scavenger c) ethanol scavenger d) ascorbic acid and ethanol scavengers e) ascorbic acid in ammonium formate buffer

The quantitative analysis of the % RCP of [$^{68}$Ga]Ga-NOTA-UBI seen on radio-HPLC for each of the radiolabelling methods in **Figure 3.3** is outlined in **Table 3**.

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**Table 3.3** Summary of peak quantification of radio-HPLC chromatograms

| | % Radiochemical purity | | |
|---|---|---|---|
| **Buffer / scavenger(s)** | **Free - $^{68}$Ga** | **Radiolytic impurities *** | **[$^{68}$Ga]Ga-NOTA-UBI **** |
| 1- Sodium acetate / 1.4% AA | 0.1 - 2.1 | 6.5 - 11.0 | 86.9 - 93.4 |
| 2- Sodium acetate / 1.6% GA | 0.1 - 12.7 | 4.4 - 13.7 | 82.8 - 86.3 |
| 3- Sodium acetate /10% EtOH | 0.1 - 1.3 | 9.3 - 12.8 | 87.7 - 90.7 |
| 4- Sodium acetate / 1.4% AA + 5% EtOH | 0.3 - 0.4 | 1.9 - 2.7 | 96.9 - 97.8 |
| 5- Ammonium formate / 1.4% AA | 0.8 - 1.5 | 2.7 -13.2 | 85.3 - 96.5 |

Notes: AA) ascorbic acid, GA) gentisic acid, EtOH) ethanol, *) values represent the sum of impurities retaining at 5.0 min (#1) and 6.1 min (#2). **) percentage radiochemical purity (HPLC; n=3)

*Impact of ascorbic acid as scavenger*

There is ample evidence in the literature of the effectiveness of ascorbic acid as a radical scavenger on its own or in combination with other scavengers.[10,18,21,22,25,31] Our results indicate that, using ascorbic acid on its own in the labelling of [$^{68}$Ga]Ga-NOTA-UBI, radiolysis was not sufficiently inhibited. **Figure 3.3a** represents a typical radio-HPLC chromatogram where ascorbic acid was used as scavenger. Impurities seen at retention time ~ 6.1 min on all radio-HPLC chromatograms represent on average 8.0 ± 2.1% (n = 3) of total counts.

The small peak seen at retention time ~ 1.8 min is most likely due to unchelated $^{68}$Ga. [$^{68}$Ga]Ga-NOTA-UBI eluted at ~ 6.7 minutes. The average RCP on radio-HPLC was 90.7% which is well below our in-house criteria of ≥ 95%. Radio-yield was acceptable with an average of 61 ± 5.4%. Average unchelated $^{68}$Ga and radiolytic impurities were 1.3 ± 0.9 and 8.0 ± 2.1 respectively. Radio-HPLC results indicate that the majority of impurities are likely due to due to radiolysis.

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*Impact of gentisic acid as scavenger*

Gentisic acid as the only scavenger did not achieve a RCP ≥ 95% on radio-HPLC. Radio-HPLC analyses showed two impurities at retention time (RT) ~ 2.5 and 6.1 min. respectively. The impurity at RT at ~ 2.5 min is most probably also due to unchelated $^{68}$Ga while the impurity seen at RT ~ 6.1 min is due to a radiolytic impurity formed during labelling (**Figure 3.3b)**. We noted a relatively high presence of unchelated $^{68}$Ga in two of the syntheses, despite optimizing the pH to 3.5 – 4.0. Interestingly, when no unchelated $^{68}$Ga was observed on radio-HPLC, the impurity due to radiolysis at RT ~6.1 min increased significantly. It is not clear if there is a correlation between unchelated $^{68}$Ga and formation of radiolytic impurities using gentisic acid as scavenger.

The presence of relatively high levels of unchelated $^{68}$Ga as well as varying levels of radiolytic impurities (ranging from 4.4 – 13%) in the labelled product, suggest that gentisic acid on its own is not a suitable radical scavenger. The average radio-yield 76 ± 7.5%, the highest of all scavengers tested in this study. The average percentage of impurities related to unchelated $^{68}$Ga and radiolysis were 7.5 ± 5.4% and 7.5 ± 4.4%, respectively. The average RCP was 85 ± 1.5% (n = 3).

*Impact of ethanol as scavenger*

The use of ethanol as a scavenger in the labelling of somatostatin analogues such as [$^{68}$Ga]Ga-DOTA-TOC, has recently attracted considerable interest. Not only does the addition of ethanol increase RCP of this radiopharmaceutical, but it also increases yield. As mentioned elsewhere, studies have also shown that it also reduces the peptide mass required for labelling, therefore increasing the specific activity of the radiopharmaceutical. Our work with [$^{68}$Ga]Ga-NOTA-UBI has shown, when ethanol was used in concentrations of 10% v/v during labelling of ubiquicidin, radio-yield significantly decreased, despite adjusting the pH to 3.5 – 4.0 with sodium acetate buffer. The average yield, using 10% v/v ethanol, decreased to only 13 ± 1.8%. The average RCP was 89.1

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± 1.4%. Reducing the ethanol content to 5 - 7% (v/v) did improve radio-yield but not radiochemical purity. Radio-HPLC analyses (**Figure 3.3c**) also indicate that most impurities seen on radio-HPLC are due to radiolysis with minimal presence of unchelated $^{68}Ga$. A possible additional impurity eluting at RT ~ 6.3 min was observed in one of the syntheses. It is not clear if this impurity is also due to radiolysis, but we grouped it together with the radiolytic impurities. Our results indicate that ethanol alone did not reduce radiolytic impurities to an acceptable level. Furthermore, we did not observe an increase in yield or specific activity as reported by Meisenheimer *et al*. and Eppard *et al.* for [$^{68}$Ga]Ga-DOTA-TOC.[24,33] The average percentage unchelated $^{68}Ga$ and radiolytic impurities were 0.4 ± 0.6 and 10.5 ± 1.6% respectively.

The results from this research also suggest that the side products observed on radio-HPLC are more likely due to radiolysis and not the result of oxidation of methionine during the heating step. Lower activities used in the manual labelling method did not result in the formation of the side products. Our findings further suggest that the use of ethanol to reduce radiolysis can be very dependent on the type of peptide.

*Impact of ascorbic acid and ethanol as combined scavengers*

The best RCP, determined by radio-HPLC, was achieved using a combination of ascorbic acid (5 mg) and ethanol (5% v/v). Using this combination, the RCP was consistently higher than our in-house specification of ≥ 95%. The average percentage unchelated $^{68}Ga$ and radiolytic impurities were 0.4 ± 0.05% and 2.4 ± 0.3% respectively (n = 3).

As can be seen on the radio-HPLC chromatogram (**Figure 3.3d**), impurities due to radiolysis were less than 3% when ascorbic acid and ethanol were used in combination. The presence of unchelated $^{68}Ga$ was < 1%. This combination furthermore resulted in acceptable radio-yields of 57 ± 3.8%. The average RCP was 97.3 ± 0.4%.

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*Ascorbic acid as scavenger in ammonium formate buffer*

As mentioned earlier, ammonium formate and ascorbic acid have also been successfully used to reduce radiolysis using a fractional elution method.[17] Ammonium formate is not regularly used as a buffer in our institution. However, based on the success to reduce radiolysis in combination with ascorbic acid in the fractional elution method, we opted to test this buffer scavenger combination using the cationic pre-purification method. Our results indicate that this combination was not effective in reducing radiolysis to an acceptable level. Radiochemical impurities seen on radio-HPLC (**Figure 3.3e**) were mostly due to radiolysis. Additionally, we observed the presence of impurities eluting after the main peak (RT ~ 5.7 min) on two syntheses. The average percentage unchelated $^{68}$Ga and radiolytic impurities were 1.5 ± 0.6% and 8.5 ± 4.4% respectively (n = 3). Radiochemical yield was on average 72 ± 10.3%.

In summary, the results of this study demonstrated a marked reduction of radiolytic impurities with a combination of 1.4% ascorbic and 5% ethanol , therefore an improvement of the RCP for [$^{68}$Ga]Ga-NOTA-UBI to meet our in-house release criteria of ≥ 95%.

## Recommendations

The purpose of this limited study was not to characterize the radiolytic impurities seen on radio-HPLC. It is, however, possible that our radio-HPLC method was not sufficiently sensitive to resolve other impurities close to the main peak. A further investigation into the identification and characterization of side-products should be considered, using a similar approach to that adopted by Mu *et al*.[36] Our study also did not investigate all possible combinations of scavengers or buffer-scavenger combinations. This may produce different results in reducing radiolysis. The scavengers included in this study are all safe for human administration and do not require additional analytical testing post synthesis. Further investigation into a more reliable TLC method for [$^{68}$Ga]Ga-NOTA-UBI should also be considered. This may include an investigation into different stationary phases

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and/or mobile phases similar to the work published by Larenkov and Maruk for [$^{68}$Ga]Ga-DOTA-TOC, [$^{68}$Ga]Ga-DOTA-TATE and [$^{68}$Ga]Ga-RGD$_2$. [37]

**Conclusion**

A radical scavenger combination of 1.4% ascorbic acid and ethanol (5% v/v) achieved the best results in reducing radiolysis in the labelling of [$^{68}$Ga]Ga-NOTA-UBI using a pre-purification method with 5 M NaCl. Our results also indicate that ethanol on its own was not effective in limiting the presence of radiolytic impurities. Increasing the ethanol content to > 5% significantly reduced radio-yield. Our results furthermore suggest that the use of ethanol as the only scavenger may be peptide specific.

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**Summary**

Radical scavengers were successfully used to decrease the presence of radiolytic impurities during the labelling of gallium-68 ubiquicidin. It was necessary is use a combination of two radical scavengers to effectively reduce radiolysis and ensure a radiochemical purity of greater than 95%.

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# Chapter 4

# A comparison of labelling characteristics of manual and automated synthesis methods for gallium-68 labelled ubiquicidin

### Rationale for the research covered in this chapter

The advantages of using an automated labelling method, rather than a manual method, were extensively discussed in the previous chapters. Automated labelling methods are usually based on an optimized manual method used during the development phase of radiopharmaceuticals, but they are not merely a scale-up of the original method performed by a piece of equipment.. Besides the higher activities used, the ingredients in the labelling reaction can differ. It is therefore important to ensure that important labelling characteristics are not negatively affected by the automation process.

In this chapter, the labelling characteristics of a manually prepared product are compared with those resulting from two automated methods developed during this research. The important similarities and differences in labelling characteristics between the manual and automated methods are highlighted.

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# A comparison of labelling characteristics of manual and automated synthesis methods for gallium-68 labelled ubiquicidin

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**Highlights**

- Optimized manual and automated synthesis methods for [$^{68}$Ga]Ga-NOTA-UBI were compared.
- Automated methods were more robust than a manual method.
- Operator radiation exposure was considerably less for automated synthesis methods.

## Abstract

Gallium-68 labelled 1,4,7-triazacyclononane-1,4,7-triacetic acid ubiquicidin (NOTA-UBI) is currently investigated as a PET radiopharmaceutical for the imaging of infections. The aim of this

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study was to compare the labelling characteristics of an optimized manual radiosynthesis method with those of optimized automated synthesis methods. Data from this study suggest that automated radiosynthesis of [$^{68}$Ga]Ga-NOTA-UBI provides a higher degree of robustness and repeatability than the manual method. Our results also suggest that for our full-scale automated synthesis, radical scavengers should be considered to reduce radiolysis. Automated synthesis methods have the advantage of markedly reducing radiation exposure to operators. Standardised automation also makes the synthesis more reliably compliant with Good Manufacturing Practice guidelines.

**Keywords:** Ga-68, automated synthesis, manual synthesis, NOTA-UBI, tin-dioxide generator, radiation exposure

## Introduction

The development of synthesis methods for novel radiopharmaceuticals often entails testing of different manual radiolabelling procedures on a small scale. Small-scale labelling has the advantage that it limits the radiation exposure to the operator (De Decker and Turner, 2011). Initial experiments are usually repeated several times to assess the impact of various parameters such as pH, incubation temperature and time, type and volume of buffer etc. on the success of the radiosynthesis. Optimizing radiometal-based synthesis methods may also include the evaluation of bifunctional chelators to determine the best radiometal-chelator-ligand complex. Once optimal labelling conditions have been determined and satisfactory results achieved, the next phase typically comprises evaluation of the robustness and repeatability of the radiosynthesis evaluation. This phase often includes up-scaling the quantity of reagents together with an increase in radioactivity used. Scaling-up is required to determine if labelling results can be reproduced using sufficient radionuclide for one or more patients. In theory, labelling results obtained from manual labelling methods should correlate well with those obtained from automated syntheses.

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Ubiquicidin 1-59 is a 6.6 kD linear cationic peptide with antimicrobial properties. It has been shown to be present in low concentration in various organs such as the colon mucosa, epithelial cells of the human airways and also macrophages (Hiemstra et al., 1999; Tollin et al., 2003). It is present intra-cellularly and only released during acute infection or severe cell damage. Ubiquicidin (UBI) has been found to affect a spectrum of pathogens (Brouwer et al., 2006). Various peptide fragments of UBI 1-59 have been synthesized, including UBI 1-18, UBI 18-35, UBI 18-29, UBI 29-41 and UBI 31-38. Brouwer's study also showed that the synthetic UBI derivatives containing amino acids 29-41 or 31-38 had the best targeting properties which could possibly be adopted in as a tool for non-invasive nuclear imaging techniques such as single-photon emission tomography (SPECT) and positron emission tomography (PET). Researchers have since then developed a number of procedures that can be used for radiolabelling of ubiquicidin fragments (Bhatt et al., 2018, 2017; Bhusari et al., 2019; Brouwer et al., 2006; Vilche et al., 2016). Peptide fragments UBI 29-41 and UBI 31-38 were successfully labelled with gallium-68 ($^{68}$Ga) and technetium-99m ($^{99m}$Tc). Preclinical investigations in mice by Brouwer et al. have shown that the $^{99m}$Tc-labelled fragments accumulated only in sites of infection.

There are a number of approaches that can be used in the production of gallium-68 ($^{68}$Ga) radiopharmaceuticals for clinical application (Velikyan, 2015). Automated and semi-automated radiosyntheses of $^{68}$Ga-radiopharmaceuticals have the advantage of enabling compliance with Good Manufacturing Practice (GMP) guidelines (Vis et al., 2015).

In 2014, synthesis of gallium-68-labelled UBI 29-41 (i.e. [$^{68}$Ga]Ga-NOTA-UBI) using a manual radiolabelling technique was first reported (Ebenhan et al., 2014) and a module-based automated radiosynthesis has recently been developed (Le Roux et al., 2020). The use of automated synthesis modules to label radiopharmaceuticals with $^{68}$Ga has been reported by several authors (Decristoforo, 2012; Malizia et al., 2012; Schopf et al., 2018; Vis et al., 2015). Well-known buffers such as sodium acetate, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) and formic acid are commonly used in $^{68}$Ga-labelling (Bauwens et al., 2010; Sasson et al., 2010; Velikyan et al., 2008).

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Various methods described in the literature compare manual with automated syntheses but to the best of our knowledge no head-to-head comparison of a manual synthesis with two automated methods for the same radiopharmaceutical exists. The aim of this study was to evaluate how the labelling characteristics of a manual synthesis procedure for preparing [$^{68}$Ga]Ga-NOTA-UBI compare to those of two different automated synthesis procedures.

## Methods

*Material and Preparations*

Analytical or pharmaceutical grade reagents were used for both manual and automated synthesis procedures. Gallium-68 eluates were obtained from a $^{68}$Ge/$^{68}$Ga-generator (iThemba LABS, Somerset West, South Africa) using 0.6 M HCl (ABX, Radeberg, Germany). Both the generator and the GRP automated synthesis unit (Scintomics, Fürstenfeldbruck, Germany), were housed in a NMC Ga-68 hot cell (Tema Sinergie, Faenza, Italy). All disposable material for the synthesis module was compliant with GMP standards. The routine preparation and general radiosynthesis protocols using a module for the production of [$^{68}$Ga]Ga-NOTA-UBI were previously reported (Le Roux et al., 2020).

Freeze-dried batches of UBI 29-41, conjugated to either 1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetra-acetic acid (DOTA) or 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) or 1,4,7-triazacyclononane,1-glutaric acid-4,7-acetic acid (NODAGA), were obtained from GL Biochem (Shanghai, China) and stored at -80 °C as 25 µg aliquots suitable for small-scale, manual radiolabelling. For the automated methods, 2 mg of NOTA-UBI (ABX, Radeberg, Germany) was dissolved in 2.0 ml of ultra-pure water to render a stock solution with a concentration of 1 mg/ml. The stock solution was sub-divided in 50 and 100 µl aliquots and frozen at -20 °C. A sodium acetate trihydrate (NaOAc) solution (2.5 M) was used as a buffer for manual preparations while 1.0 M NaOAc or 1.0 M ammonium formate were used in the automated synthesis methods. Based on initial

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experiments, aliquots of NOTA-UBI were prepared for up-scaled production at 50 µg (all automated syntheses using generator eluate fractionation) and 100 µg (all automated syntheses including eluate pre-processing); to meet the criteria for optimal [$^{68}$Ga]Ga-NOTA-UBI preparation under the different radiolabelling conditions. Ascorbic acid and pharmaceutical grade ethanol were obtained from North East Pharmaceutical Group (Midrand, South Africa) and Merck (Kenilworth, NJ, USA), respectively. PS-$H^+$ cartridges (ABX, Radeberg, Germany) were used for eluate pre-processing. C18 Sep-Pak cartridges (Waters, Milford, MA, USA) were used for purification post-radiolabelling if required.

*Radiolabelling*

**Manual radiolabelling:** Small-scale and up-scaled manual radiosyntheses were used as references, adopting the conditions described previously (Ebenhan et al., 2018, 2014) using $^{68}$Ge/$^{68}$Ga-generator eluate fractionation. The first set of experiments investigated the $^{68}$ Ga-radiolabelling of DOTA-UBI, NODAGA-UBI and NOTA-UBI to find the most suitable chelator-ligand complex. Concentrations (µg/ml) of 4, 8 16, 32, and 64 for each chelator-ligand complex were labelled with micro-scale quantities of gallium-68. Briefly, 180 µl of a buffered $^{68}$Ga-solution (pH 3.5 - 4.0) was added to either of the NOTA-, NODAGA- and DOTA-UBI solution. The labelling mixtures were heated for 10 min at 80 °C, followed by a cooling period of 5 min. The scaled-up radiolabelling conditions remained the same, however, 2 ml $^{68}$Ga-activity was mixed with 50 µg NOTA-UBI. The NOTA-UBI concentration of the reference manual labelling was 19.6 µg/ml.

**Automated radiolabelling:** Method 1 used fractional generator elution while an eluate pre-purification step with a strong cationic ion exchanger was used for automated method 2. Importantly, both manual and automated methods were optimized for type of buffer used, peptide mass and use of radical scavengers, if required.

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**Automated method 1:** $^{68}Ge/^{68}Ga$-generator eluate fractionation was performed with 0.6 M HCl and a 2 ml fraction was used for the synthesis. The pH of the eluate was adjusted to 3.5 - 4.0 using 2.0 ml of a 1.0 M ammonium formate buffer. The buffered eluate mixture was slowly added to the reaction vial containing 50 µg of NOTA-UBI and 350 µl ascorbic acid (1.4%). The NOTA-UBI concentration in this labelling mixture was 11.2 µg/ml. The mixture was heated for 10 min at 90°C, cooled for one minute and purified using a C18 Sep-Pak cartridge. [$^{68}Ga$]Ga-NOTA-UBI was desorbed from the C18 matrix using a mixture of ethanol/saline (50% v/v) and ultimately filtered through a 0.22 µm filter into a sterile vial.

**Automated method 2:** The $^{68}Ge/^{68}Ga$-generator was eluted with 10 ml 0.6 M HCl. The eluate was further diluted to 18 ml with ultra-purified water and slowly passed over a PS-$H^+$ cartridge where most of the $^{68}Ga$-activity was trapped and purified from co-eluted metals. Subsequently, 1.5 ml of a 5.0 M NaCl solution was used to desorb the $^{68}Ga$-activity from the PS-H+ matrix into a reaction vessel containing 100 µg NOTA-UBI buffered in 1.3 ml 1.0 M NaOAc. The reaction vessel also contained a radical scavenger combination of 350 µl ascorbic acid 1.4% and 170 µl ethanol. This radiolabelling mixture, with a NOTA-UBI concentration of 28.4 µg/ml, was further processed as described above in method 1.

*Qualitative Analysis*

**Radio-high performance liquid chromatography (HPLC)**

Following completion of the automated methods the radiochemical purity (RCP) was determined using a Shimadzu Nexera XR HPLC system (Kyoto, Japan), which included a variable wavelength photodiode array detector and a gamma detector (Raytest, Straubenhardt, Germany). The analysis was performed at a wavelength of 254 nm. The solvent gradient method consisted of mobile phases (v/v) A (0.1 % trifluoroacetic acid (TFA) in water) and B (0.1% TFA in acetonitrile) using a Waters C18 analytical column (4.6 mm x 250 mm x 5µm) as stationary phase (0 – 2 min: 5% B, 2 - 18 min:

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65% B, 18 - 24 min: 5% B). The flow rate was set at 2.0 ml/min. The column temperature was kept at 40 °C throughout the analysis. The retention time for [$^{68}$Ga]Ga-NOTA-UBI was 6.0 - 6.9 minutes whereas free $^{68}$Ga-activity was retained for 1 - 2 minutes.

**Radio-instant thin-layer chromatography (ITLC)**

A glass microfiber chromatography strip impregnated with silica gel (ITLC-SG, Varian, USA) was used as stationary material. The mobile phase consisted of 0.1 M sodium citrate (pH = 5.0). Distribution of radioactivity was determined using a radio-chromatographic scanner (Lablogic, Sheffield, United Kingdom) allowing gamma-counting of the full length of the ITLC-SG strip. Radiochemical purity was determined by peak analysis (counts-per-minute/area under the curve). [$^{68}$Ga]Ga-NOTA-UBI remained at the origin whereas unchelated $^{68}$Ga was separated by migrating to the mobile phase front.

*Product stability*

Stability at room temperature of [$^{68}$Ga]Ga-NOTA-UBI prepared by both automated methods was assessed following sterile filtration and dilution to 15 ml with phosphate buffered saline. The radiochemical purity was determined with radio-ITLC and radio-HPLC analyses at the end of synthesis (EOS) and at 180 min after EOS.

*Additional quality control measures*

**Table 4.1** presents a summary of additional quality control procedures performed after module-based synthesis to ascertain if the final product complied with our in-house release criteria.

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**Table 4.1** Quality control procedures and release criteria for automated radiosynthesis methods

| 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 min | Dose calibrator |
| pH of final product | 6.0 – 8.0 | pH strips |
| Bacterial endotoxins | < 10 EU/ml | Endosafe PTS |
| Residual ethanol content | < 10% v/v | Calculation |
| Sterile product filtration | ≥ 3.45 bar | Filter integrity test |
| Bacterial growth | Sterile (pass) | Broth incubation |
| Germanium breakthrough | < 0.001 % | Dose calibrator |

*Operator Radiation Exposure*

All radiolabelling techniques were performed using appropriate radiation protection and shielding. The guidelines of the European Pharmacopoeia (8.0) were followed for preparation of the $^{68}$Ga-radiopharmaceuticals. The fully-shielded, automated synthesis module was remotely operated. The $^{68}$Ge/$^{68}$Ga-generators used for this part of the study had a similar age of three months. Operator radiation exposure was recorded using an electronic X-ray and gamma personal dosimeter (PM1610, Polimaster, Belarus) (energy range of 29 keV – 10 MeV; 0.1 µSv increments) attached to the outside of the lab coat. The whole-body radiation dose was determined by recording the dosimeter reading at the beginning of the elution until end of synthesis, including mimicking the dispensing of two patient doses in a shielded laminar flow unit.

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## Results and discussion

Prior to this comparative study, each of the automated synthesis methods was optimized for peptide mass, buffer type and volume as well as scavenger addition. This resulted in different NOTA-UBI concentrations for the 3 labelling methods. Experimental results during the development of the automated synthesis methods showed the formation of a hydrolytic impurity seen on HPLC (data not shown) which required the use of an appropriate radical scavenger. The manual radiolabelling did not necessitate the use of a scavenger.

*Choice of chelator-UBI complex*

The optimal combination of azamacrocyclic chelators (NOTA-NODAGA and DOTA) and UBI was identified by gradually decreasing the chelator-UBI concentration during manual radiolabelling to 4 µg/ml (**Figure 4.1**). The best % RCP obtained using the NOTA moiety was compared to that of NODAGA- and DOTA-UBI ($p < 0.05$). To determine the optimal labelling concentration that yielded a RCP > 90%, chelator-UBI concentrations (µg/ml) of ≥8, ≥16 and ≥ 32 for NOTA-UBI, NODAGA-UBI and DOTA-UBI were evaluated. Such a direct comparison has not yet been described in the literature, however, experimental work conducted by Guérin et al. also suggested the use of NOTA-functionalized peptides for PET imaging (Guérin et al., 2010). Tri-azamacrocyclic bifunctional chelators such as NOTA and NODAGA have both been suggested as viable alternatives to DOTA based on the formation of thermodynamically stable complex with Ga(III) ions. Maximal interaction is achieved due to optimal $N_3O_3$ denticity and a smaller ring size than that of DOTA. Subsequently, the successful synthesis of NODAGA-UBI with $^{68}Ga$ was also reported (Bhatt et al., 2017).

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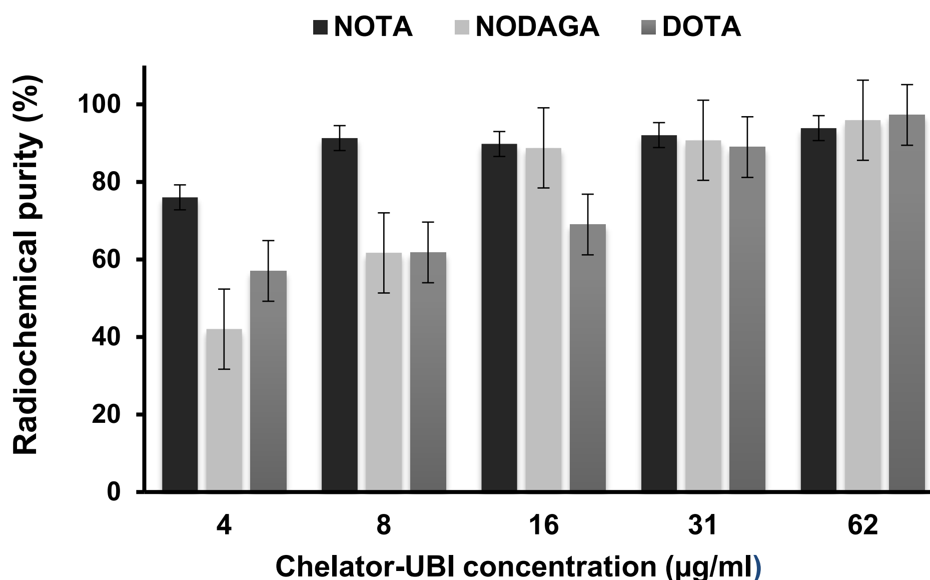


**Figure 4.1** $^{68}$Ga-radiosyntheses of UBI conjugated to NOTA, NODAGA or DOTA as azamacrocyclic bifunctional chelators (n = 3); radiolabelling was performed in the presence of NaOAc, pH 3.5 – 4, at 80 °C for 10 min. The % radiochemical purity was determined using ITLC.

A total mass of 25 µg NOTA-UBI was deemed sufficient for an up-scaled manual labelling to account for precursor losses due to glassware adherence and possible slower reactivity in the larger reaction volume. **Table 4.2** summarises the results obtained with the manual synthesis method versus the two automated synthesis methods. The $^{68}$Ga-activity concentration for the manual radiolabelling was 458 - 806 MBq/ml, considered to be an acceptable range of radioactivity for radiation protection and efficient dose preparation. For automated methods, the average $^{68}$Ga-starting activities were 1483 ± 16 MBq.

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**Table 4.2** Comparison of the manual reference method with the automated synthesis methods

| | **Manual radiolabelling (n = 9)** | **Automated method 1 (n = 3)** | **Automated method 2 (n = 3)** |
|---|---|---|---|
| Volume of $^{68}$Ga-activity (ml) | 1.0 | 2.0 | 10.0 |
| Type of eluate pre-processing | EF | EF | SCX |
| Average starting activity (MBq) | 587 ± 149$^{\$}$ | 1498 ± 73 | 1467 ± 85 |
| Residual radioactivity: PS-H $^{+}$ (%)$^{\#}$ | - | - | 8.8 ± 2.0 |
| Buffering molarity and type | 2.5 M NaOAc | 1.0 M $NH_4HCO_2$ | 1.0 M NaOAc |
| Volume of buffer used (ml) | 0.278 | 2.0 | 1.5 |
| Total mass NOTA-UBI (μg) | 25 | 50 | 100 |
| Incubation time (min)/ (°C) | 10 – 15/ 80 | 10/ 90 | 10/ 90 |
| Radiosynthesis time (min)† | 31 ± 7 | 32 ± 2 | 38 ± 2 |
| % Radiochemical yield $^{\#}$ | 65.5 ± 22.6 | 63.2 ± 1.5 | 57.3 ± 3.8 |
| % radiochemical purity (ITLC) | 97.1 ± 1.9 | 98.9 ± 0.3 | 99.3 ± 0.1 |
| % radiochemical purity (HPLC) | 97.5 ± 0.8 | 96.4 ± 0.9 | 97.3 ± 0.5 |
| [$^{68}$Ga]Ga-NOTA-UBI yield (MBq) | 473 ± 234 | 690 ± 22 | 580 ± 99 |
| Molar activity (MBq/nmol) | 20.4 ± 11.4 | 27.6 ± 0.9 | 11.4 ± 1.9 |
| Retained activity C18 SPE (%) | 10.0 ± 8.9 | 1.6 ± 1.4 | 2.5 ± 1.5 |

Notes: EOS = End of synthesis, $NH_4HCO_2$ = ammonium formate; NaOAc = sodium acetate tri-hydrate; EF = eluate fractionation; SCX = eluate processing using a strong cationic exchange matrix; † = time measured from start of elution of generator; # = decay-corrected data

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*Radiosynthesis time*

The manual method had the shortest radiosynthesis time (31 ± 7 min) while the radiosynthesis time for the cationic pre-purification method was the longest (38 ± 2 min). Both automated synthesis methods included a 6-minute step to condition the C18 cartridge prior to generator elution (not reflected in the synthesis time). The added pre-purification step, which utilizes all eluted $^{68}$Ga-activity, increased the synthesis time by 6 minutes. Pre-purification usually also increases the molar activity by decreasing the volume of the labelling mixture. The loss of activity on the PS-$H^+$ cartridge was less than 10% of the eluted activity from the generator. Despite this longer synthesis time, it is deemed necessary to include this step in the synthesis methods where a $^{68}$Ge/$^{68}$Ga-generator eluate with known high levels of metal impurities is used (Chakravarty et al., 2013). It is well-known that certain metal impurities such as Zn, Fe and Cu may have a detrimental effect on the synthesis of peptides with $^{68}$Ga-chloride (Velikyan, 2015).

The increase in radiosynthesis time resulted in about 10% decrease in radiochemical yield when compared to the automated fractional elution method (method 2).

*Choice of buffering agent*

Manual synthesis may be susceptible to operator influences such as the speed at which the solvents and reagents are introduced during the various synthesis steps or between different syntheses. In the automated synthesis methods, reagents and solvents are introduced at pre-programmed speeds in a consistent manner. The volume of the eluate and buffer used in the manual method was smaller than that used in the automated methods. The buffer volume was intentionally increased and the molarity of NaOAc adapted for automation because the synthesis module could not accurately add a volume less than 500 µl into the reaction vial. This increase in buffer volume resulted in varying peptide

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concentrations for the three synthesis methods. Variable peptide concentrations used in the automated methods still resulted in acceptable radiochemical purity and yield.

*Radiochemical purity*

Experimental results during the development of the automated synthesis methods showed the formation of a radiolytic impurity seen on HPLC (data not shown). The average starting $^{68}$Ga-activity for the manual method was 587 ± 149 MBq while for automated methods it was about 2-3 times higher (1483 ± 81 MBq). The higher $^{68}$Ga-activities used in the automated methods made them more prone to radiolysis. In order to reduce radiolysis it was necessary to use radical scavengers in the automated methods. The addition of radical scavengers successfully reduced the radiolytic impurities and increased RCP to ≥ 95%. The manual method did not require the use of a scavenger. Besides lower starting activities, differences in peptide mass and volume of labelling mixture may have contributed to prevention of radiolysis. Herein, original data is provided, suggesting that the radiochemical purity of both automated methods was more robust and repeatable than that observed with the manual method. The average percentage radiochemical purity (TLC and HPLC) for both the manual and automated procedures was above the required 95%.

*Percentage radiochemical yield and losses of product activity*

The manual method resulted in varying radiochemical yields (42 - 89%), amongst others due to inconsistency in following the labelling protocol. However, this is not uncommon for manual radiosynthesis. The average percentage radiochemical yield of module-based preparations of [$^{68}$Ga]Ga-NOTA-UBI showed a markedly smaller variation (< 4%) than the manual method (22%). There was a large difference in the percentage activity retained on the C18 cartridge when the manual method is compared to automated methods. The manual method retained 4-5 times more activity than the automated methods, despite matching labelling conditions. A possible reason can

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be that more colloids are formed during the manual method which are retained on the C18 cartridge during product purification. It should be noted that the average RCY from all methods was satisfactory, providing sufficient radioactivity to potentially prepare one or more patient doses despite differences in generator age.

*Effect on final product quality and stability*

The molar activity of the different radiosynthesis methods ranged from 11.4 MBq/nmol to 27.6 MBq/nmol. This wide range of molar activities did not affect the quality of the final product. Radio-ITLC and radio-HPLC results indicated that [$^{68}$Ga]Ga-NOTA-UBI, labelled with both automated methods, remained stable at room temperature for at least 180 min (data not shown). Unpublished data also confirmed that the manually labelled [$^{68}$Ga]Ga-NOTA-UBI was stable for up to 180 min post synthesis.

*Radiation exposure to operator*

Radiation exposure to operators is influenced by several factors such as exposure time, shielding and distance from the radiation source, as well as the degree of automation of the radiosynthesis. Module-based syntheses have a clear advantage over manual methods in this regard. **Table 4.3** provides a summary of radiation exposure readings recorded during manual and module-based methods. In general, exposure levels for the manual method were higher despite using a lower starting activity (about 60 % less). In order to compare the exposure from manual and automated syntheses, we normalised the effective doses to starting activity. The normalized effective dose for the automated method was 0.002 µSv/MBq compared to 0.04 µSv/MBq for the manual method. This is a 20-fold reduction in radiation exposure. This data confirms the added advantage of automation regarding radiation exposure of operators in the day-to-day production of radiopharmaceuticals in a clinical setting.

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**Table 4.3** Comparison of whole-body radiation exposure: manual versus automated methods

| Method (n=5) | $^{68}$Ge/$^{68}$Ga-generator shelf-life (days) | Synthesis time (min) | Effective dose (µSv) | Normalized effective dose (µSv/MBq) |
|---|---|---|---|---|
| Manual | 109 ± 7.9 | 32.2 ± 7.5 | 25.2 ± 6.2 | 0.04 ± 0.05 |
| Automated* | 101 ± 51 | 36.9 | 2.3 ± 0.4 | 0.002 ± 0.01 |

*) data from method 1 and 2 was combined.

Automated synthesis modules are usually housed in hot cells which also contribute to lower operator radiation exposure and lower background radiation. Even without a hot cell, the hands-off set-up is the most important reason for the reduced radiation exposure seen with the automated methods, as there is no need for the operator to remain in the vicinity of the high radioactivity. It can be argued that a major advantage of manual syntheses is the possibility to intervene in the labelling procedure if necessary. However, operator intervention makes it more difficult to comply with GMP standards.

In a scenario where an operator uses the manual labelling method daily, the whole-body effective dose could exceed 6 mSv per year. Higher starting activities or manual labelling several times per day could lead to a whole-body effective dose close to the annual limit of 20 mSv per year (averaged over 5 years) as published by the International Commission for Radiological Protection's guidance for occupational exposure (International Commission on Radiological Protection, 2003).

*Additional quality control and compliance with release criteria*

Pharmacopoeias provide the legal and scientific benchmark for delivering high quality medicines, including radiopharmaceuticals. Novel radiopharmaceuticals, such as $^{68}$Ga-labelled ubiquicidin, do not have pharmacopoeial monographs. This study used the European Pharmacopoeia's published monograph for Gallium ($^{68}$Ga) Edotreotide Injection as a guide for the addressing release criteria for $^{68}$Ga-labelled ubiquicidin, as the synthesis of these two radiopharmaceuticals are very similar. Both automated methods fulfilled our in-house criteria for release of a radiopharmaceutical for human administration (**Table 4.1**). Manual radiolabelling met with the release criteria in three consecutive

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preparations. Even though it may be generally compliant with GMP, a weakness of the manual method is that it is less robust. GMP plays an integral part in the production of radiopharmaceuticals intended for clinical use. Consistent and reproducible results produced by automated synthesis methods make them the method of choice for producing radiopharmaceuticals in a GMP-compliant manner. Batch records produced by the module software are usually GMP-compliant, providing a method to monitor labelling steps and conditions. The module software furthermore provides a mechanism for reliable traceability of the radiopharmaceutical manufacturing process.

## Conclusion

The aim of this work was to compare the product characteristics and pharmaceutical quality of optimized automated and manual methods.

Our results showed a high degree of robustness and repeatability using a Scintomics GRP synthesis unit. It was necessary to include scavengers for both automated methods to reduce radiolysis. Automated synthesis methods furthermore have the clear advantage of reducing radiation exposure to operators and facilitating production of radiopharmaceuticals in a GMP compliant manner.

**Declaration of competing interest:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Summary**

The labelling characteristics of the automated method were more robust and repeatable when compared with the manual method. Both automated methods required the use of radical scavengers to reduce the formation of radiolytic impurities. This comparison also confirmed the added advantage module-based labelling have in reducing radiation exposure to the operator.

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# Chapter 5

# The use of HEPES-buffer in the production of gallium-68 radiopharmaceuticals

### Rationale for the research covered in this chapter

HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) is a zwitterionic buffer that is extensively used in the radiolabelling of gallium-68 radiopharmaceuticals to create optimal labelling conditions. A pharmacopoeial limit regulates the HEPES content in radiopharmaceutical preparations. The limit is strict, and a lack of toxicity is cited as a reason for the strict limit.

In this chapter, current available data on the toxicity of HEPES in animals and humans are presented in the form of technical note.

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## Technical note: The use of HEPES-buffer in the production of gallium-68 radiopharmaceuticals – time to consider the strict pharmacopoeial limits?

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**Abstract**

HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) is a buffer that is used in the radiolabelling of gallium-68 compounds. The beneficial effects of HEPES on specific activity in bioconjugates have been well described. Current strict regulations on the HEPES content in radiopharmaceuticals limit its use when intended for parenteral administration.

This technical note summarizes data from the literature on the toxicity of HEPES in dogs after intravenous infusion and the subsequent use in humans. We also highlight the use of HEPES in an Unites States Food and Drug Administration (FDA) labelled intravenous drug formulation. Regulatory institutions may consider this data to review current strict limits.

**Use of HEPES in pharmaceutical products**

Radiosynthesis of gallium-68 compounds requires a labelling mixture with a pH of 3.5 – 5.0 to ensure good complexation of gallium-68 with the precursor. Buffers such as 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) and sodium acetate are extensively used in the labelling of radiopharmaceuticals with gallium-68. These buffers are reported to have low metallic complexation properties making them suitable to adjust pH during labelling and also reduce formation of colloids (Bauwens et al., 2010).

HEPES, a zwitterionic buffer, is listed as a Good's buffer with a pKa values of 3.00 and of 7.55 (Good et al., 1966). Velikyan et al. illustrated the beneficial effect of HEPES on specific activity in radiolabelling bioconjugates (Velikyan et al., 2004). A number of references also indicate that adjusting the pH with HEPES during radiolabelling with gallium-68 provides the optimum results in terms of molecular activity, reproducibility, reliability and versatility (Bauwens et al., 2010; Eppard et al., 2014; Pfaff et al., 2018; Velikyan et al., 2004). The European Pharmacopoeia prescribes a strict limit for the HEPES content in radiopharmaceuticals intended for intravenous

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administration (European Pharmacopeia, 2017). However, the method described in the European Pharmacopoeia to test for HEPES in radiopharmaceutical preparations has proven to be unreliable (Antunes et al., 2020; Pfaff et al., 2018; Sasson et al., 2010). The low limit has hampered the use of HEPES in gallium-68 radiopharmaceuticals, despite its superior buffering properties. A lack of acute toxicological data, especially after intravenous administration, is generally cited as the main reason for these strict limits (Bauwens et al., 2010; European Pharmacopeia, 2017; Sasson et al., 2010).

A chronic tolerance study of 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (product code TVZ-7), published in 1997 by Theodore et al., provides important data on HEPES toxicity in beagle dogs after intravenous infusion (Theodore et al., 1997a). The animals were monitored for pharmacological or toxicological effects from increasing doses of 5 – 500 mg/kg HEPES administered over an extended period of 148 days. Initially doses were administered daily via intravenous infusion but then changed to alternate days because of a subjective observation of stress at doses reaching 300, 350 and 400 mg/kg. Routine clinical pathology evaluations consisted of complete blood count and blood chemistry. Histopathology (bone marrow and liver biopsies) was done at the end of the highest dose segment when clinical effects were observed. Important observations from the study included the following:

a) Vomiting, a pharmacological effect of HEPES administration, occurred in the first two segments of the study but subsided when HEPES was administered before feeds.
b) When intravenous (IV) doses approached 400 mg/kg (4000 mg for a dog with an average weight of 10 kg), significant changes in the hematopoietic and reticuloendothelial system were observed in some dogs. These included hypercellular bone marrow and extramedullary hematopoiesis.

The study however concluded that no severe adverse effects are associated with chronic intravenous administration of HEPES in doses ranging from 5 – 500 mg/kg. In a patient with an average weight of 70 kg, this would amount to a maximum dose of 35 000 mg (35 g).

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A more significant publication is the preliminary evaluation of a fixed dose of HEPES in humans by Theodore et al. The investigators report on the use of HEPES to evaluate its potential beneficial effects in clinical cancer (Theodore et al., 1997b). In this study setup, most subjects received an average fixed daily dose of 5000 mg, administered intravenously for 2 weeks. Thereafter, the same dose was administered three times per week for 2 more weeks. Patient response was evaluated at 4 weeks. A maintenance dose of 5000 mg, administered two to three times per week, was thereafter instituted. This study reported minimal side-effects and toxicity, similar to what was reported in the study with dogs.

The current limit for HEPES in radiopharmaceutical preparations specified by the European Pharmacopoeia is 200 µg/V, where V represents the maximum injected dose in millilitre. This very strict limit translates to a maximum HEPES amount of 200 µg per dose. Labelling of specific radiopharmaceuticals may require different volumes and concentrations of HEPES as reported by Antunes et al. (Antunes et al., 2020). Using these examples, in a worst-case scenario where no HEPES is removed during the labelling process, a total single dose between 283 and 714 mg HEPES would be intravenously administered to a patient. This translates to a HEPES dose of 4.0 – 10.2 mg/kg for a 70 kg patient. The highest single dose is much lower than the maximum mg/kg dose used in the chronic toxicity study in dogs. This is also approximately 14 % of the average dose administered to humans in the preliminary fixed dose study by Theodore et al. It is known that a considerable fraction of HEPES is removed during the purification process (Mueller et al., 2012; Mukherjee et al., 2015; Pfaff et al., 2018). The HEPES content is therefore significantly lower than the doses used in the Theodore toxicity study and still far below the chronic dose of 400 mg/kg where significant changes in the hematopoietic and reticuloendothelial systems were observed in dogs.

In 2015 the FDA approved ONIVYDE ™ (Merrimack Pharmaceuticals), a topoisomerase 1 inhibitor which also contains HEPES as a buffering system. The pharmaceutical formulation contains 4.05 mg/ml HEPES in a 10 ml vial solution, to be diluted in 500 ml dextrose 5% for intravenous

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administration (Baker and Levien, 2017; Merrimack, 2015; Theodore et al., 1997a). The recommended dosage is 70 $mg/m^2$ Ironectan (4.3 mg/ml) resulting in an average male (1.9 $m^2$) receiving a total amount of 133 mg of the drug and 126 mg of HEPES. This amount of administered HEPES is much higher than the recommended 200 µg per dose for gallium-68 based radiopharmaceuticals. Limiting the HEPES content to 200 µg/V, as advised currently by the European Pharmacopoeia, may be a far too strict limit.

To conclude: The expected HEPES amounts used in radiopharmaceutical preparations is significantly less than the amount that was tolerable in other approved pharmaceuticals, in particular the HEPES doses given to dogs as part of the chronic toxicity study published by Theodore et al. The HEPES levels administered intravenously within a radiotracer formulation is also considerably lower than the amount given to cancer patients which was considered safe based on their clinical toxicology screening. It is not plausible for patients scheduled for Nuclear Medicine investigations to receive a daily intravenous dose of HEPES exceeding 5000 mg, which was well-tolerated in humans. Taking all factors into account, regulatory institutions may consider reviewing the strict limit for HEPES currently applying to radiopharmaceutical preparations.

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**Summary**

Available data suggest that the current pharmacopoeial limit for HEPES may be too strict. The HEPES content in radiopharmaceuticals preparation is far lower than the doses administered in animals and also much lower than the doses used in the human studies. The data presented in can be used by regulatory institutions to reconsider the current strict limit.

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# Chapter 6

# Thin-layer chromatography for instant analysis of the radiochemical purity of gallium-68 labelled ubiquicidin

## Background

Quality control of radiopharmaceuticals is a critical step in the production of radiopharmaceuticals intended for clinical application. Radiopharmaceuticals must be tested for acceptable quality against a set of criteria prior to administration in humans [1, 2]. The synthesis and formulation process of gallium-68 radiopharmaceuticals must adhere to certain specifications for physiological acceptability, chemical and radiochemical characteristics, as well as product sterility and purity [3]. Most PET radiopharmaceuticals have a relatively short half-life and it is important to perform quality control within a short time to establish if the labelled product complies with the release criteria. However, it is not always possible to complete all quality control tests before injecting the radiopharmaceutical to patients. For example, the confirmation of sterility of the radiopharmaceutical will only be available after an incubation period of 14 days. Typical tests that are performed in the quality control of radiopharmaceuticals include the following [4]:

- Test for appearance
- Test for pH
- Test for bacterial endotoxins
- Test for filter integrity
- Test for sterility
- Test for radiochemical purity (TLC and HPLC)
- Test for radionuclide purity

Quality control of [$^{68}$Ga]Ga-NOTA-UBI was extensively investigated and discussed in this study[17]. The finished product was subjected to several quality control procedures to establish if the radiopharmaceutical complied with the in-house release criteria. Results from these tests showed

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that [$^{68}$Ga]Ga-NOTA-UBI complied with the set of in-house criteria, but from this work it was clear that a thin-layer chromatography (TLC) method to identify gallium-68 colloids in the labelled product required further investigation.

TLC is regarded as a fast and effective method to determine the radiochemical purity of radiopharmaceuticals [5]. The advantage of TLC is that the analysis can be completed in a relatively short time, which is important when dealing with radiopharmaceuticals with short half-lives. This method also does not result in large quantities of solvent waste that need to be disposed of. TLC or instant thin-layer chromatography (iTLC) to determine the radiochemical purity of gallium-68 radiopharmaceuticals usually comprises of a two-strip method. The iTLC method used in the analysis of gallium-68 labelled DOTA-NOC serves as a good example [6, 7]. This method uses iTLC-SG together with the following mobile phases to separate the labelled product from possible radioactive impurities:

- 0.1 M HCl or 0.1 M sodium citrate buffer (pH 5.0) to separate free gallium from colloid and labelled compound (free gallium-68 migrates to the solvent front while colloidal impurities and the labelled product remain at the origin)
- A mixture of 1.0 M ammonium acetate/methanol (50:50 v/v) to separate colloidal impurities and free gallium from the labelled product (colloidal impurities and free gallium-68 remain at the origin while labelled product migrate with the solvent front)

Silica-gel based iTLC (iTLC-SG) paper (in combination with specific mobile phases) is the predominately used stationary phase to distinguish between radiochemical impurities in peptide-based labelling of gallium-68 radiopharmaceuticals like [$^{68}$Ga]Ga-DOTA-TATE, [$^{68}$Ga]Ga-DOTA-CP04 and gallium-68 labeled exedin [8–10]. Larenkov and Maruk reported the use of a single strip using 4% TFA as a mobile phase to evaluate the radiochemical purity of several gallium-68 BCA-peptides [11].

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iTLC-SG was also used in our analysis of [$^{68}$Ga]Ga-NOTA-UBI as free gallium can be detected with a sodium citrate mobile phase. This method has been extensively validated at our institution (data not shown). A typical chromatogram of this method can be seen in **Figure 2.3**, Chapter 2. The presence of gallium-colloids in [$^{68}$Ga]Ga-NOTA-UBI could not be detected with the ammonium acetate/methanol with iTLC-SG strip combination. As the gallium-68 colloidal impurities remain at the origin with this method, and the labelled product had a retardation factor (Rf) of 0 - 0.1, distinction between the different species was not possible. A typical example of such a chromatogram is presented in **Figure 6.1** in the results section of this chapter. Adapting the combination of the mobile phase to ratios of 70/30 (v/v) and 30/70 (v/v) still did not result in a significant change in the Rf of [$^{68}$Ga]Ga-NOTA-UBI.

According to Decristoforo *et al.*, a final C18 purification step after radiolabelling of gallium-68 peptides removed colloidal impurities from the labelling mixture [12]. The two automated synthesis methods developed for labelling ubiquicidin with gallium-68 both made use of a C18 purification step [17]. The preparation of gallium-68 radiopharmaceuticals from cold kits is becoming more widely used and has the advantage that there is no need for the use of an automated synthesis module. This can reduce production costs considerably and is therefore an attractive alternative to meet routine demand for radiopharmaceuticals. To reduce radiation exposure to the operator, some institutions may also opt to omit a C18 purification step after using a kit-based radiolabelling procedure. For instance, the instructions for NETSPOT®, a cold kit for the preparation of gallim-68 DOTA-TATE injections, does not mention a C18 purification step as part of the procedure [13].

Recently, a kit-based labelling method for gallium-68 ubiquicidin was published by Bhusari *et al.* [14]. This method does not include a C18 purification step but, like most radiopharmaceutical syntheses, did include a final filtration step using a 0.22 µm pore size membrane filter. It is not clear if this step is supposed to remove colloidal impurities from the labelling mixture. In the labelling of fragments of NODAGA-ubiquicidin with gallium-68, Bhatt *et al.* specifically mention the use of a 0.22 µm pore size filter to remove colloidal impurities from the labelling mixture [15]. It should be

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noted that particles smaller than the filter pores will probably not be removed from the labelling mixture by filtration. The TLC method used by Bhatt *et al.* consisted of a mobile phase of 15% HCl in methanol to detect the presence of colloidal impurities and 0.1 M sodium citrate pH 5.0 to detect free gallium in the analysis of gallium-68 labelled NODAGA ubiquicidin [15]. ITLC-SG or Whatman 3 MM paper was used as stationary phase in both instances. Gallium-68 colloid is normally retained in the liver and can influence diagnostic accuracy of the investigation [16]. Institutions that decide not to include a post labeling purification, should ensure that a reliable quality control procedure exists to detect and accurately quantify colloidal impurities if present in the final product. TLC methods are often used for this purpose.

**Problem statement**

TLC methods may not be capable to distinguish between all impurities present in radiopharmaceutical preparations in all instances. In addition, a TLC method developed for a specific compound may not always be suitable for other products labelled with the same radionuclide. In the TLC analysis of [$^{68}$Ga]Ga-NOTA-UBI, the combination of a mobile phase of methanol and ammonium acetate (50:50 v/v) with iTLC-SG as stationary phase could not separate colloidal impurities from the labelled product. There is thus a need for a method that allows investigators to distinguish between [$^{68}$Ga]Ga-NOTA-UBI and colloidal impurities on the same TLC strip.

**Aim**

The aim of this research was to find a suitable TLC method for gallium-68 ubiquicidin that can separate colloidal impurities from the labelled product. This work therefore aimed to:

1. Assess several mobile phases in combination with iTLG-SG to identify a method that can distinguish between gallium-68 colloidal impurities and the labelled product on one TLC

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strip. In this scenario, colloidal impurities would usually remain at the origin while the labelled products should migrate away from the origin.

2. Establish if C18 purification and membrane filtration (0.22 µm pore size membrane) on their own are able to quantitatively remove colloidal impurities from the labelling mixture.

## Methods

### *General methods*

Prior to testing the combination of specific stationary and mobile phases, an 'impurity' mixture was first assessed using a known method that can distinguish between free gallium-68 and colloidal gallium-68 [8]. This investigation focused on using iTLC-SG (Varian, Lake Forest, USA) strips. The following reagents, consumables, chromatography mobile phases, and instruments were used: sodium citrate, ammonium acetate, methanol, tri-fluoro-acetic acid, ethyl-acetate, acetone (Sigma Aldrich, St Louis, USA), concentrated HCl, acetonitrile, butan-2-one, pH strips with 0.5 unit increments, 0.22 µm sterile vented filter (Merck, Massachusetts, USA), saline (B. Braun, Melsungen, Germany), sterile empty glass vials (GE Healthcare, Chicago, USA), 0.6 M HCl (ABX, Radeberg, Germany), iThemba LABS $^{68}Ge/^{68}Ga$-generator (iThemba LABS, Somerset West, South Africa), Scan-RAM™ radio-TLC scanner (Lablogic, Sheffield, UK). C18 (tC short) Sep-Pak cartridges (Waters, Milford, USA), and a Curiementor 3 dose calibrator (PTW, Germany). Double deionized water was obtained from a Merck-Millipore Direct Q3 water purification system (Merck, Massachusetts, USA),

To create an impurity mixture that contained both free gallium-68 and colloidal gallium-68, a $^{68}Ge/^{68}Ga$-generator was eluted with 0.6 M HCl. The eluate (1 ml) was transferred into a separate sterile empty glass vial. The pH of this solution was adjusted to pH 6.0 – 6.5 with 2.5 M sodium acetate solution, to induce the formation of colloids according to the method suggested by Petrik *et al.* [16]. The mixture was incubated at room temperature for 15 minutes. To confirm the presence

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of free- and colloidal gallium-68, iTLC-SG strips (85 mm x 8.5 mm) were used for TLC analyses. The mobile phase was prepared by diluting 3.4 ml HCl (37% m/m) to 10 ml with analytical grade methanol (15% HCl in methanol). Approximately 5 µl of the impurity mixture was spotted at 15 mm from the lower edge of the strip (origin), which was then immediately placed in a mobile phase consisting of sodium citrate buffer pH 5.0. The mobile phase was allowed to migrate up to 5 mm from the top edge of the strip (solvent front) and was immediately removed from the developing chamber. The strip was dried and distribution of the radioactivity along the strip was visualized using a radio-TLC scanner. The background and visible peaks on the resultant radio-TLC chromatogram were evaluated. Free gallium-68 migrated with the solvent front while colloidal gallium-68 remained close to the origin.

NOTA-UBI was labelled with gallium-68 as previously described using a Scintomics automated synthesis module [17]. The radiochemical purity evaluation with HPLC confirmed the presence of > 95% [$^{68}$Ga]Ga-NOTA-UBI after radiolabelling. Pre-conditioning of the C18 cartridge was performed with 5 ml ethanol, followed by 10 ml purified water.

A series of experiments were conducted to address each of the aims listed earlier:

Experiment 1 to address Aim 1

To find a suitable TLC method to identify the presence of colloids in gallium-68 ubiquicidin a number of sub-experiments were conducted which included the following:

*Experiment 1.1*

This experiment consisted of evaluating several mobile phases using [$^{68}$Ga]Ga-NOTA-UBI to establish the retardation factor (Rf) of the labelled product on iTLC-SG as the stationary phase. This experiment was performed as an initial screening test, to identify a potential suitable TLC method where the Rf of [$^{68}$Ga]Ga-NOTA-UBI was approximately 0.5 – 1.0. Any colloidal impurity would be anticipated to have a Rf of 0.0 – 0.1.

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*Experiment 1.2*

In this investigation an impurity mixture that contained both gallium-68 colloids and free gallium-68 was tested using several mobile phases with iTLC-SG. The purpose of this experiment was to find a mobile phase that could successfully distinguish between gallium-68 colloids and free gallium-68. Based on the results of this experiment, only mobile phases that could distinguish between these two species were considered for further evaluation in experiment 1.3.

*Experiment 1.3*

In this set of experiments, [$^{68}$Ga]Ga-NOTA-UBI was spiked with a colloid/free gallium-68-containing impurity mixture (prepared as described earlier) and analyzed with iTLC-SG. Mobile phases identified in experiment 1.2 were used.

Experiment 2 to address Aim 2

The impurity mixture containing gallium-68 colloids was prepared and the presence of radioactive colloids was confirmed as described above. This mixture (1 ml) was passed over a pre-conditioned C18 cartridge and the purified effluent was collected in a glass vial. The cartridge was further rinsed with 5 ml purified water, which was collected in the same vial. TLC analysis was then performed on the combined effluent to identify the presence of colloids using iTLC-SG as stationary phase and 0.1 M sodium citrate buffer pH 5.0 as mobile phase. On three occasions, the C18 cartridge was further washed with 2 ml Milli-Q water and this wash water was collected in a glass vial for further TLC analysis.

To evaluate the effectiveness of membrane filtration to remove colloidal impurities, 1 ml of the same eluate mixture used above was passed through a 0.22 µm pore size membrane filter. The filter was then rinsed with 5 ml purified water. The filtered mixture was collected in glass vial and analyzed with the TLC method described earlier.

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## Results

The results from experiment 1.1 to 1.3 are listed in **Tables 6.1 – 6.3** below. Typical chromatograms of TLC analyses performed on the eluate mixture and [$^{68}$Ga]Ga-NOTA-UBI using iTLC-SG in combination with the different mobile phases are shown in **Figure 6.1 a-e** below.

*Results experiment 1.1*

**Table 6.1** Retardation factor [$^{68}$Ga]Ga-NOTA-UBI using thin-layer chromatography in combination with various mobile phases and iTLC-SG as stationary phase

| Mobile phase | Rf of [$^{68}$Ga]Ga-NOTA-UBI # |
|---|---|
| Ammonium acetate/ methanol (50:50 v/v) | 0.1 - 0.3 |
| Ammonium acetate/methanol (30:70 v/v) | 0 - 0.1 |
| Ammonium acetate /methanol (70:30 v/v) | 0.2 |
| HCl (conc)/methanol (5:95 v/v) | 0 - 0.1 |
| HCl (conc)/methanol (10:90 v/v) | 0 - 0.1 |
| 3% Tri-fluoro acetic acid | 0 - 0.1 |
| 4% Tri-fluoroacetic acid | 0 - 0.1 |
| 5% Tri-fluoro acetic acid | 0 - 0.1 |
| 15% HCl in methanol | 0.65 † |

†: well-defined peak shape
# purified using C18 cartridge

Based on the results displayed in **Table 6.1** [$^{68}$Ga]Ga-NOTA-UBI 29-41 only migrated clearly beyond the origin in 15% HCl in methanol on iTLC-SG as stationary phase and formed a characteristic peak with a Rf = 0.65 (n = 2). In all other tested mobile phases, there was almost no migration of [$^{68}$Ga]Ga-NOTA-UBI from the origin (Rf 0 – 0.3).

*Results from experiment 1.2*

The results from these experiments displayed in **Table 6.2** showed that only 15% HCl in methanol and 4% TFA are mobile phases that potentially distinguish between colloidal impurities and free gallium-68, however, no colloidal impurities could be detected. The control confirmed the presence

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of both gallium-68 species and provided an appropriate separation of free gallium-68 from colloidal gallium-68.

**Table 6.2** Results from TLC analysis of impurity mixture in various mobile phases on iTLC-SG

| **Mobile phase** | **Impurities separated (Y/N)** | **Rf of separated impurities** |
|---|---|---|
| 0.1 M Sodium citrate pH 5 buffer (control) | Yes | Colloid: Rf = 0 – 0.1<br>Free gallium-68: Rf = 0.70 |
| Ammonium acetate methanol (50:50 v/v) | No | |
| Butane-2 one/ethyl acetate (50:50 v/v) | No | |
| Water/methanol (50:50 v/v) | No | |
| Ethyl acetate | No | |
| HCl pH 5 | No | |
| Butane-2 one | No | |
| Acetone | No | |
| Methanol | No | |
| Methanol/saline (50:50 v/v) | No | |
| Methanol saline ( 5:1) | No | |
| Methanol saline (1:5) | No | |
| Saline | No | |
| 4% TFA | Yes | Colloid: not detected<br>Free gallium-68: Rf = 0.70 |
| Acetonitrile /water (50:50 v/v) | No | |
| 15% HCl in methanol | Yes | Colloid: not detected<br>Free gallium-68: Rf = 0.65 |

*Results from experiment 1.3*

**Table 6.3** Rf values of [$^{68}$Ga]Ga-NOTA-UBI using citrate buffer and 15% HCl in methanol

| **Product** | **Mobile phase** | **Rf** |
|---|---|---|
| Impurity mixture | 0.1 M Sodium citrate pH 5.0 | coll: 0 - 0.1, free: 0.70 |
| Impurity mixture | 15% HCl in methanol | coll: nd, free: 0.70 |
| [$^{68}$Ga]Ga-NOTA-UBI | 15% HCl in methanol | [$^{68}$Ga]Ga-NOTA-UBI: 0.65 |
| [$^{68}$Ga]Ga-NOTA-UBI (sp) | 15% HCl in methanol | Coll: nd,[$^{68}$Ga]Ga-NOTA-UBI: 0.65 |
| [$^{68}$Ga]Ga-NOTA-UBI | 0.1 M sodium citrate pH 5.0 $^{\#}$ | [$^{68}$Ga]Ga-NOTA-UBI: 0 - 0.1 |

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**Figures 6.1a-e** Chromatograms of the impurity mixture and [$^{68}$Ga]Ga-NOTA-UBI with iTLC-SG and different mobile phases. In these figures the Y-axis represents the measured radioactivity (counts) and the X-axis represents the migration distance (0-100 mm).

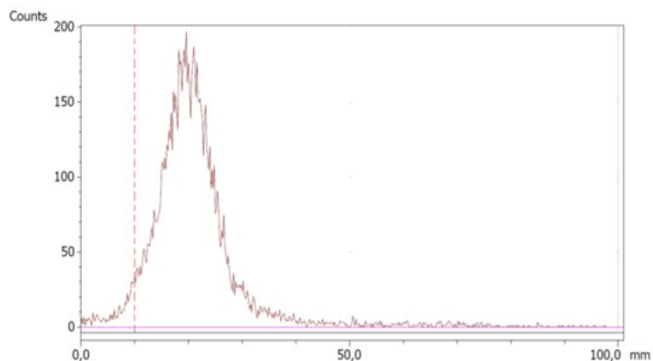


**Figure 6.1a** Typical chromatogram of [$^{68}$Ga]Ga-NOTA-UBI using iTLC-SG and ammonium acetate/methanol (50:50 v/v)

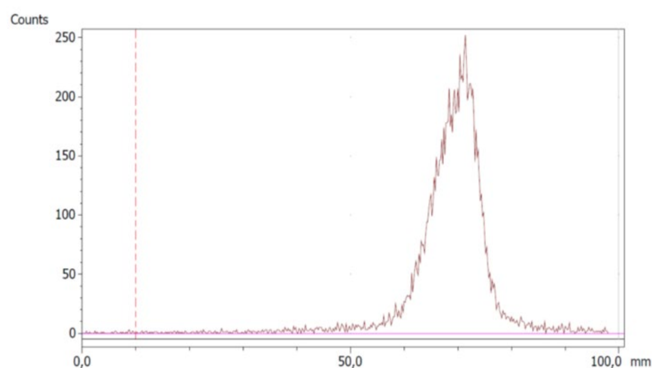


**Figure 6.1b** Typical chromatogram of [$^{68}$Ga]Ga-NOTA-UBI using iTLC-SG and 15% HCl in methanol

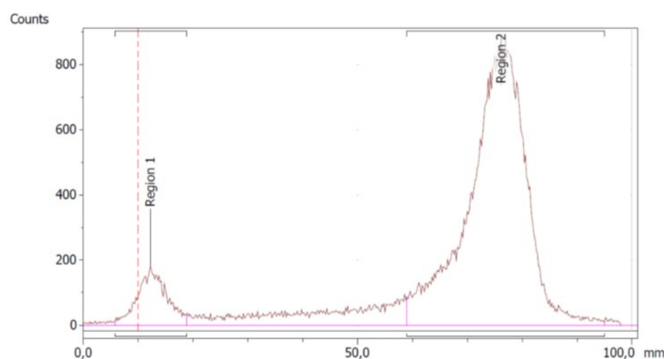


**Figure 6.1c** TLC chromatogram of impurity mixture using iTLC SG and sodium citrate (Region 1 = gallium-68 colloids; region 2 = free gallium-68)

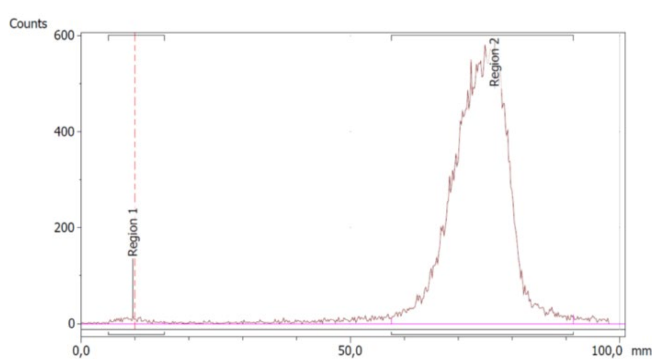


**Figure 6.1d** TLC chromatogram of impurity mixture using iTLG-SG and 15% HCl in methanol (region 1 = gallium-68 colloids, region 2 = free gallium-68)

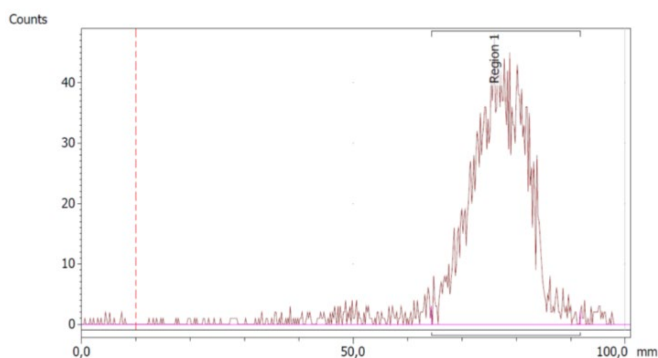


**Figure 6.1e** Typical TLC chromatogram of [$^{68}$Ga]Ga-NOTA-UBI spiked with the prepared impurity mixture using iTLC-SG and HCl in methanol (region 1 = [$^{68}$Ga]Ga-NOTA-UBI)

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The results from this experiment confirmed that a mobile phase consisting of 15% HCl in methanol was not able to detect the colloidal impurities in a spiked sample of [$^{68}$Ga]Ga-NOTA-UBI.

*Results from experiment 2*

Results of the TLC analyses performed on the mixture which were purified using a C18 cartridge or a membrane filter are listed in **Table 6.4** below.

**Table 6.4** Results from iTLC-SG analysis of impurity mixture following C18 purification and membrane filtration (mobile phase = 0.1 M sodium citrate buffer pH 5)

| **Sample (impurity mixture)** | **Colloidal gallium-68 (%)** |
|---|---|
| Crude (control, n = 3) | 9.46 ± 1.93 |
| C18 SPE purification (n = 5) | 4.62 ± 0.37 |
| Membrane filtration 0.22 µm (n = 4) | 4.93 ± 2.06 |

Both techniques, i.e. C18 purification and membrane filtration, reduced the amount of colloidal gallium-68 by 2-fold which was significant compared to the crude (unprocessed) samples. A quantitative reduction of the colloid impurity (about 10% is present the crude sample) was not achieved.

**Discussion**

*Experiment 1.1*

The 15% HCl in methanol was the only mobile phase in which [$^{68}$Ga]Ga-NOTA-UBI migrated a significant distance from the origin on iTLC-SG; it therefore has potential to distinguish between gallium-68 colloids and labelled product (**Figure 6.2**). If present, any gallium-68 colloidal impurity would remain at the origin. A single strip TLC method using 4% TFA and iTLC-SG suggested by Larenkov and Maruk was not further investigated as the Rf of [$^{68}$Ga]Ga-NOTA-UBI (Rf = 0.1 - 0.2)

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was too close to that of colloidal impurities. Altering the TFA contents to 3% and 5% did not result in any significant changes in the Rf of [$^{68}$Ga]Ga-NOTA-UBI.

*Experiment 1.2*

In this experimental setup mobile phases such as 15% HCl in methanol and 4% TFA showed potential to separate gallium-68 colloids from free gallium-68 using iTLC-SG as the stationary phase. With all these mobile phases, free gallium-68 clearly migrated almost to the solvent front, similar to its behaviour in sodium citrate (control, **Figure 6.3**). Gallium-68 colloids were expected to remain at the origin. However, TLC analyses detected very little, and in some cases, no gallium-68 colloids with the 15% HCl in methanol mobile phase. This observation was made despite the confirmed presence of gallium-68 colloids in the mixture. It is important to note that the same impurity mixture was used for chromatography with sodium citrate and 15% HCl in methanol. The chromatogram in **Figure 6.4** shows the presence of small amounts of a colloidal impurity, which is markedly less than what was detected when sodium citrate was used as the mobile phase. In certain cases, no colloidal impurities could be detected (data not shown). The results from these experiments suggest that the higher acidity of 15% HCl in methanol ($pH < 2.5$) (compared to pH 5.0 for sodium citrate) probably dissolves most of the colloidal impurities present in a sample. The pH of 15% HCl in methanol solution falls within the pH range where insoluble $Ga(OH)_3$ can become soluble again, therefore dissolving any colloidal impurities in the test solution. The presence of colloidal impurities can therefore not be detected, making this method unreliable and not suitable for routine use.

*Experiment 1.3*

Based on findings from experiments 1.1 and 1.2 it was a plausible validation step to spike [$^{68}$Ga]Ga-NOTA-UBI with the eluate mixture that contained both radioactive impurities. Analyzing the TLC chromatogram of spiked [$^{68}$Ga]Ga-NOTA-UBI using 15% HCl in methanol and iTLC-SG, any colloidal gallium-68 impurities should be visible at the origin while [$^{68}$Ga]Ga-NOTA-UBI (and free gallium-68) should migrate with the mobile phase. The TLC chromatogram of the spiked [$^{68}$Ga]Ga-

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NOTA-UBI sample showed no presence of colloidal gallium-68 at the origin (**Figure 6.5**). [$^{68}$Ga]Ga-NOTA-UBI (and free gallium-68) migrated a significant distance (Rf = 0.7) as expected, confirming what was demonstrated in experiments 1.1 and 1.2. Overall, the results suggest that a mobile phase consisting of 15% HCl in methanol can therefore not be used to identify or quantify the presence of colloidal impurities in [$^{68}$Ga]Ga-NOTA-UBI preparations.

*Experiment 2*

It is known from the chemistry of gallium that insoluble $Ga(OH)_3$ is formed at a pH range of 3 – 9 [18]. Under acid conditions, $Ga^{3+}$ is stable in an aqueous solution, but at a pH range of 3 - 9 insoluble $Ga(OH)_3$ may be present in the solution as a colloidal impurity. According Morgat *et. al.* this insoluble species can occur up to pH 9. At pH > 9.5, formation of a soluble gallate ion $Ga(OH)_4$ is induced [18]. Labelling of radiopharmaceuticals with gallium-68 using bifunctional chelators such as DOTA is predominantly performed at a pH range of 3.5 - 5.0. At this pH range, insoluble gallium-68 can form when the labelling requires high specific activities, i.e. when the amount of precursor is low [10, 19]. Peptide labelling, rather than colloid formation, is enhanced in the presence of higher precursor amounts. It is therefore important to test for the presence of gallium-68 colloids in the final product solution.

It has been widely accepted that C18 purification will remove colloidal impurities [12, 20]. In kit-based formulations of gallium-68 radiopharmaceuticals, C18 purification often does not form part of the preparation of the radiopharmaceutical. Results from this study however indicate that a C18 purification procedure does not remove all colloidal impurities from the product solution after the initial purification step. Using TLC analysis, the gallium-68 colloids present in the effluent after purification amounted to 4.6 ± 0.4%. This is 48.8% of the amount of colloidal impurities initially present in the impurity mixture. Moreover, it should be stated that C18 purification usually consists of two steps. In the first step, the labelled product is retained on the C18 cartridge while soluble

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hydrophilic impurities are diverted to waste. During this step, smaller colloids that are not retained by the C18 cartridge will be collected in waste. Subsequent elution of the labelled product from the C18 cartridge will most likely not contain any colloidal impurities. It is important to note that the experimental set-up in this study was different to that of a normal radiosynthesis. In this experiment, the rinse liquid was analyzed as opposed to analyzing the final product as in a normal synthesis.

Sterilization of gallium-68 radiopharmaceuticals is usually performed using a sterile 0.22 µm pore size membrane filter. The use of membrane filtration to remove colloidal impurities is suggested by Bhatt *et al.* To the best of our knowledge, membrane filtration to remove colloidal impurities, has not been proven in the literature. TLC analysis of a test solution known to contain colloidal impurities showed that the filtered solution still contained colloidal impurities after membrane filtration. The gallium-68 colloids present in the solution amounted to $4.9 \pm 2.1\%$ of the activity on the strip. This value represents 52.1% of the amount of colloidal impurities that was initially present in the eluate mixture. Compared to data described by Bhatt *et al.*, the result presented in this study demonstrates that membrane filtration is not an effective technique to remove colloidal impurities from gallium-68 labelled radiopharmaceuticals. The results rather suggest that C18 purification may be a more effective way of removing colloidal impurities from a solution. The results of this study also differ from the results reported by Decristoforo *et al.* in which only 14% reduction in colloidal impurities in [$^{68}$Ga]Ga-DOTA-TOC was achieved with C18 purification step [12]. It should be emphasized that our research intentionally created a solution that contained colloidal impurities. It is therefore possible that the quantity and size of colloids may differ from normal gallium-68 labelling procedures.

**Conclusion**

Despite testing several mobile phases on iTLC-SG, a reliable method to identify and quantify gallium-68 colloidal impurities could not be established. The frequently used mobile phase

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consisting of a 1 M ammonium acetate/methanol (50:50) mixture cannot distinguish between gallium-68 colloids and [$^{68}$Ga]Ga-NOTA-UBI. Using 15% HCl in methanol as the mobile phase may underestimate of the amount of colloids present in a final product solution. This study has emphasized the relevance of a C18 purification step in the absence of a suitable method to test for gallium-68 colloids in certain gallium-68 radiopharmaceuticals such as [$^{68}$Ga]Ga-NOTA-UBI. Future work should be aimed at identifying a reliable and sensitive method that can quantify the presence of colloidal gallium-68 in [$^{68}$Ga]Ga-NOTA-UBI or similar radiopharmaceuticals.

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# Chapter 7

# Discussion and Conclusion

Radiopharmacies situated in relatively small clinical environments such as Nuclear Medicine departments usually have limited access to sophisticated equipment in contrast to research and manufacturing pharmacies. This can limit access to newer radiopharmaceuticals that are not commercially available. This study investigated the in-house synthesis and analysis [$^{68}$Ga]Ga-NOTA-UBI as a first model peptide using fairly limited equipment.

Several important findings are reported in this study. A number of recommendations for future research are provided.

## Summary of findings

The achievements of this study can be summarized as follows:

- Two different fully automated methods for the labelling of [$^{68}$Ga]Ga-NOTA-UBI have been successfully developed, optimized and validated. These methods can both be used for the efficient and safe synthesis of [$^{68}$Ga]Ga-NOTA-UBI under GMP-compliant conditions.
- The reduction of radiolytic impurities using radical scavengers during the labelling process was a key aspect for both automated methods. In particular, following cationic eluate pre-purification, a combination of at least two radical scavengers were required to reduce the formation of radiolytic impurities to an acceptable level. This work also revealed, contrary to recent reports in the literature regarding other gallium-68 labelled peptides, that using ethanol as a sole scavenger did not improve radiochemical yield or radiochemical purity of [$^{68}$Ga]Ga-NOTA-UBI preparations. The results from this research also suggest that the side products observed on radio-HPLC are more likely due to radiolysis and not the result of oxidation of methionine during the heating step. Lower activities used in the manual labelling method did not result in the formation of the side products.

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- The in-depth comparison of a manual labelling method with two automated methods for [$^{68}$Ga]Ga-NOTA-UBI supported the automated methods using a Scintomics GRP synthesis unit. Those methods showed a high degree of robustness and repeatability and furthermore had the clear advantage of reducing radiation exposure to operators and facilitating production of radiopharmaceuticals in a GMP-compliant manner.
- It is evident from data available in the literature that the toxicity of HEPES to humans and animals may be overestimated. Clearly, the quantities of HEPES used in the labelling of gallium-68 radiopharmaceuticals are far less than the HEPES doses described in published studies. It is therefore reasonable to argue that regulatory institutions should reconsider the current strict limits.
- The importance of a reliable and sensitive method to detect the presence of gallium-68 colloids in [$^{68}$Ga]Ga-NOTA-UBI preparations is highlighted. This work also emphasizes the importance of a purification step to routinely minimize the potential occurrence of gallium-68 colloids in gallium-68 radiopharmaceuticals such as [$^{68}$Ga]Ga-NOTA-UBI. This work may also be relevant to other radiolabelled peptides.

**Limitations and future work**

A few limitations of this study were identified and are listed below. Suggestions for further work are provided.

- The scope of this study did not include [$^{68}$Ga]Ga-NOTA-UBI synthesis with an automated method which combined a generator eluate pre-purification step with HEPES as buffering agent.
- It is possible that the radio-HPLC method that was used in the analysis of [$^{68}$Ga]Ga-NOTA-UBI was not sufficiently sensitive to resolve possible product related impurities close to the main peak. A further investigation aiming to better identify by-products should be

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considered. This could ideally form part of a radioanalytical study based on the findings of this work.

- It is not feasible to investigate all possible combinations of scavengers or buffer-scavenger combinations, but the tested scavengers showed success. Other combinations than those described may also reduce radiolysis and further improve the peptide labelling conditions.
- The possibility of reducing total synthesis time by increasing the flow rates in the post-heating steps during the automated labelling of [$^{68}$Ga]Ga-NOTA-UBI was not investigated. It is recommended to investigate further optimization of the synthesis, which may lead to an increase in the amount of radiolabelled product.
- The ITLC method used for quality control analyses of [$^{68}$Ga]Ga-NOTA-UBI could not distinguish between colloidal impurity and the labelled compound. This study only investigated iTLC-SG as the stationary phase and other stationary phases should also be evaluated. It is recommended that a reliable and sensitive TLC procedure be developed.

The influence of varying the pH and temperature on the labelling characteristics of [$^{68}$Ga]Ga-NOTA-UBI was extensively investigated by Ebenhan et al. during the development of the manual labelling method. It was not deemed necessary to reinvestigate these parameters for the purpose of this study. A graphical presentation of the effect of varying pH, temperature and incubation time on labelling efficiency of [$^{68}$Ga]Ga-NOTA-UBI is provided in addendum I.

The findings in this research can be translated to the development of other radiopharmaceuticals using alternative chelators and radionuclides for e.g. zirconium-89 ($^{89}$Zr) and copper-64 ($^{64}$Cu). This may include an investigation into finding the most suitable chelator for the specific radionuclide, choice of a buffer to adjust the pH of the labelling mixture, whether heating is required or not, and if necessary, considering the use radical scavengers to improve the RCP of the labelled product. Labelling monoclonal antibodies with zirconium-89 can be used as example. It is well known that antibodies are heat sensitive and the labelling should therefore not include a heating step which can damage the antibodies. A further aspect to consider is the choice of a suitable chelator.

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Desferrioxamine B (DFO) is a hexadentate chelator and the octadentate derivative has been suggested as a suitable chelator for labelling with zirconium-89 due to the oxiphilic properties of the $Zr^{4+}$ metal ion [1]. The longer half-life of zirconium-89 (t½ = 78 hours) is suitable for imaging radiolabelled antibodies because of their slower pharmacokinetics. Copper-64 on the other hand has a shorter half-life (t½ = 12.7 hours) than zirconium-89 and has also been successfully used in labelling [$^{64}$Cu]Cu-DOTA-TATE for somatostatin receptor imaging.

UBI fragments are cation-rich and *in-vitro* and pre-clinical studies have shown preferential binding to bacterial cells. This radiopharmaceutical is considered to be non-toxic and unlikely to cause adverse effects. First in human studies were encouraging, confirming its potential usefulness as a radiopharmaceutical for infection imaging [2]. There has been rapid development in the field of infection imaging with an emphasis on distinction between infections and sterile inflammation, and also targeting specific properties of bacteria and fungi as a method to detect specific bacterial or fungal infections.

The use of radiolabelled polysaccharides with fluorine-18 has also attracted some interest [3]. A transport mechanism for the transport of maltose and maltodextrins that is specific to bacteria has made it possible to image bacterial infections using maltose labelled fluorine-18. The potential of labelling polysaccharides with gallium-68 is therefore also a possibility due to the chemical properties of Ga (III).

Siderophore-based imaging of *A. fumigatus* is based on radiolabelling of fusarine C and a tri-acetylated derivative triacetylfusarine C (TAFC) which are secreted by this fungus. A specific siderophore iron transporter (SIT) type is responsible for transport of the chelator complex into the fungus [4]. These transporters appear to be confined to fungi only and in certain instances limited to specific species. Specific imaging of *P. aeruginosa* infection can be achieved by labelling pyoverdine (PVD) with gallium-68. *Pseudomonas* species are known to exclusively produce PVD. [$^{68}$Ga]Ga-PVD has been successfully used to image *P. aeruginosa* infections even in the presence

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of a low bacterial burden. This radiopharmaceutical has also shown high specificity and sensitivity to detect *Pseudomonas* infections [4]. These radiopharmaceuticals however still need translation into clinical practice.

A recent systematic review by Auletta *et al*. discussed several PET radiopharmaceuticals for specific bacterial imaging. This review specifically mentioned that both [$^{68}$Ga]Ga-NOTA-UBI 29-41 as well as [$^{68}$Ga]Ga-NOTA-UBI 31-38 have been already been used in human studies with promising results [5]. Other PET radiopharmaceuticals that have also been used in humans include iodine-124 labelled 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil ([$^{124}$I]FIAU, but this radiopharmaceutical was neither specific nor sensitive to identify active bacterial infections [6]. Studies in humans with fluorine-18 labelled fluorodeoxysorbitol [$^{18}$F]FDS were able to distinguish between bacterial infections and sterile inflammation [7]. [$^{68}$Ga]Ga-NOTA-UBI 29-41 is a promising candidate in a small group of radiopharmaceuticals investigated for infection imaging with PET.

## Conclusion

Two different automated labelling methods for [$^{68}$Ga]Ga-NOTA-UBI were successfully developed in a hospital radiopharmacy environment using the synthesis module that is routinely used for production of [$^{68}$Ga]Ga-DOTA-NOC and [$^{68}$Ga]Ga-PSMA. In our institution, pre-purification of gallium-68 eluates prior to a radiosynthesis is preferred. It was therefore considered important to optimize one of the labelling methods to accommodate this step as part of the production method.

The effect of scavengers to reduce radiolysis can only be evaluated using HPLC analysis. It is therefore important that radiopharmacies that aim to develop new radiolabelling protocols have access to HPLC at least during the developmental stage of new protocols. This study highlights which steps in the production protocol need to be optimized. Not only does this include optimization of the labelling method, but it also involves evaluation of quality control procedures such as thin-

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layer chromatography. It is also essential that information on GMP-compliant methods is discussed to provide guidance on the safe production of radiopharmaceuticals for human use.

The knowledge gained from this study can be used as an important starting point for future optimization of production methods of other radiopharmaceuticals at our institution. This study may serve as an important guide to other radiopharmacies based in clinical settings regarding successful optimization of production methods.

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## Addendum A

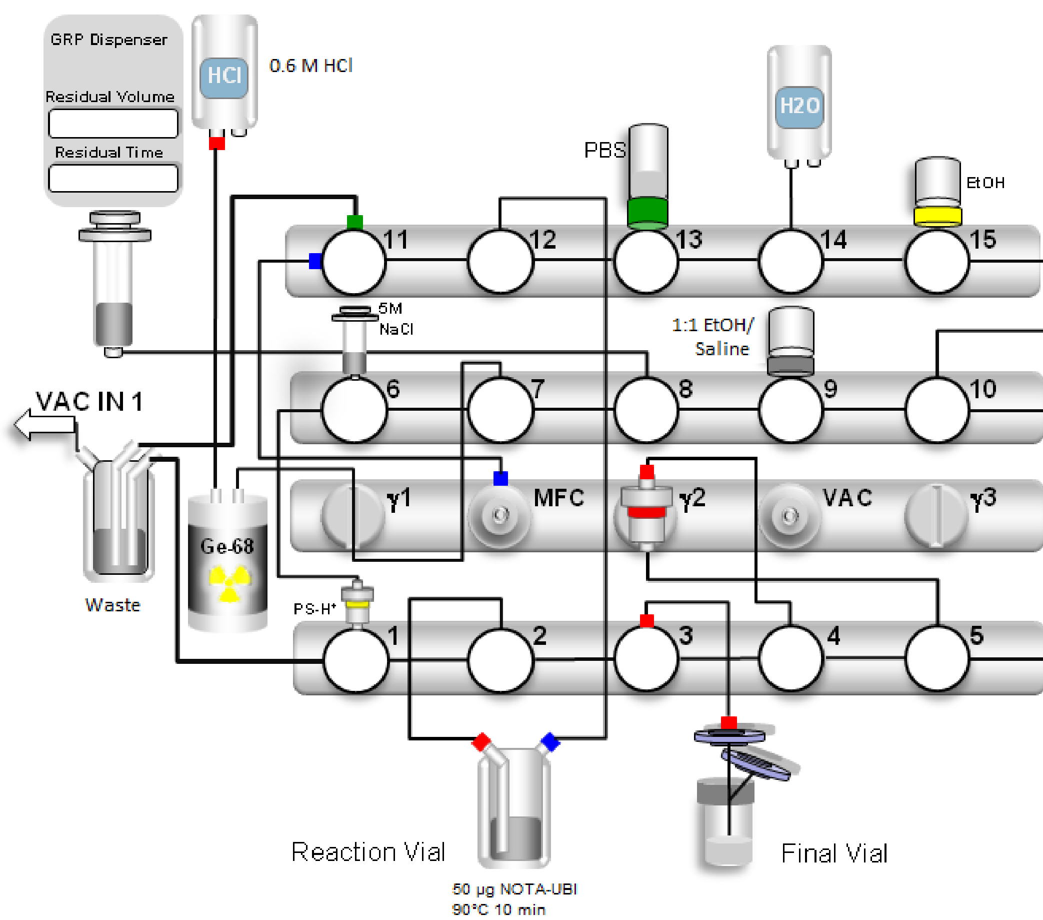


**Figure A1** Typical Scintomics module set-up for the synthesis of NOTA-UBI 29-41 using a $^{68}$Ge/$^{68}$Ga-

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**Addendum B**

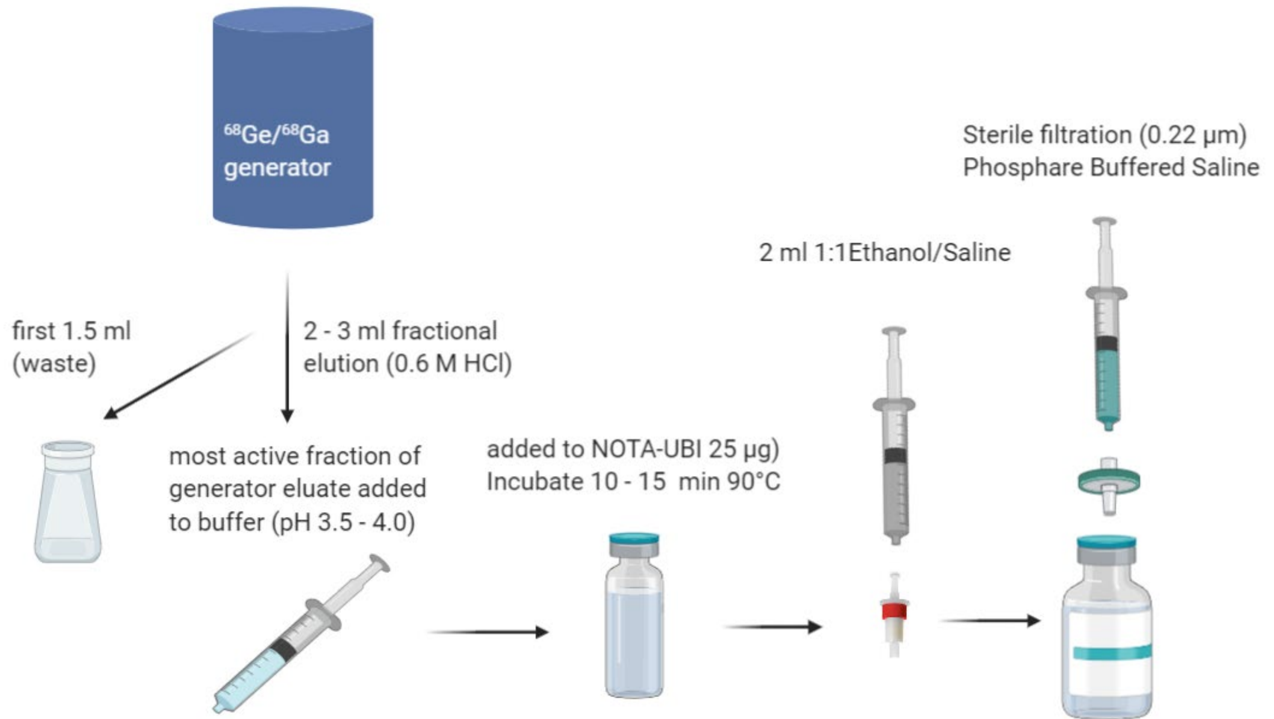


**Figure B1** Simplified steps of manual labelling method.

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## Addendum C

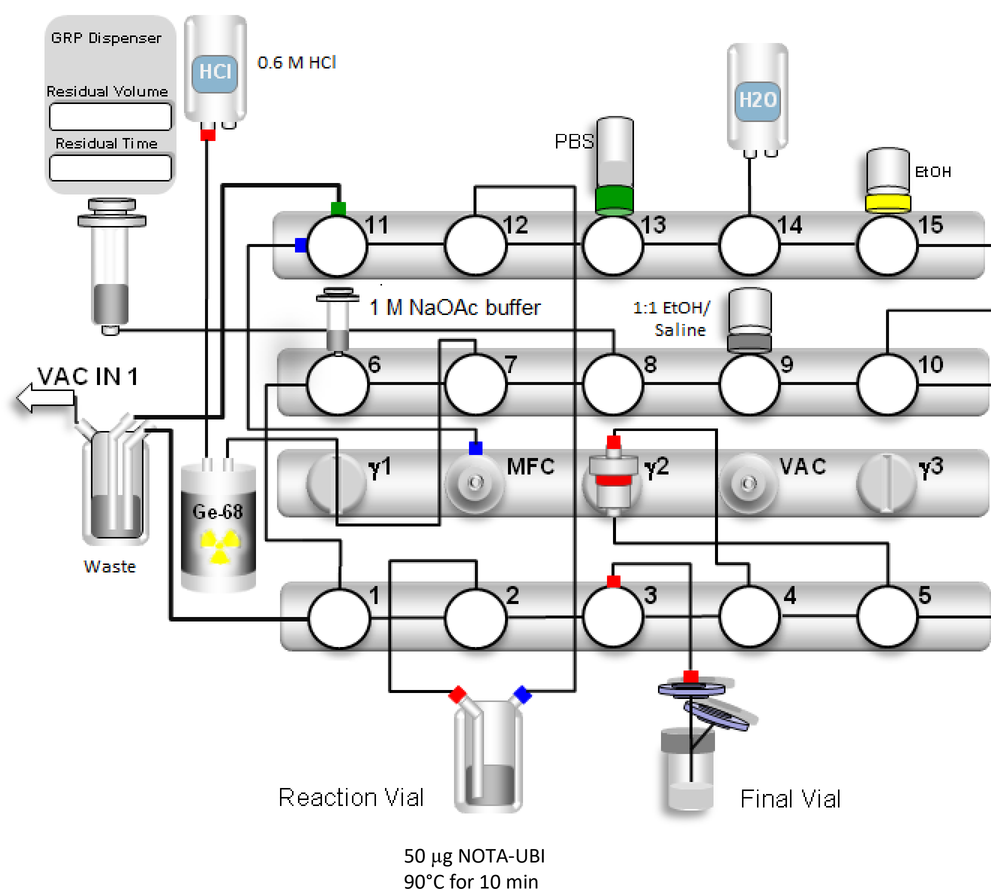


**Figure C1** Typical Scintomics module set-up for a fractional elution labelling method.

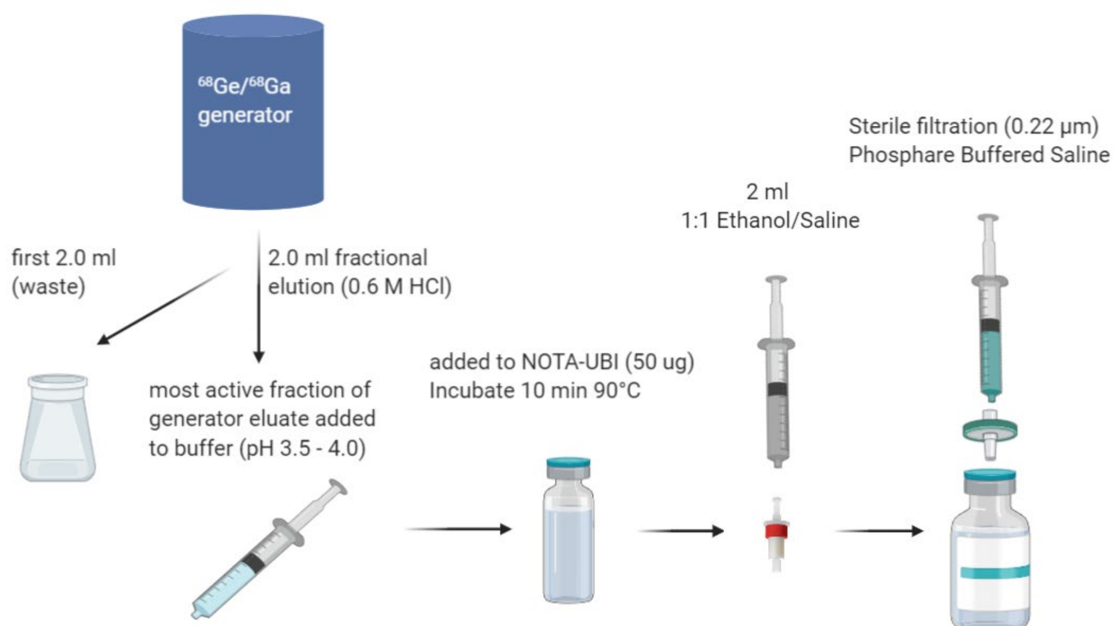


**Figure C2** Simplified steps of the fractional elution method labelling method.

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## Addendum D

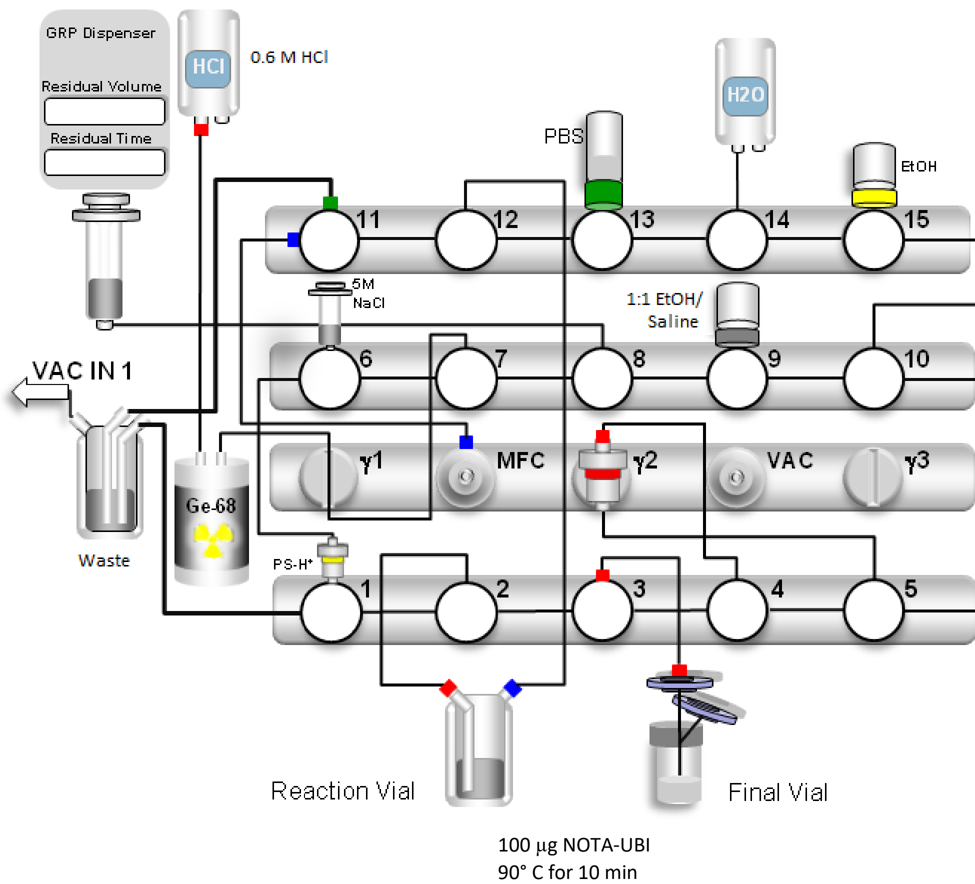


**Figure D1** Typical Scintomics module set-up for a cationic pre-purification labelling method.

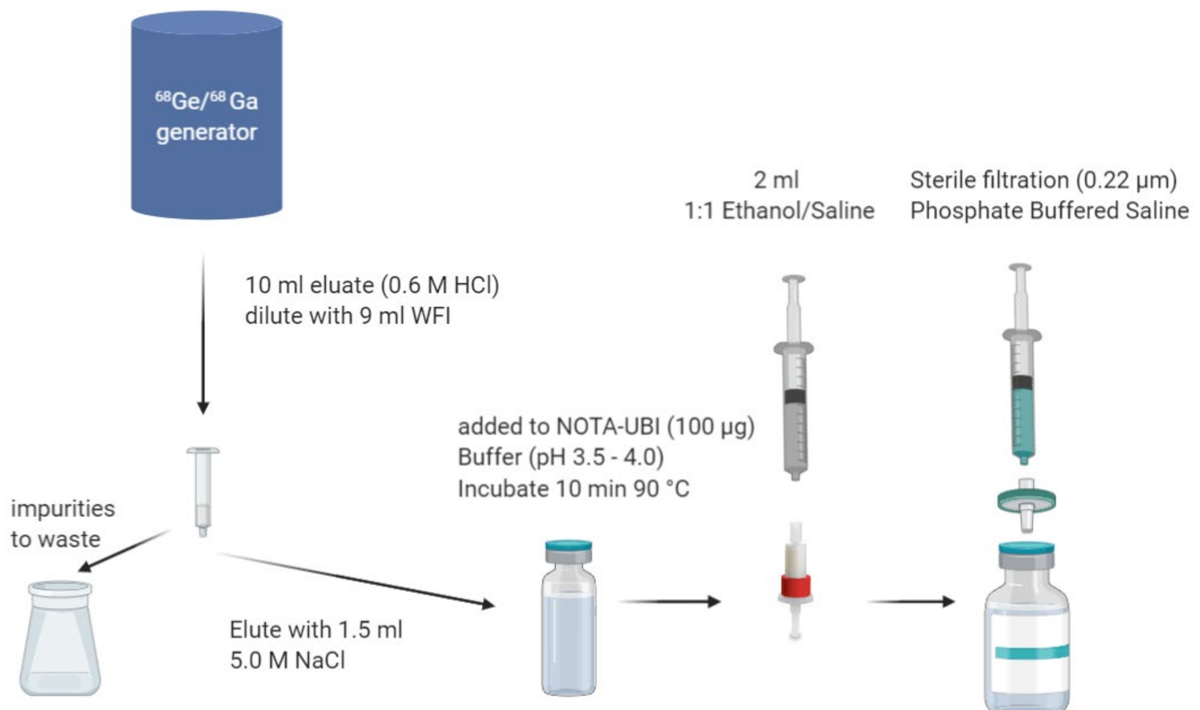


**Figure D2** Simplified steps of a cationic pre-purification labelling method.

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**Addendum E**

Published manuscript:

Le Roux J, Rubow S, Ebenhan T, Wagener C. An automated synthesis method for $^{68}$Ga-labelled ubiquicidin 29–41. *J Radioanal Nucl Chem*. 2020;323(1):105-116.

**https://link.springer.com/article/10.1007/s10967-019-06910-1**

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## Addendum F

**Le Roux, JS, Mnr [jsleroux@sun.ac.za]**

**From:** Robert Dannals <onbehalfof@manuscriptcentral.com>
**Sent:** Tuesday, 9 June 2020 22:06
**To:** Le Roux, JS, Mnr [jsleroux@sun.ac.za]
**Subject:** JLCR-20-0059 successfully submitted

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09-Jun-2020

Dear Mr Le Roux,

Your manuscript entitled "Impact of radical scavengers on the radiolabelling characteristics and purity of Gallium-68-ubiquicidin" has been successfully submitted online and is presently being given full consideration for publication in Journal of Labelled Compounds and Radiopharmaceuticals.

Your manuscript number is JLCR-20-0059. Please mention this number in all future correspondence regarding this submission.

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Sincerely,

Journal of Labelled Compounds and Radiopharmaceuticals Editorial Office

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**Addendum G**

Published manuscript:

Le Roux J, Rubow S, Ebenhan T. A comparison of the labelling characteristics of manual and automated synthesis methods for gallium-68 ubiquicidin. *Applied Radiation and Isotopes,* https//doi.org/10.1016/j.apradiso.2020.109452

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# Addendum H

## Nuclear Medicine Communications

### Technical note The use of HEPES-buffer in the production of gallium-68 radiopharmaceuticals – time to reconsider strict Pharmacopoeial limits?

--Manuscript Draft--

| | |
|---|---|
| **Manuscript Number:** | NMC-11-3643 |
| **Full Title:** | Technical note The use of HEPES-buffer in the production of gallium-68 radiopharmaceuticals – time to reconsider strict Pharmacopoeial limits? |
| **Article Type:** | Technical Note |
| **Keywords:** | HEPES; gallium-68; radiopharmaceuticals; limits; toxicity; human use |
| **Corresponding Author:** | Jannie le Roux, M.Sc<br>Stellenbosch University Faculty of Medicine and Health Sciences<br>Stellenbosch, Western Cape SOUTH AFRICA |
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| **Corresponding Author's Secondary Institution:** | |
| **First Author:** | Jannie le Roux, M.Sc |
| **First Author Secondary Information:** | |
| **Order of Authors:** | Jannie le Roux, M.Sc |
| | Janke Kleynhans, PhD |
| | Sietske Rubow, PhD |
| **Order of Authors Secondary Information:** | |
| **Manuscript Region of Origin:** | SOUTH AFRICA |
| **Abstract:** | HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) is a buffer that is used in the radiolabelling of gallium-68 compounds. The beneficial effects of HEPES on specific activity in bioconjugates have been well described. Current strict regulations on the HEPES content in radiopharmaceuticals limit its use when intended for parenteral administration.<br>This technical note summarizes data from the literature on the toxicity of HEPES in dogs after intravenous infusion and the subsequent use in humans. We also highlight the use of HEPES in a FDA labelled intravenous drug formulation. Regulatory institutions may consider this data to review current strict limits. |

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## Addendum I

Effect of varying incubation temperature, pH, incubation duration and compound molarity on the labelling efficiency of [$^{68}$Ga]Ga-NOTA-UBI 29-41 and [$^{68}$Ga]Ga-NOTA-UBI 30-41

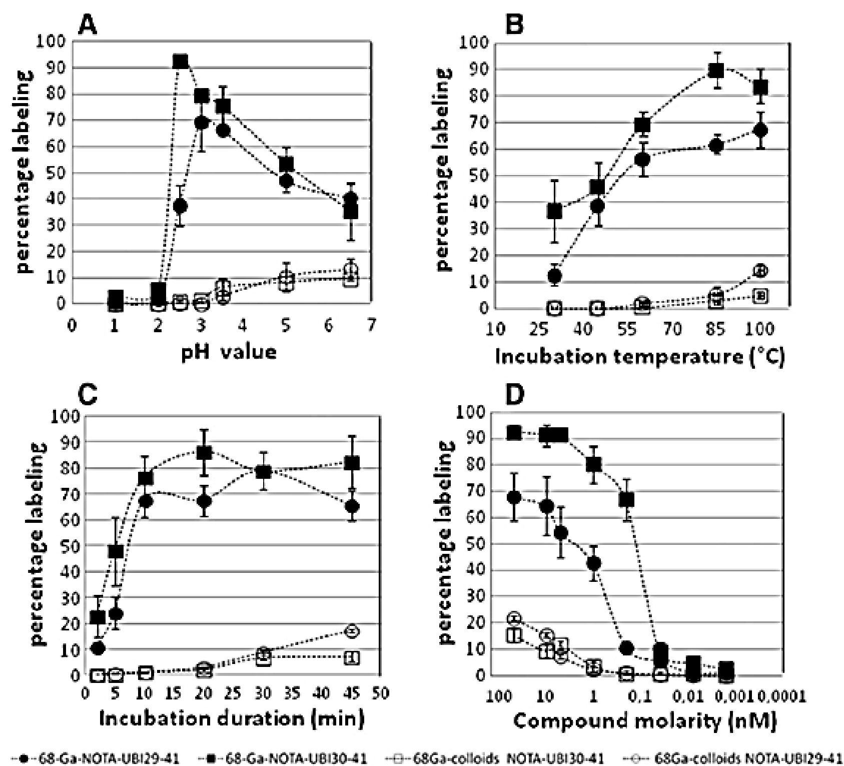


**Figure I1** The effects of various factors of $^{68}$Ga-labelling for NOTA-UBI including a) sodium acetate buffer molarity, b) incubation temperature, c) incubation duration and d) NOTA-peptide concentration (n > 3) (reprinted with permission from Ebenhan et al, 2014, ©Elsevier 2014).