

Qualification of in-house prepared ^{68}Ga RGD in healthy monkeys for subsequent molecular imaging of $\alpha_v\beta_3$ integrin expression in patients

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Preface

I hereby acknowledge and declare that this project was my own work performed and that all references are referenced and declared as such as well as acknowledgements made to people who were a part of this project in different phases and fields and tasks performed, in mentor or supportive roles. In addition, no conflict of interest exists. Acknowledgement was given to co-workers in terms of the references and directly indicating their contribution.

“Whoever wishes to pursue properly the science of medicine must proceed thus...For if a physician knows these things well...; so he will not be at loss in the treatment of diseases...”

Hippocrates

"There is no blue without yellow and without orange."

Van Gogh

“You treat a disease, you win, you lose. You treat a person, I guarantee you, you'll win, no matter what the outcome.

Our job is improving the quality of life, not just delaying death.”

Hunter Patch Adams

**“Theranostics is a revolutionary
Approach that promises improved
Therapy selection on the basis of
Specific molecular features of disease,
Greater predictive power for adverse
Effects due to improved patient-
Specific absorbed dose estimates,
And new ways to objectively monitor
Therapy response...”**

Prof. M.M. Sathekge

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Abstract

Introduction: Targeted pharmaceuticals for labelling with radio-isotopes for very specific imaging (and possibly later for targeted therapy) play a major role in Theranostics which is currently an important topic in Nuclear Medicine as well as personalised medicine. There was a need for a very specific lung cancer radiopharmaceutical that would specifically be uptaken in integrin $\alpha_v\beta_3$ expression cells to image patients using a Positron Emission Tomography- Computed Tomography (PET-CT) scanner.

Background and problem statement: Cold kits of c (RGDyK)–SCN-Bz-NOTA were kindly donated by Seoul National University (SNU) to help meet Steve Biko Hospital's need for this type of imaging. These cold kits showed great results internationally in labelling with a 0.1 M $^{68}\text{Ge}/^{68}\text{Ga}$ generator ($t_{1/2}$ of ^{68}Ge and ^{68}Ga are 270.8 days and 67.6 min, respectively). However the same cold kits failed to show reproducible radiolabeling with the 0.6 M generator manufactured under cGMP conditions at iThemba LABS, Cape Town and distributed by IDB Holland, the Netherlands.

Materials and methods: There was therefore a need for producing an in-house NOTA-RGD kit that would enable production of clinical ^{68}Ga -NOTA-RGD in high yields from the IDB Holland/iThemba LABS generator. Quality control included ITLC in citric acid to observe labelling efficiency as well as in sodium carbonate to evaluate colloid formation. HPLC was also performed at iThemba LABS as well as Necsa (South African Nuclear Energy Corporation). RGD was obtained from Futurechem, Korea. Kit mass integrity was determined by testing labelling efficiency of 10, 30 and 60 μg of RGD per cold kit. The RGD was buffered with sodium acetate trihydrate. The original kits were dried in a desiccator and in later studies only freeze dried. Manual labelling was also tested. The radiolabelled in-house kit's ex vivo biodistribution in healthy versus tumour mice were examined by obtaining xenografts. The normal biodistribution was investigated in three vervet monkeys by doing PET-CT scans on a Siemens Biograph TP 40 slice scanner.

Results: Cold kit formulation radiolabeling and purification methods were established successfully and SOPs (standard operating procedures) created. HPLC results showed highest radiochemical purity in 60 μg cold kit vials. ^{68}Ga -NOTA-RGD showed increased

uptake in tumours of tumour bearing mouse. The cold kit also showed normal distribution according to literature with fast blood clearance and excretion through kidneys into urine, therefore making it a suitable radiopharmaceutical for clinical studies.

Conclusion: The in-house prepared cold kit with a 4 month shelf-life was successfully tested in mice and monkeys.

Keywords: integrin $\alpha_v\beta_3$ expression, $^{68}\text{Ge}/^{68}\text{Ga}$ generator, c(RGDyK)–SCN-Bz-NOTA, xenografts, vervet monkeys, PET-CT, in-house cold kit.

Opsomming

Inleiding: Geteikende farmaseutikums vir die merking van radio- isotope vir baie spesifieke beelding (en moontlik later geteikende terapie) speel tans 'n groot rol in Teranostiek, 'n belangrike onderwerp in Kerngeneeskunde en verpersoonlikte medisyne. Daar was 'n behoefte vir 'n spesifieke long kanker merker wat opgeneem kan word in integrien $\alpha_v\beta_3$ sel ekspressie om pasiente te verbeeld met 'n Positron Emissie Tomografie-Rekenaar Tomografie (PET-RT) skandeerder.

Agtergrond en probleem: Koue kitsstelle van sikliese '(RGDyK)-SCN-Bz-NOTA' was deur Seoul Nasionale Universiteit (SNU) aan Steve Biko Hospitaal geskenk om met hul probleem te help. Die koue kitsstelle het goeie resultate gelewer internasionaal wanneer hul gemerk is met 0.1 M generator $^{68}\text{Ge}/^{68}\text{Ga}$ ($T_{1/2}$ van ^{68}Ge en ^{68}Ga is 270.8 dae en 67.6 min, onderskeidelik). Ewewel het dieselfde koue kitsstelle gefaal om herhaalbare radiomerking te lewer met die 0.6 M generator wat onder cGMP kondisies by iThemba LABS Kaapstad en versprei deur IDB Holland, Nederland.

Materie en metodes: Daar was daarom 'n behoefte vir produksie van 'n self geproduseerde RGD kitsstel en na NOTA vasgestel is as die cheleerder van keuse, was ^{68}Ga -NOTA-RGD suksesvol gemerk en gesuiwer, in die gebruik van Ga-III wat geëlueer is van die IDB Holland/iThemba LABS generator. Kwaliteits kontrole het ITLC ingesluit in vloeï mediums van sitriese suur en om merkings doeltreffendheid vas te stel en natrium karbonaat om kolloïed vorming vas te stel. HPLC is ook by iThemba LABS asook Necsca (Suid Afrikaanse Kernenergie Korporasie) uitgevoer. RGD was verkry van Futurechem, Korea. Kitsstel massa integriteit was vasgestel deur die toets van merkings doeltreffendheid van 10, 30 en 60 mikrogram RGD per koue kitsstel. Die RGD was gebuffer met natrium asetaat trihidraat. Die oorspronklike kitsstelle was gedroog in 'n dissekator en in latere studies slegs gevriesdroog. Merking met die hand was ook getoets. Die radio gemerkte self-geproduseerde kitsstele se distribusie in gesonde teenoor tumor draende muise was ondersoek deur die verkryging van xenograafs. Die normale distribusie was ondersoek in drie blou apies deur PET-RT skandering op 'n Siemens Biograph TP skandeerder te doen.

Resultate: Koue kitsstel formulering en radiomerkings en suiwerings metodes was suksesvol vasgestel en SOPs (Standaard Operateurs Prosedures) is ontwerp.HPLC het die hoogste suiwering getoon in 60 mikrogram koue kitsstel flesse. Massa bepalende studies het bevestig dat 60 mikrogram gebruik moes word in die kitsstel. ^{68}Ga -NOTA-RGD het opname getoon in die tumor draende muis. Die koue kitsstel het ook normale distribusie getoon in blou apies in verhouding tot literatuur wat verwys na vinnige bloed opruiming en uitskeiding deur die niere en blaas wat dit daarom 'n gunstige radiofarmaseutikum maak vir kliniese studies.

Gevolgtrekking: Die self geproduseerde koue kitsstel van omtrent 4 maande rakleefyd is suksesvol getoets in muise en ape.

Sleutelwoorde: integrien $\alpha_v\beta_3$ ekspressie, $^{68}\text{Ge}/^{68}\text{Ga}$ generator, c(RGDyK)-SCN-Bz-NOTA, 60 mikrogram, natrium asetaat trihidraat, xenograaf, blou apies, PET-RT skandeerder, self geproduseerde koue kitsstel.

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List of Abbreviations

A	Activity (radioactivity)
AIDS	Acquired Immuno Deficiency Syndrome
ALARA	As low a reasonably achievable
AUCC	Animal use and care committee
cGMP	current Good Manufacturing Practice
cGRPP	Current Good Radiopharmacy Practice
CT	Computed Tomography
CTI	Competitive Technologies, Incorporated
^{18}F	Fluor-18
FDG	Fluorodeoxyglucose
^{68}Ga	Gallium-68
^{68}Ge	Germanium-68
GMP	Good Manufacturing Practice
HIV	Human Immunodeficiency Virus
HPL	High pressure liquid chromatography
ITLC	Instant thin layer chromatography
^{111}In	Indium-111
K30 and K60	Refers to 30 and 60 μg of peptide in cold kit
LE	Labelling efficiency

MI	Molecular Imaging
MICAD	Molecular imaging and contrast agent database.
⁹⁹ Mo	Molybdenum-99
MRI	Magnetic Resonance Imaging
n	sample size(number)
NOTA-cyclic RGDyK	Cyclic Arg-Gly-Asp-D-Tyr-Lys-NOTA
NM	Nuclear Medicine
PET-CT.	Positron Emission Tomography- Computed Tomography
pH	Potential Hydrogen
RCP	Radiochemical purity
SD	Standard deviation
SEM	Standard error of mean
SOP/SOP's	Standard operating procedure/procedures
SPECT	Single Photon Emission Computed Tomography
SPN	Solitary Pulmonary Nodule
SUV	Standardized Uptake Value (of tumor Calculated during PET-CT processing).
SUVs	Standardized uptake values
TB	Tuberculosis

TNM	Tumour, Nodes, Metastasis
USA	United States of America
VOI/VOIs	Volume of interest/Volumes of interest

List of Symbols and Equations

$^{\circ}\text{C}$	Degrees in Celsius
$\%ID/g$	Percentage injected dose per gram
$\alpha_v\beta_3$	alpha-v beta-3
$E=mc^2$	Theory of relativity
mCi	milli Curie
ml	milli litre
r	Correlation
μ	Micro sign
μg	Microgram
μl	micro Litre
\geq	Greater than or equal to
\leq	Less than or equal to
$<$	Less than
$>$	Greater than

Chapter 1: Introduction and problem statement

1.1 Introduction

Medical radio isotopes play a major role in new developments and patient management.

“There is something about the ‘radium isotopes’ that is so remarkable that for now we are telling only you...perhaps you can suggest some fantastic explanation”...Otto Hahn (1938) as cited in Bodanis (2000).

Ten years ago lung cancer was already known as the leading cause of death among different cancers already almost ten years ago (Chen et al., 2005). Countries that are economically developed still has cancer as the number one killer and in developing countries it is the second reason for death according to global cancer statistics (Jemal et al., 2011). It is therefore also geographically important to look at the statistics in Southern Africa. According to global statistics lung cancer is increasing in some countries including countries in Africa, (Jemal et al., 2011). Lung cancer incidence in South Africa did not decline even in a 9 year period (Bello et al., 2011). Furthermore cancer in itself has been projected a seventy eight percent increase by 2030 in South Africa (Health24 press release, 2013).

Even though cancer is treated worldwide, South Africa and Africa is unique in its current epidemiologic pattern. South Africa has some of the highest Human Immunodeficiency Virus (HIV) infection rates in the world (Sathekge and Buscombe, 2011) Acquired Immune Deficiency Syndrome (AIDS) related malignancies are increasing. Advanced lung cancer for example stages III or IV is now even diagnosed in younger patients with HIV. In comparison to the general population, the incidence of lung cancer in HIV patients is 2 to 4 times higher (Pakkala and Ramalingam, 2010). About ten years ago studies by United States of America (USA) researchers already showed lung cancer as one of the risk cancers 4 to 27 months after onset of AIDS (Mbulaiteye et al., 2003).

Lung cancer in itself has some challenges in diagnosis especially when wanting to diagnose a Solitary Pulmonary Nodule (SPN). It is important to view lung anatomy to

understand the complex structures where for example a SPN could be detected. Anatomically lungs constitute of a few structures including different lung lobes (Fig 1-1) as well as lung epithelial cells through which lung cancer usually can metastasize (Herbst, 2013). Today cancer can still metastasize aggressively according to the TNM (Tumour, Nodes, and Metastases) staging criteria and therefore the availability of imaging modalities to detect cancer as early as possible as well as very sensitively is essential. Its spread is 'crablike' as described by Hippocrates (Dos Santos, 1999) and this is relevant as it can be imaged with specific Molecular Imaging (MI) scanners.

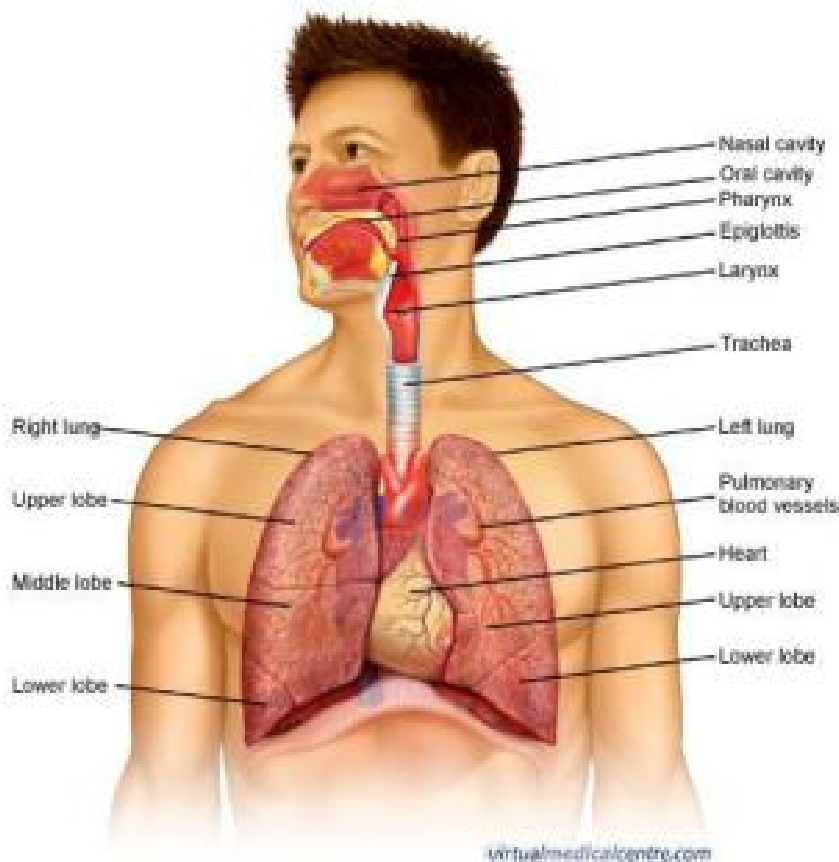


Figure 1-1: Lungs constitutes of different structures including lobes and epithelial cells (Herbst, 2013).

Figure.1-2 refers to the N of Tumour, Nodules, Metastases (TNM) staging therefore to which lymph nodes the primary tumour could spread to (American Cancer Society, 2013). PET-CT is the modality of choice for lymph node metastases detection.

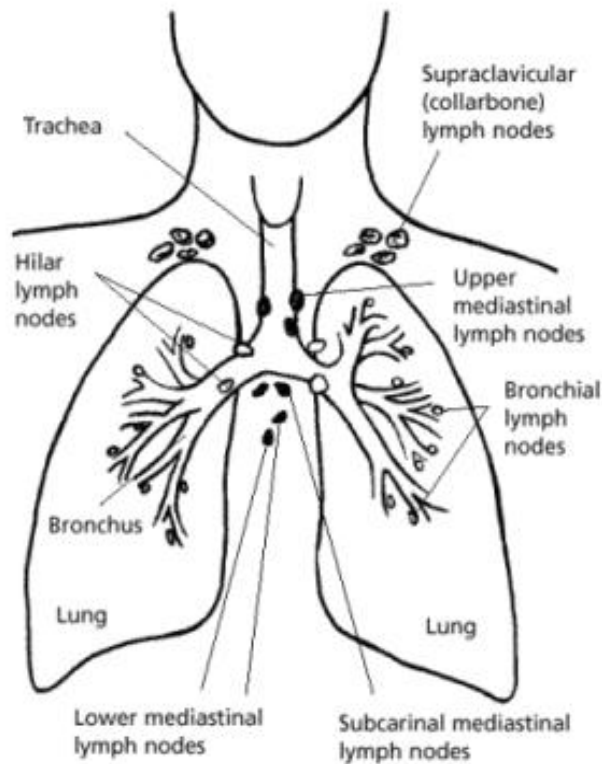


Figure 1-2: Lymph nodes refer to the N in TNM staging of cancer (American Cancer Society, 2013).

It is important to note that different modalities and therefore also clinical studies are utilized for detecting TNM status. It is in clinical studies that pharmaceuticals and radiopharmaceuticals can meet technology in the form of various imaging modalities. Several imaging modalities including gamma cameras also sometimes called single photon emission computed tomography (SPECT) scanners as SPECT is a part of gamma camera imaging, are available to investigate lung diseases as well as other non-imaging methods for example lung fine needle biopsy and blood tests. It is important to note the other possibilities as well as the route that a patient first go before visiting a Nuclear Medicine department in which radiopharmaceuticals play a major role. Basic X-rays, for example a

chest X-ray is the first imaging modality of choice when imaging is needed for a patient's lung investigation. This is to view any anatomical changes in the lungs. More advanced imaging that not only investigates anatomy but also physiology is nowadays available. The different scanner capability of detecting disease as well as the specificity and sensitivity of detection however differs a lot. It is therefore important for best patient management to utilise the correct modality especially when more physiological information is needed which can be detected with gamma cameras and other Molecular Imaging scanners. Hybrid imaging scanners, for example Positron Emission Tomography-Computed Tomography (PET-CT), plays a significant role in the management of the cancer patient. Since it is possible, by using different radiopharmaceuticals, to image tumour processes, such as angiogenesis, apoptosis and hypoxia with a PET-CT scanner. However, more clinical studies are needed to show the value of PET-CT imaging in radiotherapy planning and clinical studies are ongoing (IAEA, 2008). PET-CT could play a significant role in tumour staging in lung cancer especially with referral to TNM (Tumour Nodules Metastases) status.

Lung cancer and more specific a SPN lesion is an example of a tumour that needs very sensitive and specific detection by the imaging modality. This should be possible using molecular imaging modalities when a very specific in vitro tracer could be developed to target these specific tumours. Such tumours are over expressing integrin $\alpha_v\beta_3$ which still needs a specific radiopharmaceutical that could help to detect these cells with a PET-CT scanner. Several cancers have been investigated with various radiopharmaceuticals. In lung disease, clinical studies on differentiation of benign from malignant SPN could not conclude that the most currently available used radiopharmaceutical in South Africa in PET-CT imaging, Fluorine-18-Fluorodeoxyglucose (^{18}F -FDG) can distinguish between the two types of nodules in Tuberculosis (TB) (Sathekge et al., 2010). Such nodules could be followed up by PET-CT when a specific radiopharmaceutical could be developed for this purpose because the Standard Uptake Value (SUV) of the tumour could then be quantified. This is possible because the radiopharmaceutical tumour uptake could be measured by post processing imaging software if the correct parameters was also available, for example patient weight, injected dose and volume of interests drawn around applicable organs and the tumour. The liver, because of its more uniform homogeneous tissue, is usually used as

a standard in quantification process. Acquisition time and injected time also plays a role, however SUV could be calculated by using injected dose radioactivity concentration in a specific organ at a specific time point or as reference to whole body uniform distribution of the radiotracer(but at the same time point and at a specific image frame,therefore also requiring decay correction), (Standardized uptake value, 2014).

SUV is usually of significance when helping with the patient follow up, for example during or after radiotherapy or chemotherapy. Evaluation and measurements are possible with PET-CT imaging and data processing. PET-CT scanners are usually utilized in conjunction with radiotherapy planning because the different sets of images can give a lot of information on both the anatomy as well as the metabolic status of a tumour. This is best shown in imaging when patients could be injected with the best radiotracer that could possibly be visualized by the PET detector of the PET-CT scanner. Radiotracers that are used for PET-CT imaging as well as therapy could also play a role in Theranostics (diagnosis and therapy work together). This could be a better way of patient management when radiopharmaceuticals could be utilised more often in diagnosis as well as therapy, for example when an imaging tracer could be administered in higher dosage for patient therapy.

Therefore there is a need for more clinical studies to be done on various radiopharmaceuticals, including on integrin expression $\alpha_v\beta_3$ in angiogenesis (also page 10 &11).Molecular Imaging could play an enormous role in therapy and drug delivery systems when angiogenesis could be detected. Tumours smaller than 2 cubic millimetres do not yet have a blood supply, however blood supply formation directly could show tumour progression and possibility to metastasize. Dr Folkman already showed from 1970's a strategy to stop cancer growth by investigating angiogenesis inhibitors (Angiogenesis in cancer, 2014). Although there is continuous development of PET radiopharmaceuticals, they are not yet available or developed everywhere in the world. Some radiopharmaceuticals are not qualified yet for human administration and not yet manufactured under current Good Manufacturing Practice (cGMP) conditions. The smaller number of PET-CT scanners installed in Southern Africa is limited to availability of cyclotron produced isotopes and only registered radiopharmaceuticals, for example ^{18}F -FDG is available. Cyclotrons are costly to set up if infrastructure is unavailable, especially

in Africa. Therefore research on a Germanium-68/Gallium-68 ($^{68}\text{Ge}/^{68}\text{Ga}$) generator for investigating a variety of radiopharmaceuticals is a favourable alternative. Currently with most studies being performed with Cyclotron produced isotopes, a generator is suggested to be used supplementary to Cyclotrons, or in geographical areas which have no nearby Cyclotron. A generator's purchase is once a year in comparison to a Cyclotron which is installed once and can be utilised at least 15 to 20 years. A Cyclotron set up could be at least 20 million rand (Siemens Healthcare) in comparison to a generator with consumables which would be low below 1 million rand depending on consumables used when purchasing the iThemba Labs generator which should be replaced at least once or just less than once a year.

The radiation dose per patient would be much cheaper when produced in a $^{68}\text{Ge}/^{68}\text{Ga}$ generator than when cyclotron produced. The labelling of a radiopharmaceutical using the generator is much easier and faster than the complex chemistry involved in cyclotron produced isotopes and then synthesizing radiopharmaceuticals. Running costs of a cyclotron, for example all the consumables needed, is also much more expensive. Generators are also much more accessible than for example setting up cyclotrons. They are much smaller therefore only laboratory space is needed. The cost in this case would more be focused on a cGMP environment than the actual generator cost. Generators can also be developed in a shorter time than setting up and installing a cyclotron. In the case of the cyclotron set-up, the cGMP costs are also much higher due to the size of the laboratories as well as the additional equipment needed. This also includes additional cyclotron chemistry solutions.

1.2 Problem statement and aim of the study

Since the available radioisotopes for PET-CT imaging are expensive, it is also only available close to cyclotrons and cannot distinguish between two types of nodules with regards to TB nodules or Tumour nodule in a Single Pulmonary Nodule, the study's purpose was to develop a new, cheaper and geographically more available pharmaceutical for the purpose of radiolabeling and PET-CT imaging (scanning) in lung cancer with the advantage and probability of investigating other cancers in future with over expression of $\alpha_v\beta_3$ integrin. Cold kits for radiolabeling in a cheaper way were donated but failed to show

consistent labelling with $^{68}\text{Ge}/^{68}\text{Ga}$ generator and therefore an in-house cold kit was formulated.

1.3 Research objectives

In light of the poor labelling results achieved with the imported cold kits (see Chapter 2 for more details) an in-house cold kit was developed, evaluated in the laboratory and evaluated preclinically as preparation for future use in humans in order to meet the purposes stated above.

The objectives of this study were to:

- Create a SOP (Standard operating procedure) for kit formulation. This kit should be able to label specific with the 0.6 M $^{68}\text{Ge}/^{68}\text{Ga}$.
- Create a SOP for ^{68}Ga -RGD labelling and purification.
- Conduct a preclinical study in xenografted mice for comparing the biodistribution of healthy versus tumour bearing mice.
- Conduct a preclinical imaging of ^{68}Ga -RGD in healthy monkeys for the subsequent molecular imaging of $\alpha_v\beta_3$ integrin expression in patients.

Chapter 2: Background and literature review

2.1 Radiopharmaceuticals and cGMP in Molecular Imaging

2.1.1 Imported pharmaceutical kits and radio isotope production generator.

2.1.1.1 'Cold kit' pharmaceuticals and integrin expression cell detection.

Cold kits in Nuclear Medicine (NM) for example in the Radiopharmacy: It refers to the non radio active ingredients/ raw materials in small amounts not resulting in pharmaceutical or therapeutic effect when used for diagnosis only, therefore when used in small amounts only as well as used with diagnostic radiation dosage only. Radio isotopes are labelled to pharmaceutical 'cold kits' to form a radiopharmaceutical as seen in Figure 2-1 below:

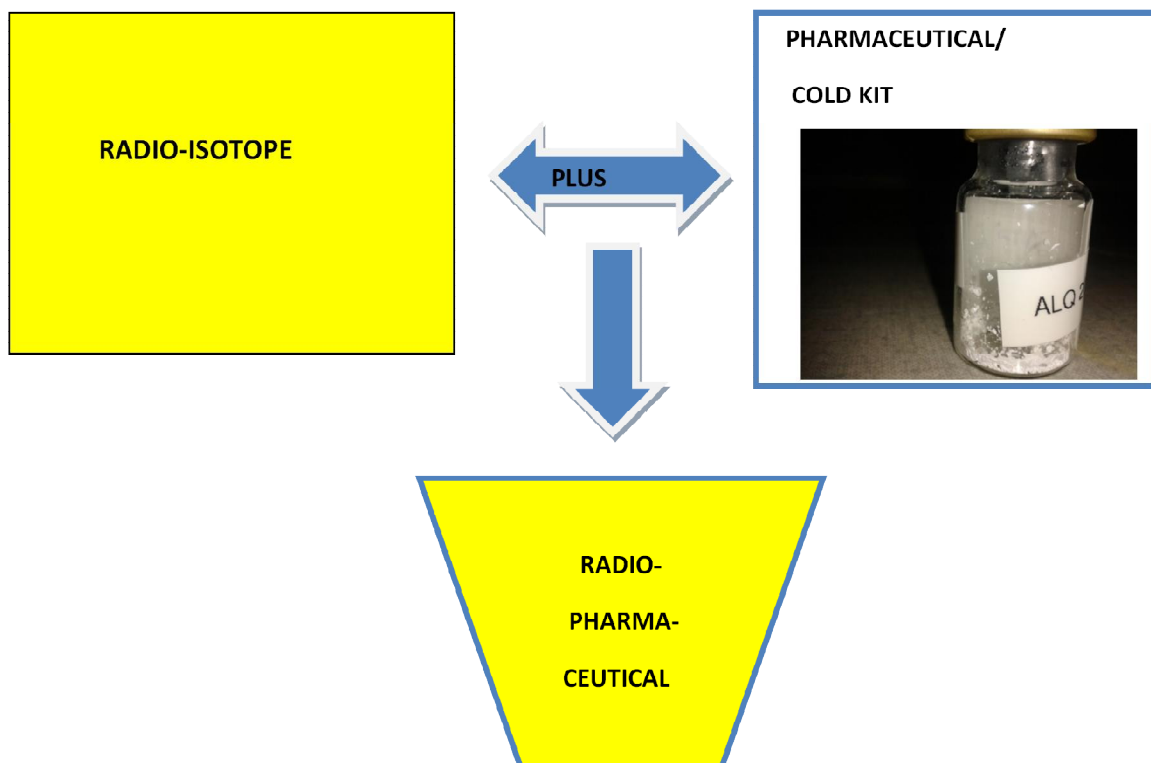


Figure 2-1: Schematic image of radiopharmaceutical basic labelling process.

'Cold-kit' pharmaceuticals and the development thereof play an enormous role in disease management. It is in physiological preclinical and clinical imaging studies that pathology could be best expressed when the actual in vitro diagnostic and or therapeutic ingredients could be more specific for disease detection. This could assist with overall disease management for example when a diagnostic radiotracer could also become in larger therapeutic amounts (radio isotope) a therapeutic probe for cancer treatment.

Cold kits of Arg-Gly-Asp (RGD) were originally kindly provided by Seoul University for labelling with ^{68}Ga with the purpose of imaging patients with tumours over expressing integrin $\alpha_v\beta_3$ for example lung cancer. Imaging of lung cancer especially SPN is still a need and especially in South Africa where there is limited PET radiopharmaceuticals available. ^{68}Ga labelled imported 'cold kits' have been investigated in this study. Jeong provided methodology (further described in Chapter 3) in labelling the same kits with their different types of $^{68}\text{Ge}/^{68}\text{Ga}$ generator as well as send results via email. The cold kits donated that were labelled in this study failed to show consistent reproducible results in labelling with the $^{68}\text{Ge}/^{68}\text{Ga}$ generator currently used in South Africa.

Integrin $\alpha_v\beta_3$ over expression on endothelial cells in tumours plays a major role in tumour angiogenesis as in Figure 2-2 (Weiss and Cheresh, 2011). This can include various different tumours. Angiogenesis and tumour growth is supported by the expression of integrins on many cell types, playing a role in detecting integrin physiology as well as playing a therapeutic role; the importance of these cells are that integrin $\alpha_v\beta_3$ is expressed on tumour cells and not on normal endothelial cells, (Weiss and Cheresh, 2011). Figure 2-2 also contains an image of fibroblasts: It is of significance to note that fibroblast growth factor or tumour necrosis factor that leads to angiogenesis needs the function of integrin $\alpha_v\beta_3$; $\alpha_v\beta_5$ (also illustrated on the figure 2-2) is required when vascular endothelial growth factor or transforming growth factor stimulates angiogenesis (Weiss and Cheresh, 2011). Pericytes are also illustrated in Figure 2-2. It is important to note that pericytes are removed from blood vessels when new blood vessels

formation starts (Danhier et al., 2012). For this study lung cancer will be the focus as this was the clinical need of Steve Biko Hospital, although some other cancers could also over express this integrin for example breast, prostate and pancreatic cancer. Lung cancer was a concern specific in HIV patients more than ten years ago (Poweles et al., 2003). Lung cancer was even then the mostly diagnosed cancer in the world (Baum et al., 2004). Nowadays the total patient management could change when more specific and sensitive diagnostic and therapeutic radiotracers could be investigated because as shown before, lung cancer is still a major problem. The specific need was for a radiotracer that could be uptaken in e.g. lung cancer that has over expression of Integrin $\alpha_v\beta_3$ cells and be more specific in diagnosing a SPN lesion.

In the modernised world, lung cancer is still the leading cause of death among different cancers in Europe and worldwide (Malvezzi et al., 2013).The need for lung tracers therefore has been increased over many years. An opportunity to investigate a new cold kit emerged.

Seoul University Korea kindly donated a batch of cold kits NOTA-RGD, buffered with sodium carbonate to Pretoria Academic Hospital (Steve Biko Hospital) to label for imaging over expression of integrin $\alpha_v\beta_3$ cells.

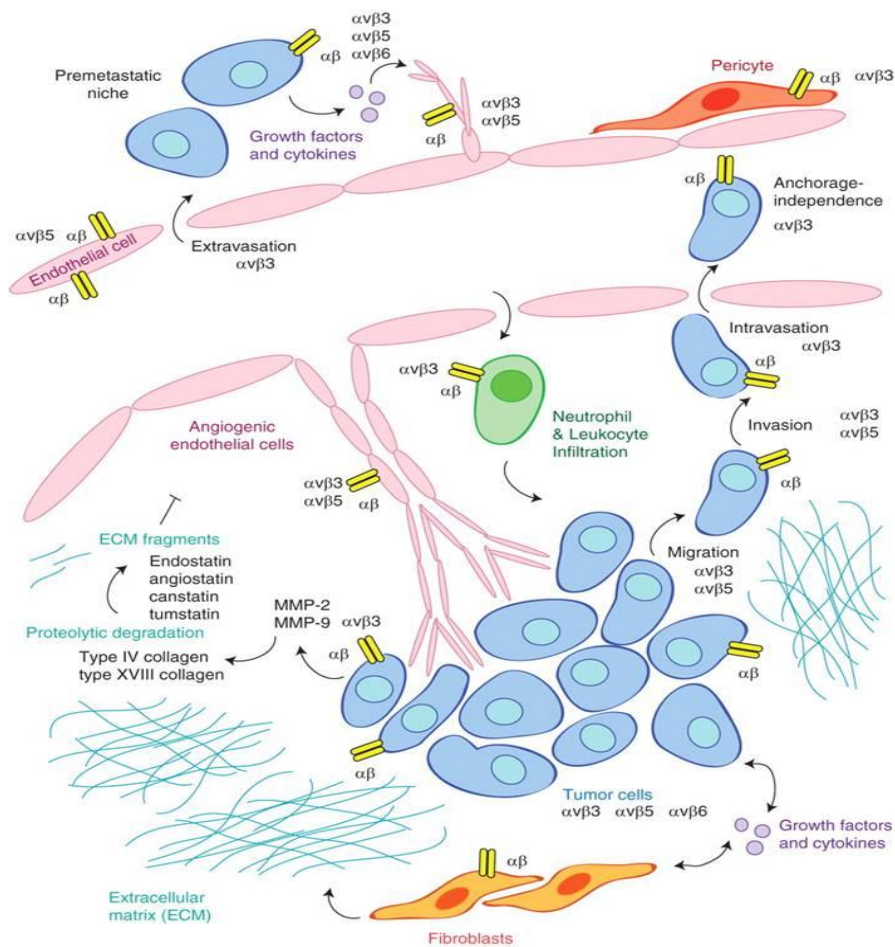


Figure 2-2: Integrin expression cells (Weis and Cheresh, 2011).

Integrin $\alpha_v\beta_3$ is an adhesion molecule involved in physiological and pathological angiogenesis as well as tumour invasion and metastasis. Therefore, it is considered an important target for molecular imaging and delivery of therapeutics for cancer, and there is a strong interest in developing novel agents interacting with this protein. Integrins also plays a much bigger role in tumor cells than just tumour growth. Its purpose is quite invasive as seen in Figure 2-3 below that shows how it also is involved in tumour progression including survival, migration and invasion and proliferation (Desgrosellier and Cheresh, 2010). It is important to know about metastases possible pathways in order to know how to image such cancers for example that involves integrin $\alpha_v\beta_3$ over expression.

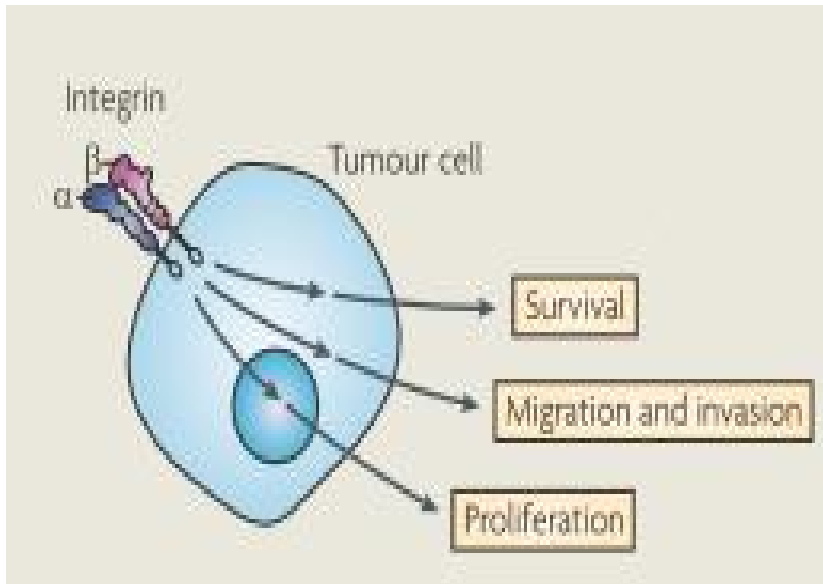


Figure 2-3: Integrins influencing the tumour cell in various ways (Desgrosellier and Cheresh, 2010).

PET-CT investigations in South Africa are currently dependant on the most available PET-CT radiotracer ^{18}F -FDG. Studies have already been done internationally for labelling ^{18}F with RGD (Arg-Gly-Asp) peptide (Lee et al., 2006; Beer et al., 2007). However this is still ^{18}F produced in a more expensive and less available cyclotron facility. Clinically there is an essential need for more novel radiopharmaceuticals. The PET principle is based on a PET scanner that can detect positron emitters that were administered to a patient. Positron events are registered after annihilation of electrons and positrons, meaning that coincidence events could be detected. This means detection of 511 keV photons moving in 180° opposite directions towards the PET detector that consists out of very specific crystal technology in order to detect the events. A line of response or source is therefore possible to locate (http://en.wikipedia.org/wiki/Positron_emission_tomography, 2014).

The $^{68}\text{Ge}/^{68}\text{Ga}$ generator is a reliable source for ^{68}Ga (IDB Holland bv operating instructions/iThemba labs; Zhernosekov et al., 2007). It is a positron emitter and ^{68}Ga as a metal can be labelled e.g. to various peptides. This positron emitter is

becoming more easily available. In the case of investigating integrin expression in tumor, RGD (Arg-Gly-Asp) was investigated in this study. Gallium labelling with proteins plays a major role in future of PET-CT tracer molecules (Wängler et al., 2011). However for this study, only RGD was labelled with a radio isotope, ^{68}Ga . ^{68}Ga has radio physical properties making it favourable for PET-CT imaging with high positron yield. It has a 68 min half-life (close to ^{18}F -FDG that has a 109.8 min half-life) and 89% positron emission (Blom et.al., 2012). It has a fast blood clearance and is rapidly uptaken in the target areas. It has also showed superior choice of preferred metal for labelling for example when compared with Indium-111-labelled peptides (Antunes et al., 2006). A chelator is needed to the macromolecule/ peptide on the one side to a metal (radio metal) ion on the other side therefore a chelator forming the most stable complexes was investigated. Chelator absence would lead to insufficient or no radiolabeling.

PET-CT using a new angiogenesis tracing radiopharmaceutical (^{68}Ga -labelled) was investigated in this study with NOTA as chelator. ^{68}Ga -NOTA-RGD may differentiate tumours with angiogenesis and tumours without angiogenesis with very high diagnostic accuracy. In addition, PET-CT scans using RGD-labelled isotopes may show higher diagnostic value as it provides structural and functional information in the same setting as compared to existing modalities for detection of angiogenesis, therefore, likely to detect far more cases of angiogenesis positive tumours.

RGD does not only have great affinity for integrin expression cells, but also have been used for cancer therapy as RGD targeted nanoparticles (Weis & Cheresh, 2011). It has also been used as gold nanoparticles for tumor targeting, (Arosio et al., 2011) and therefore has a variety of functionality and possibilities also for future use. RGD has been described a non-invasive radiotracer for radiolabeling purposes to image integrin $\alpha_v\beta_3$ in preclinical and clinical studies. Therefore radiotracers such as ^{68}Ga -DOTA-RGD as well as the newer tracer ^{68}Ga -NODAGA-RGD could be compared and showed that ^{68}Ga -NODAGA-RGD could also be used as alternative to ^{18}F -labelled RGD (Knetsch et al., 2011). This also

shows the variety and flexibility of RGD tracers. Therefore in this study labelling with NOTA was an interesting alternative. NOTA has been proven in literature also due to smaller than DOTA that its blood clearance is faster than DOTA and that it showed more stable labelling complexes and low serum binding (Joeng et al., 2009). NOTA has also been proven superior as chelator to DOTA in ^{64}Cu labelling for PET-CT imaging (Zhang et al., 2011).

2.1.1.2 Cold kits and ^{68}Ga radiolabeling problem

'Cold kit' pharmaceuticals and integrin expression cell detection:

Two batches of cold kits were tested; one kindly donated by Seoul National University (SNU) and one purchased from SNU/Jeong till in house prepared kit had been formulated.

Therefore an in house prepared kit, qualified specific for the use with this generator has been utilized also for preclinical imaging. The near future purpose is also clinical PET-CT imaging. RGD kit constitution differs from centres for example a specific manufacture's kit (Seoul National University) from an international site contained 10 microgram RGD. Another according to a study performed in Austria, 40 microgram (Knetsch et al., 2013) and currently in house prepared RGD kit, at least 30 microgram is used for labelling efficiency. RGD is the peptide of choice for this study. Knetsch also presented this publication in the 29th International Symposium, 2010 in Austria (Knetsch et al., 2010)

NOTA-SCN-RGD kits were kindly donated to Nuclear Medicine Department Steve Biko Hospital South Africa. The cold kits were supplied by Seoul National University (SNU), however did not show good labelling with 0.6 M HCl elution. Five kits that showed labelling were not tested on HPLC and unfortunately kit stability could not yet be tested. However In house prepared NOTA-RGD kit was prepared for labelling with 0.6 M HCl eluted ^{68}Ga from a SnO_2 -based $^{68}\text{Ge}/^{68}\text{Ga}$ generator.

RGD- NOTA kit labelled with ^{68}Ga has been well described by Jeong as a ^{68}Ga labelled tracer for angiogenesis evaluation (Jeong et al., 2008). The same kit formulated for labelling with 0.1 M $^{68}\text{Ge}/^{68}\text{Ga}$ generator therefore in this study failed to show consistent labelling with 0.6 M $^{68}\text{Ge}/^{68}\text{Ga}$ generator. Jeong's method described a purification method of c (RGDyK)–SCN-Bz-NOTA (NOTA-RGD) before labelling to ^{68}Ga . Due to clinical and financial need for using more ^{68}Ga labelled PET/CT tracers, the preparation on an inhouse kit was investigated.

2.1.2 Peptide labelling with stable chelators and 'lock and key' structure

It was important to note that during a target like a peptide receptor labelling with a ligand, the 'lock and key' structure remains as in Figure 2-4 (Jamous et al., 2013) for the purpose of stable labelling which is essential for quality of imaging when injecting a radiopharmaceutical into a patient. In this study, we also found that NOTA labelled stable with ^{68}Ga and the RGD peptide target, therefore other chelators were not further explored. "If, as in most reported cases (for instance DOTATATE), the NOTA or DOTA chelator is linked to the peptide via one of the acid groups (as is also reported herein) the conjugation ability of the chelator is reduced as compared to linking to the peptide via the carbon backbone. However studies have shown that the chelation is sufficient for *in vivo* use and stable for 24 h even if challenged with a 10^4 fold molar excess of DTPA ", (de SA et al, 2010), (Ferreira et al., 2010), (Kubicek et al., 2010).

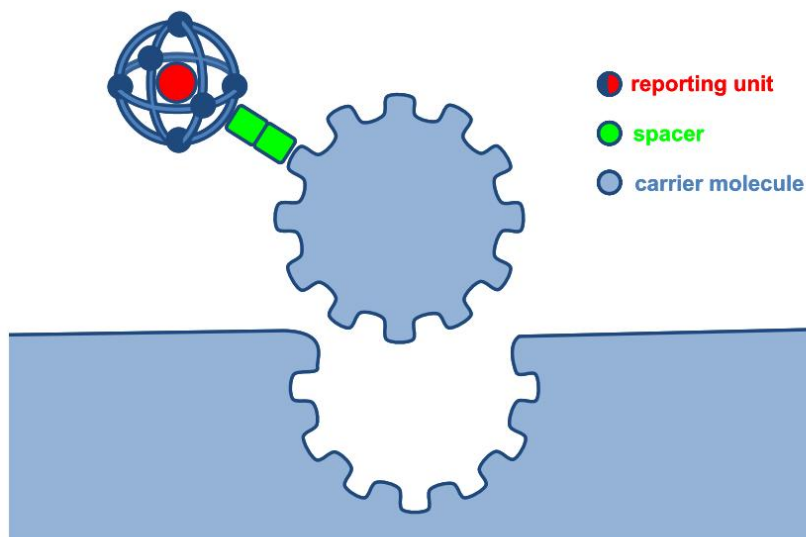


Figure 2-4: Peptide receptor binding with a ligand (Jamous et al., 2013).

'Lock-and-key' in this regards means that on the surface of tumour cells are receptors for example where there are over expression of the integrin $\alpha_v\beta_3$ cells. The receptors have an area like an opening in which the peptide could fit into; therefore like a key (peptide) that could fit into a keyhole (opening on receptor cells).

It is emphasized that according to literature too ^{68}Ga labelling for example Ga(III) forms an exceedingly stable complex with the NOTA chelator (Brechbiel, 2008; Guerin et al., 2010). It is essential to look at the requirements with regard to ^{68}Ga -labelled peptides with purpose to do preclinical and clinical work. Nowadays even a hydroxypyridinone is used as bifunctional chelator for ^{68}Ga labelling, therefore not alone NOTA anymore. This is compared below in Figure 2-5 (Berry et al., 2011) with other chelators for example NOTA that has been used for the purpose of this study.

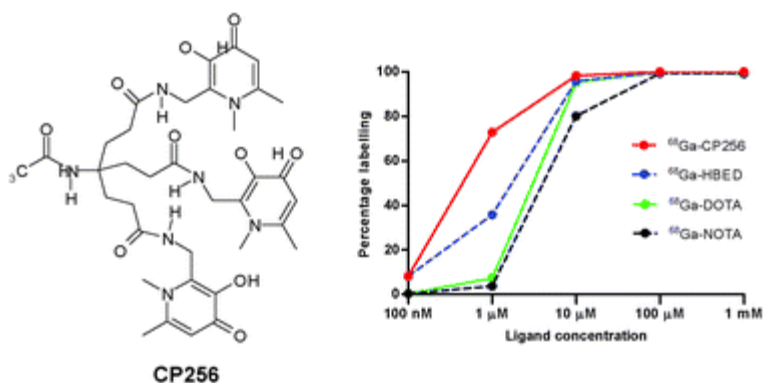


Figure 2-5: Different bifunctional chelators compared in ^{68}Ga labelling (Berry et al., 2011).

Chelators have also been described by Bartholomä (2012) including DOTA, NOTA and TETA shown in Figure 2-6 below.

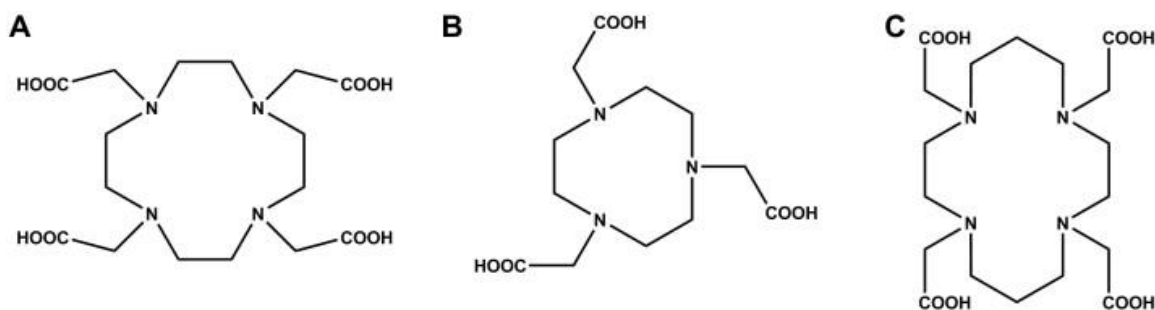


Figure 2-6: The most common bifunctional chelators in radiolabeling involving metals: A (DOTA), B (NOTA) and C (TETA), (Bartholomä, 2012).

It is important to note that the chelator should be chosen that is best to label with the radiometal. Molecular structure shape is affected by the chemical structure after labelling and therefore larger molecules for labelling purposes are preferred for example peptides (Lee, 2010). In this study the RGD peptide was used. Chelators are essential to secure radiolabeling which would without it not happen, however different chelators would be when compared show more or less stable labelled complexes.

Preclinically, a few ^{68}Ga tracers using different chelators have been used in comparison to evaluate uptake in melanoma tumour bearing mice. All tracers showed positive uptake in M21 human melanoma (M21-L is the negative control tumour) in Figure 2-7-4 below (Knetsch, 2012). It was only [^{68}Ga] Oxo-DO3A-RGD that was non-specific in tumour uptake. [^{68}Ga]Oxo-DO3A-RGD as well as [^{68}Ga] NS₃-RGD showed high uptake in liver and kidneys 60 minutes post injection and therefore is not suitable for clinical imaging (Knetsch et al., 2011).

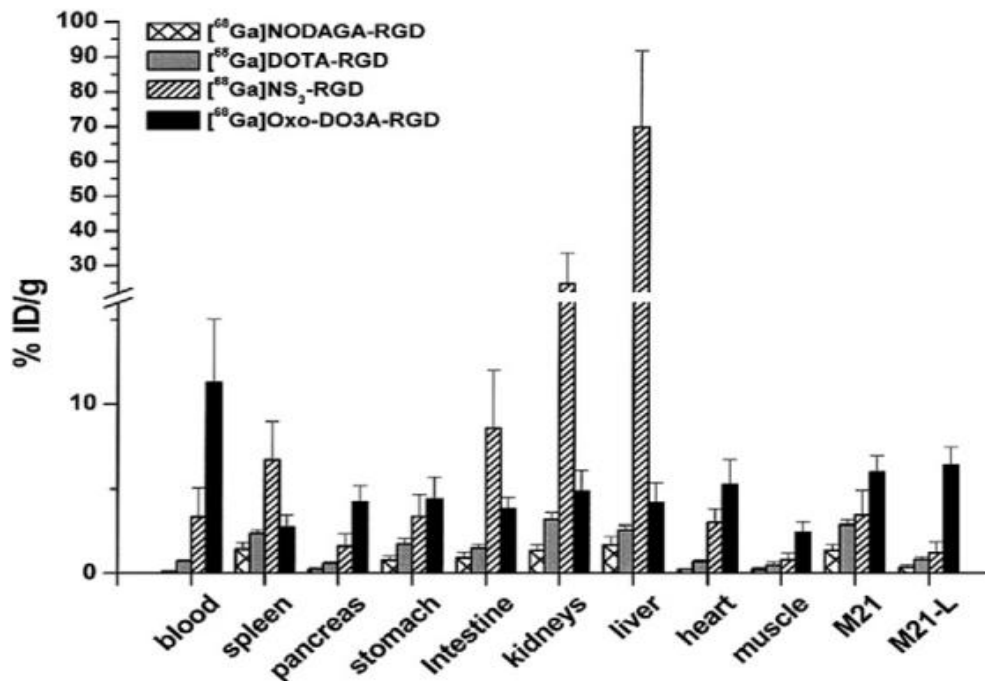


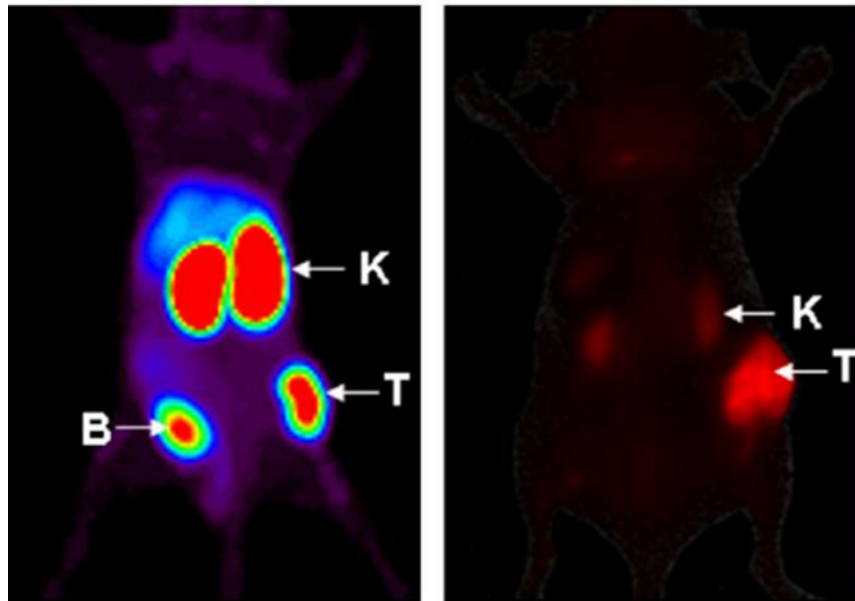
Figure 2-7: Various ^{68}Ga -RGD tracers compared (Knetsch et al., 2011).

In above Figure 2-7, ^{68}Ga -NOTA-RGD that was labelled in this study was not compared; however it was important to view the comparison of other chelators for future labelling opportunities.

^{68}Ga has been labelled with RGD for mice feasibility studies (Jeong et al., 2008) and then administered to humans in a study to evaluate tumor angiogenesis

(Baum et al., 2008). Other isotopes could be investigated further for example ^{64}Cu . The production of this isotope yet is more complex on Cyclotron when other targets for example Zink (Zn) Nickel (Ni) is used. The example below was already done four years ago, however Copper-64 (Cu-64) has not been used much for this purpose and is also a newer tracer regarding the fact that not many known sites produce Cu-64 specific for RGD labelling. RGD was labelled to ^{68}Ga but also Cu-64 in another study (Dumont et al., 2011).

It has been used in RGD Labelling though with good results on mice xenografts as shown in Figure 2-8 (Galibert et al., 2010) below. On top left image and scanned again 3 days later as shown on the right image. K represents the kidney, B the bladder and T the tumour. Image below, nr.12, represents the radiopharmaceutical that the tumour-bearing mouse was injected with.



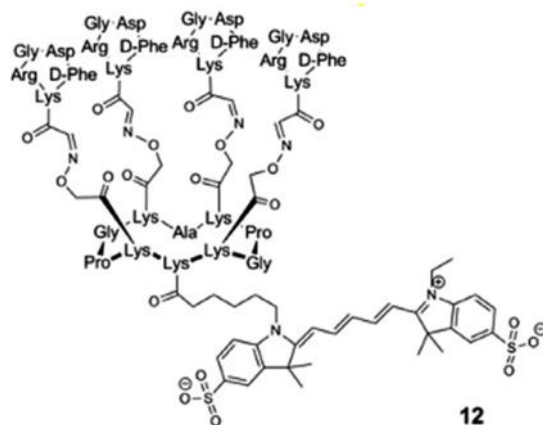


Figure 2-8: ^{64}Cu -RGD injected into tumour bearing mice (Galibert et al., 2010).

In another study where only the radiometal ^{68}Ga was labelled with RGD and various chelators, therefore no other radiometals tested, it was suggested that NOTA can label at room temperature (Blom et al., 2012). In this study this was tested with a small sample only as we followed Jeong's protocol that we knew has been tested and succeeded therefore boiled the labelled product and did not rely on room temperature only. A few years ago already various RGD multimeric types have also been labelled with ^{68}Ga -NOTA and all showed similar tumour-to-background uptake for example ^{68}Ga -NOTA-RGD, ^{68}Ga -NOTA-RGD2 and ^{68}Ga -NOTA-RGD1, (Lee et al., 2006), therefore it does not have an effect whether one chooses to use monomeric, dimeric or tetrameric. Another study investigated this too and concluded with the same findings (Dijkgraaf et al., 2010). The inhouse prepared kit might also be investigated in future for other pathology detection and not only cancer for example myocardial perfusion. Eo et al., (2013) performed a study where myocardial perfusion was investigated and if there is a need for this, myocardial perfusion could be investigated with the in-house prepared cold kit.

2.2 $^{68}\text{Ge}/^{68}\text{Ga}$ generator

The IDB Holland $^{68}\text{Ge}/^{68}\text{Ga}$ generator produced at iThemba LABS Cape Town was used for the purpose of this study.

Four different $^{68}\text{Ge}/^{68}\text{Ga}$ generators are currently available (Ballinger & Solanki, 2011). The iThemba LABS generator will be used for this study. This generator is currently produced under GMP conditions. It is distributed by IDB Holland, Netherlands.

^{68}Ga has radio physical properties making it favourable for PET/CT imaging (discussed in detail in Chapter 3) due to high positron yield. It has a fast blood clearance and is rapidly uptaken in the target areas. It has also showed superior choice of preferred metal for labelling for example when compared with Indium-111 (^{111}In)-labelled peptides in a study where ^{68}Ga -DOTA was compared with ^{111}In to see which metal has the best labelling efficiency in investigating somatostatin receptor tumours (Antunes et al., 2006). Therefore even a few years ago ^{68}Ga was already the radiometal of choice when compared to other radiometals for example ^{111}In . It is also suggested to investigate alternative chelator systems for integrin expression. ^{68}Ga labelled RGD (Arg-Gly-Asp) is able to directly trace in vivo biological processes of angiogenesis and integrin expression as ^{68}Ga -NOTA-RGD binds with high affinity to $\alpha_v\beta_3$ integrin (Lee, 2010). ^{18}F -Galacto-RGD has been compared with ^{18}F -FDG. ^{18}F -FDG was more sensitive for tumor staging, but it was suggested that more studies were needed to evaluate the role of ^{18}F -galacto-RGD in targeted molecular therapies for example with integrin $\alpha_v\beta_3$ -targeted drugs (Beer A et al., 2007). Recently ^{68}Ga -NODAGA-RGD showed promising results when compared with ^{18}F -labelled peptides as a promising alternative as the two tracers compare well with clinical purpose, however ^{18}F -labelled peptides are complex and formulation as well as more time consuming than its ^{68}Ga compared tracer (Knetsch et al., 2011). It is clear in all above comparison tracers that the formulation of an inhouse kit specific for ^{68}Ga labelling was an exciting and promising pharmaceutical. ^{68}Ge breakthrough can be absorbed in SepPak cartridge. ^{68}Ge distribution in rats was tested and showed very fast excretion of the radiotracer. No uptake was evident in any organ (Velikan, 2013) showing no harm to the patient when some breakthrough detected.

2.3 Introduction: Radiopharmaceuticals in general/conservative Molecular Imaging

Siemens Medical solutions acquired the Nuclear Medicine division of Competitive Technologies, Incorporated (CTI) in Chicago in 2005 to form Siemens Medical Solutions Molecular Imaging. Concurrently PET started in Knoxville in Tennessee in the United States of America (USA) during the 1980's. The PET development used Scintillation crystal technology that was further developed with the focus of PET and SPECT camera crystals (Melcher, 2014).

(Nowadays it is possible to visit these crystal technology factories for PET in Knoxville and for SPECT in Chicago USA).

The Nuclear Medicine division as it is known in most hospitals can investigate physiological processes on molecular level for example bone scintigraphy in the human body (or for example animal in preclinical pathology detection for example stress fracture detection in race horses). The latter is possible by also doing bone scintigraphic for example at Onderstepoort. Radiopharmaceuticals in gamma camera work could be easily prepared on site in the hot laboratory. Single photon emission isotopes can be labelled under cGMP(current Good Manufacturing Practice) and cGRPP (current Good Radiopharmacy Practice) conditions for example in laminar flow cabinet, sterile as well as only using cGMP compliant raw materials for example cold kits, generator manufactured under cGMP conditions, specific quality control equipment and quality control performed to only mention a few examples.

Hospitals with Nuclear Medicine divisions mostly have gamma cameras for SPECT (Single photon emission computed tomography) imaging of radiopharmaceuticals. Nowadays there is a limited amount of PET-CT cameras available throughout South Africa. Both types of cameras make use of radiopharmaceuticals. These radiopharmaceuticals however differ in radioisotope needed for imaging as well as different indications for specific scans and therefore pathology detection. However, both types of MI modalities, PET-CT

and SPECT(gamma cameras) as in Figure 2-9 below could investigate patient physiology and therefore radiopharmaceutical biodistribution and pharmacokinetics. The patients have different emissions of radio-isotopes due to different radiotracers that have been injected. According to the Molecular imaging and contrast agent database (MICAD), these modalities, both PET and SPECT stand for 42% and 31 % with regards to contribution made in an environment where there is still a need for more radiopharmaceuticals (Velikan, 2013).

Emission (from Patient) - Detection

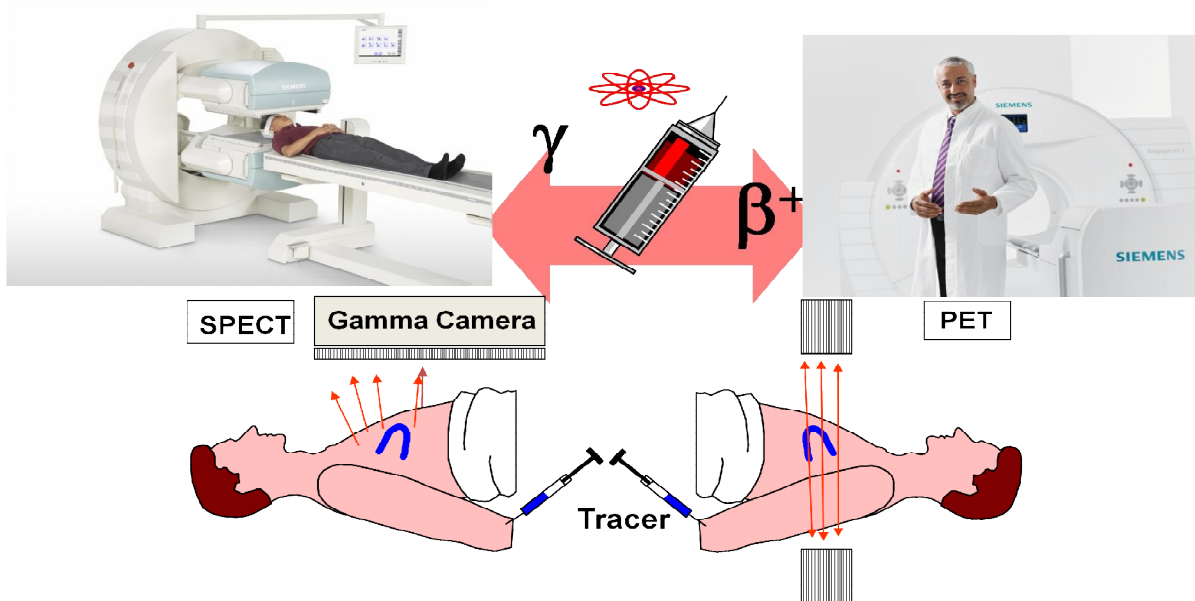


Figure 2-9: Patients with different injected radiotracers will have different emissions and different cameras for example SPECT (left) and PET-CT (right) therefore gamma emission and positron emission tracers (Siemens, 2014).

It is always crucial though to have a very fast target uptake for example specific tumour as well as blood clearance and fast excretion for example mainly through urine. It is therefore normal to see high uptake of urine in the bladder during this time as for example in Figure 2-10 below.

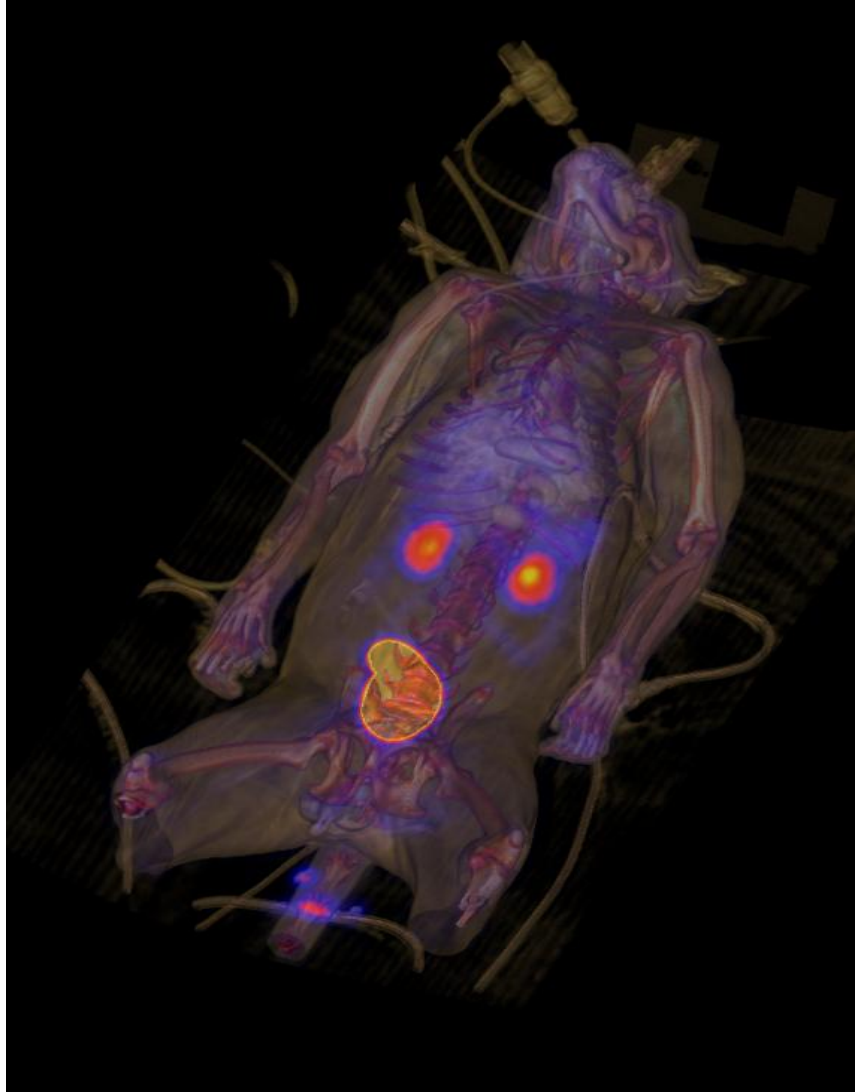


Figure 2-10: Preclinical image of full bladder and kidney uptake.

An example of a radiopharmaceutical is a cold kit labelled with a radio-isotope and this will form a radiopharmaceutical. Phosphonate for example in a cold kit such as Methylene diphosphonate (MDP) can be injected into a patient for bone imaging. An example is below in Figure 2-11 that shows patient biodistribution at least two hours after the bone injection. Only then could bone uptake throughout the skeleton can be seen. The National Comprehensive Cancer Network (NCCN) proposed PET-CT scans since 2012 for patient management instead of their previous approach of bone scintigraphy only (Xanthopoulos, 2013). This also

shows that there is a constant clinical need for more developments in radiopharmaceuticals. Bone scintigraphy is not enough anymore to answer the clinical need.

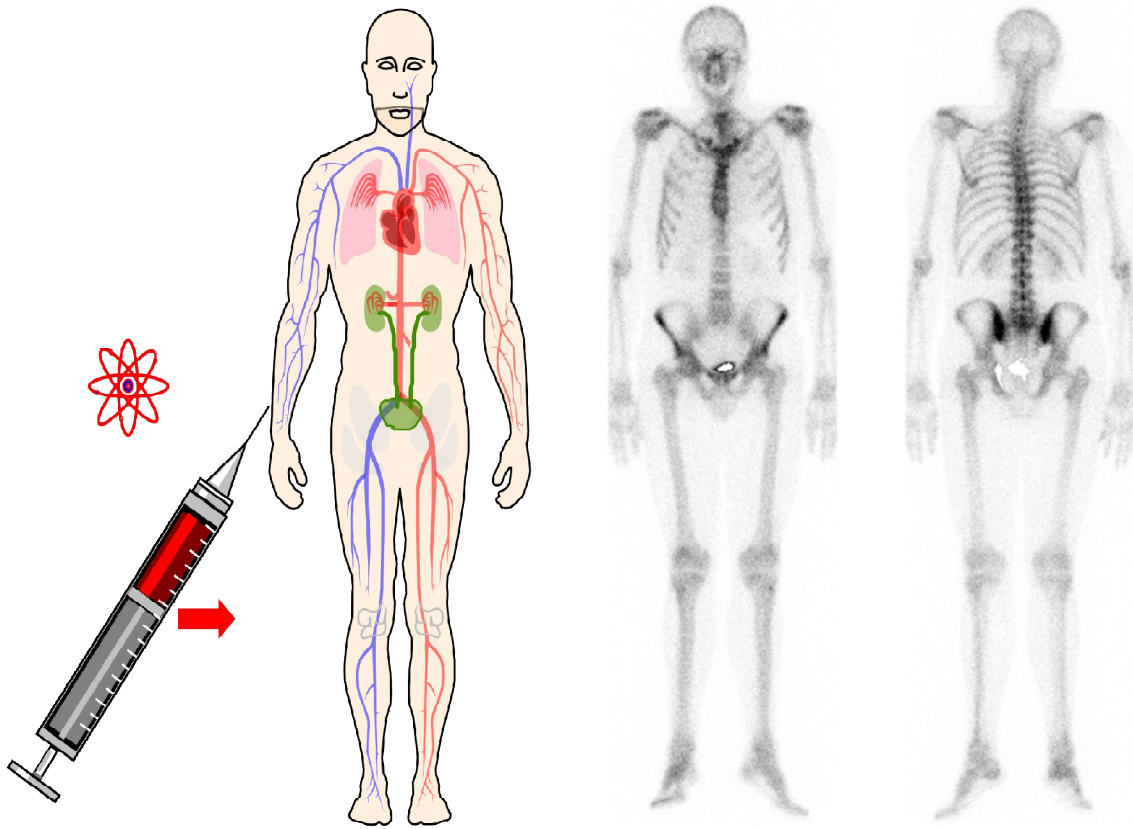


Figure 2-11: Siemens training slides of bone scintigraphic.

After labelling of ^{68}Ga -NOTA-RGD, normal radiotracer uptake and biodistribution in the whole body could be seen in Figure 2-12. Figure 2-13 shows abnormal ^{68}Ga -NOTA-RGD distribution. Abnormal uptake is specifically seen in areas of integrin over expression for example the lung lesion in the patient's chest which is clear by viewing the abnormal radiotracer uptake of the abnormal dark spots in the patient's chest area, (Jeong, 2011).

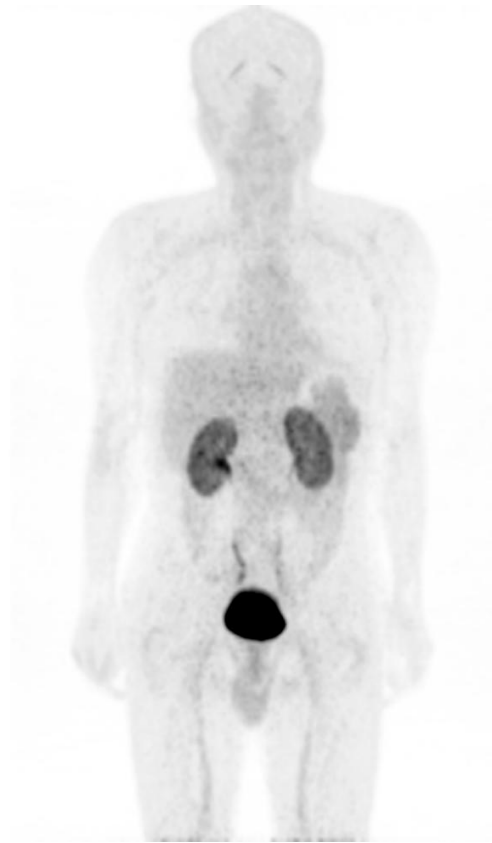


Figure 2-12: Normal distribution in ^{68}Ga -NOTA-RGD (Jeong, 2011).

In MI imaging reporting, it is essential to understand the normal distribution in clinical to better understand and report on abnormalities, because all radioactive uptake in organs are not abnormal. ^{68}Ga -NOTA-RGD is excreted through kidneys and then urine therefore bladder area. This explains a bit more increased uptake that is seen in both kidneys.



Figure 2-13: Lung lesion ^{68}Ga -NOTA-RGD (Jeong, 2011).

Nuclear Medicine departments all have hot laboratories where radiopharmaceuticals could be prepared or kept if ordered from one major Radiopharmacy. It is possible to prepare the radiopharmaceuticals at sites for gamma camera work. The process is more complex and costly on the PET-CT side and therefore positron emission radiopharmaceuticals are generally ordered from a radiopharmacy. The cost effectiveness of PET-CT has also been investigated. It was explained that despite infrastructure costs, PET-CT could actually be cost effective in saving on additional unnecessary scans when one reviews additional scans and operations that PET could prevent. Whole body dosimetry has been performed by Kim et al., (2013) in using ^{68}Ga -NOTA-RGD in 8 patients. Patients were scanned within 90 minutes post intravenous injection of

^{68}Ga -NOTA-RGD on a PET-CT scanner. Ninety minutes is also within the suggested clinical proposed time after biodistribution of three monkeys was investigated.

In the clinical study above, the findings were for acceptable effective radiation dose which is well in relation to this study's findings also regarding more activity shown in kidneys and bladder due to fast blood clearance and excretion through kidneys and bladder in all three monkeys.

^{68}Ga labelled RGD (Arg-Gly-Asp) is able to directly trace in vivo biological processes of angiogenesis and integrin expression as ^{68}Ga -NOTA-RGD binds with high affinity to $\alpha_v\beta_3$ integrin. ^{18}F -Galacto-RGD has been compared with ^{18}F -FDG. ^{18}F -FDG was more sensitive for tumor staging, but it was suggested that more studies were needed to evaluate the role of ^{18}F -galacto-RGD in targeted molecular therapies (Beer et al, 2007). ^{18}F was also labelled with RGD for Breast cancer imaging (Kenny et al., 2008). Recently ^{68}Ga -NODAGA-RGD showed promising results when compared with ^{18}F -labelled peptides as a promising alternative (Knetsch et al., 2011). The need was for a lung SPN lesion targeting tracer however it is good to know for future purposes that this tracer might be of value in more cancers for example Figure 2-14 below shows the other types of cancers that integrin $\alpha_v\beta_3$ could also be over expressing on for example prostate and pancreatic cancer.

Tumour type	Integrins expressed	Associated phenotypes
Melanoma	$\alpha v\beta 3$ and $\alpha 5\beta 1$	Vertical growth phase ^{35,172-174} and lymph node metastasis ^{173,175}
Breast	$\alpha 6\beta 4$ and $\alpha v\beta 3$	Increased tumour size and grade ¹⁷⁶ , and decreased survival ¹⁷⁷ ($\alpha 6\beta 4$). Increased bone metastasis ^{36-38,64} ($\alpha v\beta 3$)
Prostate	$\alpha v\beta 3$	Increased bone metastasis ⁷⁵
Pancreatic	$\alpha v\beta 3$	Lymph node metastasis ⁴⁰
Ovarian	$\alpha 4\beta 1$ and $\alpha v\beta 3$	Increased peritoneal metastasis ¹⁷⁸ ($\alpha 4\beta 1$) and tumour proliferation ¹⁷⁹ ($\alpha v\beta 3$)
Cervical	$\alpha v\beta 3$ and $\alpha v\beta 6$	Decreased patient survival ^{41,180}
Glioblastoma	$\alpha v\beta 3$ and $\alpha v\beta 5$	Both are expressed at the tumour-normal tissue margin and have a possible role in invasion ¹⁸¹
Non-small-cell lung carcinoma	$\alpha 5\beta 1$	Decreased survival in patients with lymph node-negative tumours ¹⁸²
Colon	$\alpha v\beta 6$	Reduced patient survival ¹⁰⁹

Figure 2-14: Different integrins and expression of $\alpha v\beta 3$ in prostate and pancreatic tumours, (Desgrosellier and Cheresh, 2010).

2.3.1 PET going back to basics of 'SPECT-type' 'Generator' produced isotope labelled with 'cold kit'

'Cold kits' are mostly available in a small, sterile and GMP compliant vial for the preparation of a radiopharmaceutical. All different studies needs different 'cold kits' even if labelled with the most general radio-isotope technetium 99m produced by the Molybdenum/Technetium generator or PET generators as in Table 2-1 (Saha., 2010). These are the most general generators available in MI. The Molybdenum-99 (⁹⁹Mo) generator produces ^{99m}Technetium. This is could for labelling with cold kits to image patients (or unlabelled for example in thyroid imaging). All 'cold kits' each have a very specific SOP for preparation. This does not only ensure the right GMP procedures but also correct radiation dose administered to patients for example administration amounts have to be according to for example patient weight.

Table 2-1: Generators in medical use

TABLE 5.1. Several generator systems useful in nuclear medicine

Parent	Parent $t_{1/2}$	Nuclear reaction	Daughter	Daughter $t_{1/2}$	Mode of daughter decay	Principal photon energy (keV) (% abundance)	Column	Eluant
^{99}Mo	66 h	Fission, $^{98}\text{Mo}(n, \gamma)$	$^{99\text{m}}\text{Tc}$	6 h	IT	140 (90)	Al_2O_3	0.9% NaCl
^{68}Ge	271 days	$^{69}\text{Ga}(p, 2n)$	^{68}Ga	68 min	β^+	511 (178)	Al_2O_3	0.005 M EDTA
^{62}Zn	9.3 h	$^{63}\text{Cu}(p, 2n)$	^{62}Cu	9.7 min	β^+	511 (194)	SnO_2 Dowex 1X8	1 N HCl 2 N HCl
^{81}Rb	4.6 h	$^{79}\text{Br}(\alpha, 2n)$	$^{81\text{m}}\text{Kr}$	13 s	IT	190 (67)	BioRad AG 50	Water or air
^{82}Sr	25.5 days	$^{85}\text{Rb}(p, 4n)$	^{82}Rb	75 s	β^+	511 (190)	SnO_2	0.9% NaCl
^{90}Sr	28.6 years	Fission	^{90}Y	64.1 h	β^-	-	Dowex 50	0.03 M EDTA

Data from Browne E, Firestone RB. Table of Radioactive Isotopes. 1st ed. New York: Wiley; 1986
 IT isomeric transition

^{99}Mo Generator for SPECT and ^{68}Ge for PET (Saha, 2010).

In PET.CT due to complexity, availability and cost of positron emission Cyclotron produced isotopes that are then synthesized to form radiopharmaceuticals emitting positron emission radiation for PET detection on the PET.CT scanner is limited.

An easier as well as cheaper way of producing positron emitting radiopharmaceuticals for specific cancer detection/evaluation/monitoring is by using the basic principle of gamma emitting radiopharmaceutical production e.g. Radioactive source e.g. Generator producing the needed radio isotope as well as labelling the isotope to a GMP compliant cold kit under GMP conditions. In this study the $^{68}\text{Ge}/^{68}\text{Ga}$ was used from IDB Holland and produced in the Western Cape. It has a very small footprint.

Of course there has been studies done in PET.CT where Cyclotron produced isotopes were labelled with 'kit like' cold kits (Lui et al., 2013). This process however is different from suggested simplified 'cold kit' labelling when a generator for radio isotope production could be available onsite. The onsite generator would also result in cheaper radiopharmaceuticals for this purpose resulting and larger patient numbers possible to investigate in a certain hospital budget.

2.4 Other imaging modalities: MRI

Molecular Imaging is the only modality with the highest sensitivity for detecting physiological changes in tumours and specific data regarding radiopharmaceutical uptake when compared with Ultrasound, Computed Tomography and Magnetic Resonance Imaging (Lee Y, 2010). However another scanning modalitie that did investigate $\alpha_v\beta_3$ tumour uptake is MRI, however MRI still is able to detect only from nanomolecular levels when compared with Molecular Imaging that can detect lesions from pico to nano molecular levels. MRI have been used to characterize $\alpha_v\beta_3$ in lung cancers (Lee et al., 2006)

Eight years ago however then probe presentation differed in different tumours and it was not concluded of method of choice .Currently molecular imaging is the only imaging modality that is able to image direct molecular processes even in sub-millimolar levels (Lee, 2010). MRI has been used in labelling magnetic-fluorescent-bioluminescent-radioisotopic particle (MFBR particle) as in Figure 2-15 (Lee, 2010) therefore providing MRI as well as scintigraphic (Molecular Imaging) information was obtained then a few years later. Jiang et al., (2009) also demonstrated even three years later that MRI has indeed a place in clinical when a special USPIO probe was designed for 1.5 Tesla MRI (not yet for 3 Tesla).The study's purpose was to explore MRI in two tumour models with A549 lung tumours.

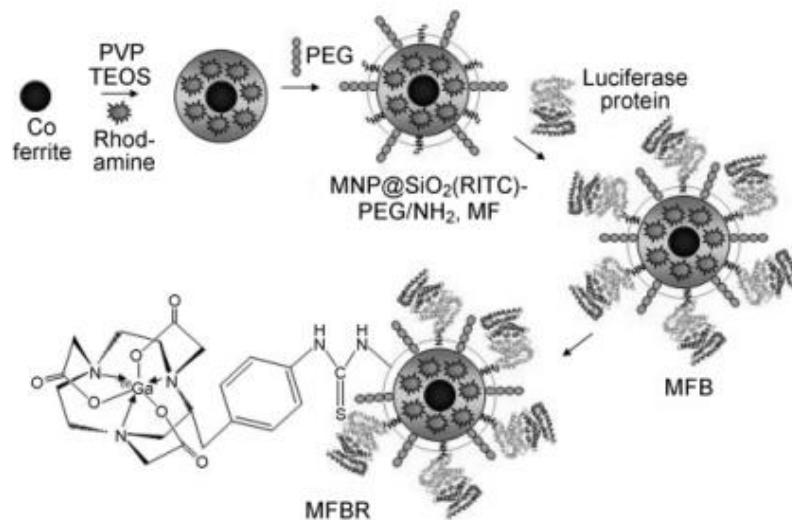


Figure 2-15: MFBR particle (Lee, 2010).

This means that multimodalities have been used in labelling the MRI particles as it contains dyes that can provide MRI as well as molecular imaging scintigraphic as well as optical signals (Lee, 2010).This could maybe rather be considered for future purposes and the focus of this reference was more on molecular imaging specific. The processes and methology is further discussed below. For future purposes RGD cyclic peptides may be used for inhibition of integrin $\alpha_v\beta_3$ receptor

and may play a major role in cancer suppression (Dechantsreiter et al., 1999). MRI have been used to characterize $\alpha_v\beta_3$ in lung cancers (Jiang et al., 2009) however probe presentation differed in different tumours and currently molecular imaging is the only imaging modality that is able to image direct molecular processes even in sub millimolar levels (Lee, 2010) and play a therapeutic role

Chapter 3: Methodology

“I have not failed 1 000 times. I have successfully discovered 1000 ways NOT to make a light bulb”...Thomas Edison

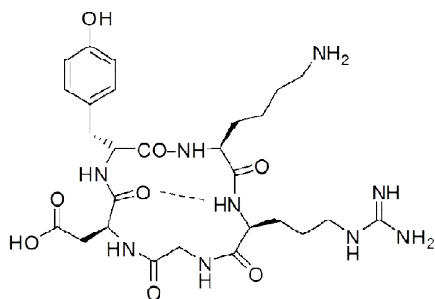
3.1 Labelling methods and procedures including with reference to SOP's (Standard operating procedures):

Radiopharmaceutical procedures including radiolabeling and synthesis, purification, quality control and radiopharmaceutical biodistribution procedures have been investigated and documented. Several experiments have been performed on imported RGD kits (Jeong, 2009) as well as in-house prepared RGD kits. The imported, kindly donated, first batch of kits showed inconsistent radiolabeling results and were not reproducible. Another batch was purchased and again failed to show consistent results. Steve Biko Hospital however still had a clinical need that was not yet met. Formulation therefore of an in-house prepared kit was conducted, especially because the new cold kit had to label with 0.6 M $^{68}\text{Ge}/^{68}\text{Ga}$ generators' eluted ^{68}Ga .

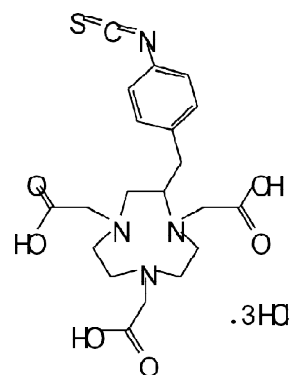
3.1.1 Radiolabeling Imported Kits: Donated kits with inconsistent results:

Seoul National University (SNU) proposed the following labelling procedure in Figure 3-1 below:

Synthesis



c(RGDyK)



**(p-SCN-Bz)-NOTA
trihydrochloride**

600 nmol cRGDyK

in 0.1 M sodium carbonate buffer (pH 9.5).

600 nmol NOTA-SCN

20 hr at room temp

HPLC purification

Figure 3-1: Jeong, Seoul National University (SNU) shared with Prof. Sathekge for labelling support.

NOTA-SCN-RGD kit (NOTA-SCN-RGD 10.70 μg and 10 nmol): Ten microgram of RGD was included in the SNU cold kit. Therefore in formulating the in-house prepared cold kit, we started tests on formulation of a cold kit with ten microgram RGD. Tests were performed on labelling efficiency with ten microgram RGD only and later adjusted by increasing the amount of RGD when inconsistent results were obtained. SNU eluted their $^{68}\text{Ge}/^{68}\text{Ga}$ generator with 0.1 M HCL. The eluted $^{68}\text{GaCl}_3$ had a pH of 4.8-5.0. The same pH was therefore aimed at obtaining in in-house kit formulation.

SNU also heated the radiolabelled RGD for ten minutes at 10 $^{\circ}\text{C}$. They shared their QC results with us. Instant thin layer chromatography (ITLC) was supposed to show results as below in Figure 3-2 according to Jeong (2009):

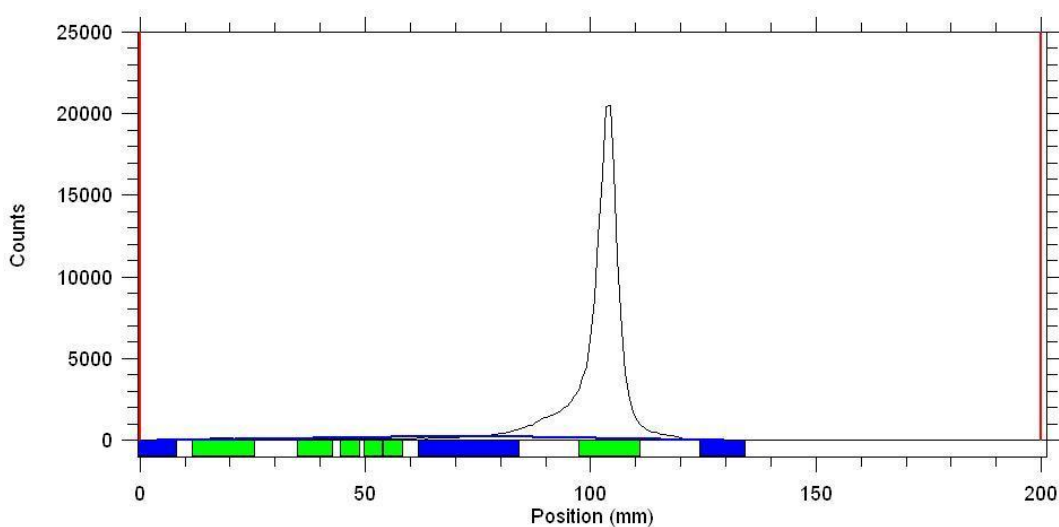
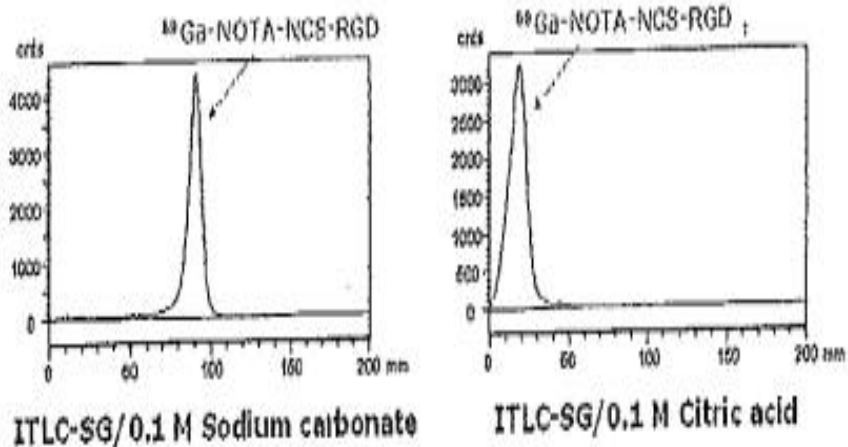


Figure 3-2: Quality control done by Seoul National University (Joeng, 2011).

It was suggested to place a drop of the ^{68}Ga -NOTA-SCN-RGD on each plate of chromatography paper. Sodium carbonate as well as 0.1 M Citric acid was used as flow medium solutions. Results are described in Chapter 4.1; the R_f value was supposed to be 1 in sodium carbonate and 0 in citric acid. HPLC was also performed and results described later. The sodium carbonate ITLC was an indication of colloid formation. The citric acid was a direct indication of labelling efficiency. Mostly in various cold kits labelled, citric acid was available and used for ITLC in the in-house prepared study performed.

At the beginning of this study when the donated cold kits were labelled, the focus was a lot on perfecting the pH of the 0.6 M $^{68}\text{Ge}/^{68}\text{Ga}$ eluted $^{68}\text{GaCl}_3$ that will be referred to ^{68}Ga for the purpose of this study).

Shukla and Mittal (2013) described the pH influence on labelling efficiency. In this study however, pH at a certain range for example 4-5 (using pH strips of up to 6 ranges) did not affect labelling efficiency and for example still did not label well. It was noted later when studies were done at iThemba labs, that adjusting the actual pH of the eluted ^{68}Ga did not have an effect on labelling. When pH was not adjusted, there was a small labelling yield but not enough labelling efficiency for preclinical or clinical studies. (It was therefore then decided on an in-house kit formulation).

^{68}Ga -NOTA-SCN-RGD was prepared in the following way after receiving the cold kits from SNU:

The $^{68}\text{Ge}/^{68}\text{Ga}$ 0.6 M generator was eluted in fractions of 1 ml each into six sterile vials. The Activity (A) in each vial was measured. Usually the highest activity was in vial number 2.

A pipette was used and 1 ml eluate was pipetted from the second vial which always had the highest activity. This was added to a clean sterile vial containing sodium hydroxide and the solution was gently mixed. The sodium hydroxide volume depended on which of the two methods further described shortly were used for example ^{68}Ga labelling or ^{68}Ga labelling dilution method therefore either 45 μl sodium hydroxide (10 M) and 45 μl sodium hydroxide (1 M) or only 450 μl of sodium hydroxide. A 0-6 pH paper strip was used to test pH.

A metal needle for venting was used. A Jelco needle however was used for labelling in order to prevent interference of the metal needle. One ml eluate was added to the RGD cold kit vial. The needles were then removed and the vial transferred to the warmbath for boiling. Boiling was always at least 5 minutes but up to 10 minutes at 90 degrees Celsius.

Quality control was performed as suggested by Jeong, during correspondence, with ITLC strips in sodium carbonate as well as citric acid flow mediums. Firstly a drop of the radiolabelled ^{68}Ga -NOTA-RGD was placed with either a 1 ml syringe or pipette onto each ITLC paper strip approximately 8 mm from the origin of the paper strip. Four

plastic small pouches were prepared. One ITLC strip was gently dried a bit with a hair dryer. It was then put into the flow mediums until it reached the front of the paper strip. It was then gently dried with a hair-dryer and cutted into four quarters. Each quarter of ITLC paper was put inside a plastic pouch and put into the Geiger-Muller counter for counting radioactivity. The percentage radioactivity of each of all quarters was calculated manually, because an ITLC scanner was not yet available for use at Steve Biko Hospital.

⁶⁸Ga Labelling RGD dilution method

The previous labelling method did not give positive labelling results even after great efforts therefore labelling possibilities and new SOP's were further investigated. Esteban Ricardo Obenaus, head of Nuclear Medicine (NM) Argentina Diagnostic Centre Buenos Aires suggested that a dilution method would ensure also the perfect pH in the eluate prior to further labelling the eluate with the RGD cold kit. The dilution of the eluate by adding 450 µl of Sodium Hydroxide (NaOH) was suppose to change the eluate of the 0.6 M Sodium Chloride (HCl) generator also to 0.1M.

⁶⁸Ge/⁶⁸Ga generator was eluted again the next day. From this day the generator was not anymore eluted in quantities for example ml only, but in drops into different sterile vials. Zero point two ml activity (A) was diluted with 1 ml sterile water. NaOH was added to this then only one ml of this buffered eluate added to the RGD cold kit. Again only Jelco needles were used (the plastic of the needle and no metal art of the needle).

This was mixed gently and heated. Quality control was performed using ITLC.

⁶⁸Ga Labelling Method:

Subsequently the labelling SOP was changed regarding the dilution and buffering method to adding 45 µl sodium hydroxide (10 M) and 45 µl sodium hydroxide (1 M) to the eluate to adjust the pH. Again a plastic needle only was used to add radioactivity into the RGD cold kit.

Eventually after testing two batches of RGD an inhouse kit was prepared. SOP's for radiolabeling as well as purification further is discussed after the radioactive isotopes and therefore after ⁶⁸Ge/⁶⁸Ga has been discussed. Herewith follows the discussion on the ⁶⁸Ge/⁶⁸Ga generator.

3.1.2 $^{68}\text{Ge}/^{68}\text{Ga}$ Generator in detail

$^{68}\text{Ge}/^{68}\text{Ga}$ Generator (Figure 3-3) was the generator of choice used in this study. This generator is produced at iThemba LABS in the Western Cape, South Africa under cGMP compliant conditions. The generator has a glass column that is made from tin-dioxide with polyethylene tubing.



Figure 3-3: IDB Holland generator produced under cGMP conditions at iThemba LABS in the Western Cape (Eckert & Ziegler, 2008).

The $^{68}\text{Ge}/^{68}\text{Ga}$ generator consists of a mother isotope ^{68}Ge which decays to its daughter, ^{68}Ga . ^{68}Ge has a half-life of 271 days, which make it perfect to keep in a hospital and nuclear medicine department for about one year. It is small and compact and easily protected behind lead in compact space (Figure 3-4) and inside a laminar flow cabinet Figure 3-5 and easy to transport. However, the daughter isotope, ^{68}Ga that is used for medical purposes, for example imaging, has a half-life of 68 min. This is favourable with its positron emission for PET-CT scanning, however less practical to transport due to short half-life. It is actually ^{68}Ga (III) that is eluted from the generator.



Figure 3-4: iThemba LABS small and compact cGMP compliant generator behind lead.

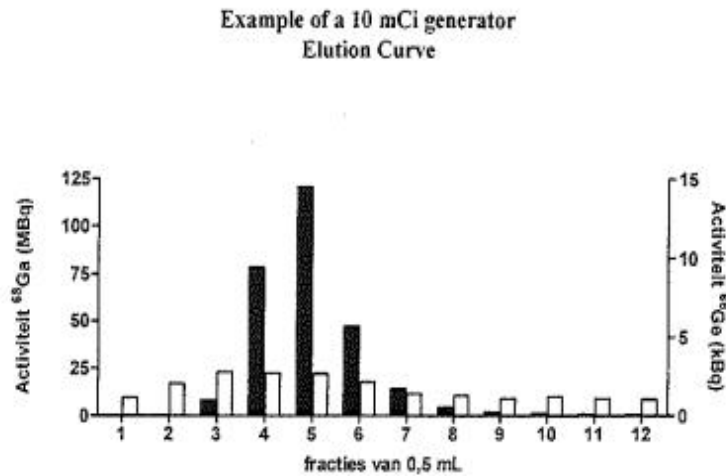
The ^{68}Ga has a specific elution profile (

Figure 3-6). This generator has no parts that are made from metal (except the outer shielding). Metal can influence the labelling efficiency and therefore also no metal is used in the radio labelling process. Needles for example are replaced with Jelco needles where only the plastic part of the syringe was used to transfer the solution from one glass vial to another. Sometimes when it was available, pipettes were utilised. This was not always available at the Hospital site however plastic syringes were in order to use as it did not interfere with labelling. It was important in this study to understand more about the radio isotopes used for the correct handling of this for practicing the As Low As Reasonably Achievable (ALARA) principle. ^{68}Ga is a positron emitter and should be handled with the same care as any other radio-isotope. In the clinical environment radiographers are more use to handling gamma emitters. However the same principles adhere. A distance should be kept from a radio-isotope source as well as handling should be as quick as possible.



Figure 3-5: Laminar flow cabinet compulsory for cGMP conditions in which $^{68}\text{Ge}/^{68}\text{Ga}$ generator is hosted.

The $^{68}\text{Ge}/^{68}\text{Ga}$ generator is suggested according to user instructions to be eluted in fractions of 0.5 ml until a labelling procedure has been established for example to know which volume of which ml fraction had to be used. Suprapure 0.6. M HCL (10 ml) was used as solution to eluate the generator. Therefore if 1.5. ml was used for radiolabeling the in-house prepared cold kit, then 8.5 ml eluted radio isotope was disregarded. It was established in this study that the second 1.5 ml had the highest activity and was added to the in-house prepared cold NOTA-RGD vial.



Elution profile with 0.6 M HCl

Figure 3-6: 0.6 M generator elution profile (IDB Holland user instructions).

It is not just important to understand fractionated elution but also the decay of the mother isotope ^{68}Ge as well as daughter isotope ^{68}Ga . The Gallium decay chart was extracted from another generator of $^{68}\text{Germanium}/^{68}\text{Gallium}$'s instructions for use below shown in Table 3-1 (Eckert & Ziegler, 2008). In all calculations for labelling efficiency the Gallium decay factors were used to calculate decay corrected values. ^{68}Ga has 89% decay by positron emission (Boschi et al., 2013) making it a favourable PET radiotracer. The table below is for all time durations from one minute to 68 minutes. Radiolabeling with this isotope will be further discussed in the following section 3.1.3. A comparison of the Eckert & Ziegler pre-purification of ^{68}Ga before radiolabeling as later in Chapter 4 compared with this study's generator, the IDB Holland/iThemba LABS generator.

TABLE 3-1: Gallium-68 decay chart directly extracted from Eckert & Ziegler’s generator User manual (Eckert & Ziegler, 2008).

Table 3-1: Half-life in minutes: Time represents 1-68 min

GALLIUM-68 DECAY CHART

Half-life 68.0 minutes

ElapsedTime in Minutes	Decay Factor	ElapsedTime in Minutes	Decay Factor
1	0.990	35	0.700
2	0.980	36	0.693
3	0.970	37	0.686
4	0.960	38	0.679
5	0.950	39	0.672
6	0.941	40	0.665
7	0.931	41	0.658
8	0.922	42	0.652
9	0.912	43	0.645
10	0.903	44	0.639
11	0.894	45	0.632
12	0.885	46	0.626
13	0.876	47	0.619
14	0.867	48	0.613
15	0.868	49	0.607
16	0.850	50	0.601
17	0.841	51	0.595
18	0.832	52	0.589
19	0.824	53	0.583
20	0.816	54	0.577
21	0.807	55	0.571
22	0.799	56	0.565
23	0.791	57	0.559
24	0.783	58	0.554
25	0.775	59	0.548
26	0.767	60	0.543
27	0.759	61	0.537
28	0.752	62	0.532
29	0.744	63	0.526
30	0.737	64	0.521
31	0.729	65	0.516
32	0.722	66	0.510
33	0.714	67	0.505
34	0.707	68	0.500

3.1.3 Radiolabeling In-house prepared kit

⁶⁸Ga-Radiolabeling of NOTA-SCN-Bz-RGD and NOTA-RGD

NOTA-SCN-Bz-RGD(1,4,7-triazacyclononane-1,4,7-triacetic-acid -isothiocyanatobenzyl-cyclic Arg-Gly-Asp-D-Tyr-Lys) cold kits were entrusted as a gift from Seoul National University College of Medicine, and labelled as described by Jeong et.al., (2008) as well as suggested by Jeong. Jeong et al., (2008) also did a study with obtaining images below (Figure 3-7) on PET-CT scanner after the mice was injected with ⁶⁸Ga-NOTA-RGD with and without cold RGDyk. Radiopharmaceutical uptake was as below and tumours marked with red arrows.

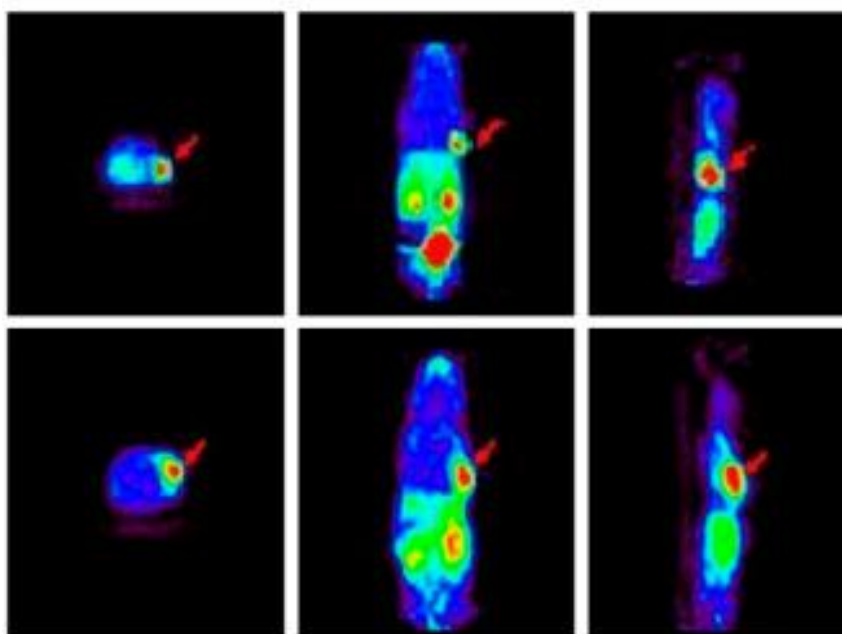


Figure 3-7: Tumour bearing mice injected with ⁶⁸Ga-NOTA-RGD including also injecting cold RGDyk (Jeong et al., 2008).

It is therefore clear that RGD does get up taken in tumour cells. Jeong also provided formulation methods as well as support to imported kit labelling. He also communicated their quality control findings via email in order for comparison with the findings in this study. NOTA-RGD (1,4,7-triazacyclononane-1,4,7-triacetic-acid-cyclic Arg-Gly-Asp-D-Tyr-Lys) was purchased from Futurechem Company (Seoul, Korea) and labelled manually for optimization using a radiolabeling procedure which amended previously

published knowledge by Rossouw and Breeman (2011) as well as Jeong's formulation and Dr Rossouw's formulation for this cold kit. In order to allow non-metallic transfer of the $^{68}\text{Ge}/^{68}\text{Ga}$ generator eluate into the reaction vial, the top of its septum was punctured by a Jelco 22G x 1" polymer catheter (Smiths Medical, Croydon, South Africa), sodium acetate trihydrate buffer salt was added to the ^{68}Ga eluate to warrant pH to 4-5 and 1.5 ml buffered ^{68}Ga -eluate was used for labelling. Following the addition of 5.6 - 33.8 μM NOTA-RGD, stirring action was performed for 20-30 seconds. The reaction vial was allowed to incubate in a temperature controlled water bath, routinely at 90 °C for 10 min .It was then allowed to cool for at least 2 min. An aliquot was retained for quality control purposes. This whole process is discussed in further detail below. More detailed methods is discussed below and SOPs are available in Annexure A (cold kit formulation) and Annexure B (radiolabeling and purification).

3.1.3.1 Certificate of analysis information

The Certificate of analysis (COA) was first obtained and explored in order to obtain more info on product purity according to HPLC performed. The RGD was purchased from **Futurechem** Company, Seoul, Korea. Important information below was obtained on this starting material that was included in the in-house prepared cold kit.

Abbreviative name: NOTA-cyclic RGDyK

Product name: Cyclic Arg-Gly-Asp-D-Tyr-Lys-NOTA

Molecular Formula: $\text{C}_{47}\text{H}_{67}\text{N}_{13}\text{O}_{14}\text{S}\cdot\text{CF}_3\text{COOH}$ (Figure 3-8 below)

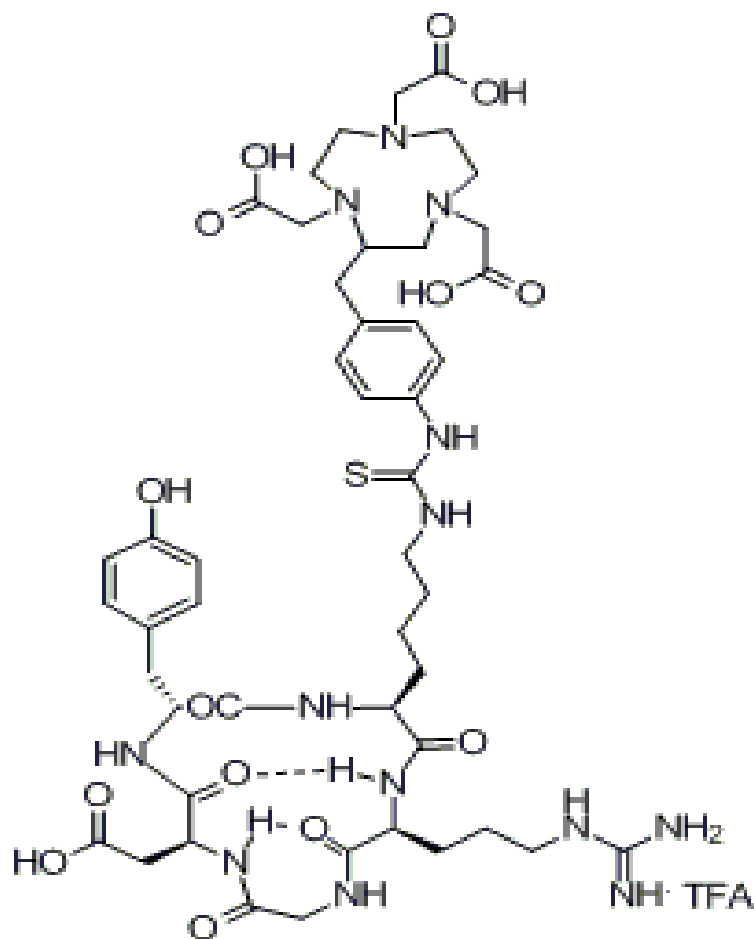


Figure 3-8: Molecular Formula: C₄₇H₆₇N₁₃O₁₄S.CF₃COOH (Futurechem).

Formula weight: 1184.18

Description: White solid

Purity(HPLC): >95%(at 240 nm)

Classification: Substance not fully tested.

It was important to know that all tests were not yet performed on this substance. This included the kits that were donated originally. They were also from Korea and were marked: 'for experimental use only'. This was therefore not signed off for South Africa as in-house prepared cold kit. This also has not yet passed cGMP compliant production conditions. Most of the physical and chemical properties were not available according to page four of the CoA. None of pH, density, bulk density, explosion limits, flammability and

autoignition temperature was available. The solubility was available and stated as soluble in water.

In this study the solubility was tested. The substance quickly dissolved in water however was not stable for more than about four weeks when frozen. The advice received from Futurechem that aliquots should be prepared for kit vial preparation and immediately freeze dried. This was therefore later done in this study. In the beginning of formulating an in-house cold kit, the aim was rather to test if under the same conditions, the cold kit would show reproducible radiolabeling with the eluted ^{68}Ga from the 0.6 M $^{68}\text{Ge}/^{68}\text{Ga}$ generator.

3.1.3.2 Peptide mass determination and shelf life

^{68}Ga -NOTA-RGD buffered with sodium acetate trihydrate and containing 60 μg has been tested up to 205 days post kit formulation date for shelf life integrity. Various peptide mass were investigated especially after one RGD vial deteriorated in terms of appearance for example most of the solid white powder disappeared and was sticky onto the glass vials inside walls. It was decided to try and get all left over powder, even though sticky, off the inside glass wall. This was used for cold kit formulation and tested for further information regarding peptide mass and deterioration influence on labelling. It was then suggested to rather use 60 μg instead of 30 μg of the RGD peptide.

Prior to this, at iThemba LABS in Cape Town, it was also agreed that different peptide masses should be investigated as well as different buffer concentrations

Test A: Influence of pH at a smaller volume: (Experiment 1: pH 3.6)

Ninety eight mg of sodium acetate trihydrate was weighed in a glass vial. The generator was milked/eluted with 0.6 M HCl as follows: Vial 1 with 1 ml, vial 2 with 2 ml, vial 3 with 6.5 ml HCl. One ml of vial 2 was added to 98 mg sodium acetate trihydrate and then 10 μl of the stock solution of NOTA-cyclic-RGDyk to obtain a 3.6 pH. The reaction volume was 1010 μl and concentration 8.4 μM . The vial was sealed and heated for ten minutes at 95 degrees Celsius. Then the vial was cooled for five minutes, uncapped and quality control performed.

A few more tests were performed up to obtaining a Ph of 4.8-5 with 147 mg sodium acetate trihydrate buffer. This was the final results for optimal labelling efficiency.

3.1.3.3 In-house prepared Cold kit raw materials, chemical structure and kit formulation.

Radiolabeling and in-house kit was prepared by using NOTA peptide from Futurechem (Futurechem RGD structure). NOTA as chelating agent has been proven to form very stable complexes with ^{68}Ga (Brechbiel, 2008). A different buffer was added: Sodium Acetate trihydrate.

The radiotracer was obtained by eluting a $^{68}\text{Ge}/^{68}\text{Ga}$ Generator (IDB Holland/iThemba LABS) elution with ten ml 0, 6 M HCl. ^{68}Ga -NOTA-RGD was purified by SepPak purification process.

Materials and instruments were used according to SOP in Annexure A for kit formulation and Annexure B for radiolabeling and purification as documented shortly below was performed according to SOP steps below in Figure 3-9.

Equipment and materials: Instruments: All equipment was obtained and operated as described in the SOP addendum A. The following equipment was therefore obtained: Sterile vials (10 ml rubber seal) were prepared, crimper for cap sealing, balance for weighing buffer, a small spatula for buffer weighing, filtration apparatus for working on during buffer weighing, a vortex (it was tested to be working when the power was switched on), various pipettes for example 100-1000 μl and 10-100 μl , a labelling device to print labels, a forceps to put rubber seals back onto the sterile vials, a freezer and a freeze drier.

Chemicals and raw materials: The raw materials and chemicals that were obtained were NOTA-RGD powder (from Futurechem) or aliquots of this peptide if this was already previously prepared, suprapure water, sodium acetate trihydrate buffer and a 10-100 μl pipette and tips.

Step one was that sterile vials from NTP were set ready for adding buffer salts. Step two was that the buffer (sodium acetate trihydrate) was weighed and transferred into all sterile vials via small spatula. Step three was that the NOTA-RGD was prepared by removing it from the fridge where according to the package insert of Futurechem it was supposed to be kept 1 $^{\circ}\text{C}$ as maximum therefore it was kept at a cooler temperature of minus 2-8 $^{\circ}\text{C}$.

For the preparation of NOTA-RGD Kits (SOP Annexure A) 10 sterile vials on filtration paper in laminar flow cabinet was prepared (unsealed rubber). A small spatula was used and buffer weighed on the sensitive Balance for each vial (147 mg sodium acetate trihydrate).

Buffer (Sodium Acetate Trihydrate). NB each time use forceps to put rubber seal cap back on each vial. In order to prevent moist inside the vials and buffer, each time after the buffer was weighed, a small forceps was used to put the rubber cap immediately back onto a vial.

RGD-NOTA raw material was prepared by using 100 µl/ 100µg suprapure water to dissolve the peptide in water. Therefore the 100-1 000µl pipette was used to transfer 1 000µl suprapure water to 1000 µg NOTA-RGD. It was then immediately vortexed. Visually it was important to ensure that the solution was clear as water and no more white powder visible. NOTA-RGD was transferred to each of the buffer containing sterile vials. The 10-100µl pipette was used to transfer 60 µl (therefore 60 µg NOTA-RGD) to each of the 10 prepared vials and then it was sealed only with rubber caps. It was noted that a small volume of liquid was mixed with a larger volume of salt (buffer). It was still vortexed leading to formation of a thicker liquid, which was vortex to be visible at the bottom of the vial. The vials were stored in the freezer for about two hours in order for the liquid to freeze before the vials were put into a freeze drier. The vials were freeze dried overnight. All vials were inspected and all vials contained a white powder of freeze dried NOTA-RGD. The vials were then sealed properly with rubber and metal caps. The vials were then stored at about minus 20 °C. Transportation was only carried out when the vials were packed with an ice pack.

In-house prepared kit radiolabeling and purification NOTA-RGD (Addendum B): Sterile cGMP kit vials were produced at Radiochemistry; The South African Nuclear Energy Corporation (Necsa, Pelindaba) using 5 ml certified sterile pyrogen-free sealed borosilicate glass vials provided by NTP Radioisotopes. After peptide dissolution in Millipore water aliquots of 60 µl of the 1 mg/ml NOTA-RGD stock solution were transferred into glass vials (K60 kits 33.8µM). Alternatively, kits with reduced NOTA-RGD content were used (K30 16.8 µM); all kits were supplemented with 147 mg of sodium acetate trihydrate buffered salts as described above. Steps for radiolabeling and purification were provided from the SOP (Annexure B) below in Figure 3-9.

Labeling ^{68}Ga with NOTA-RGD cold kit

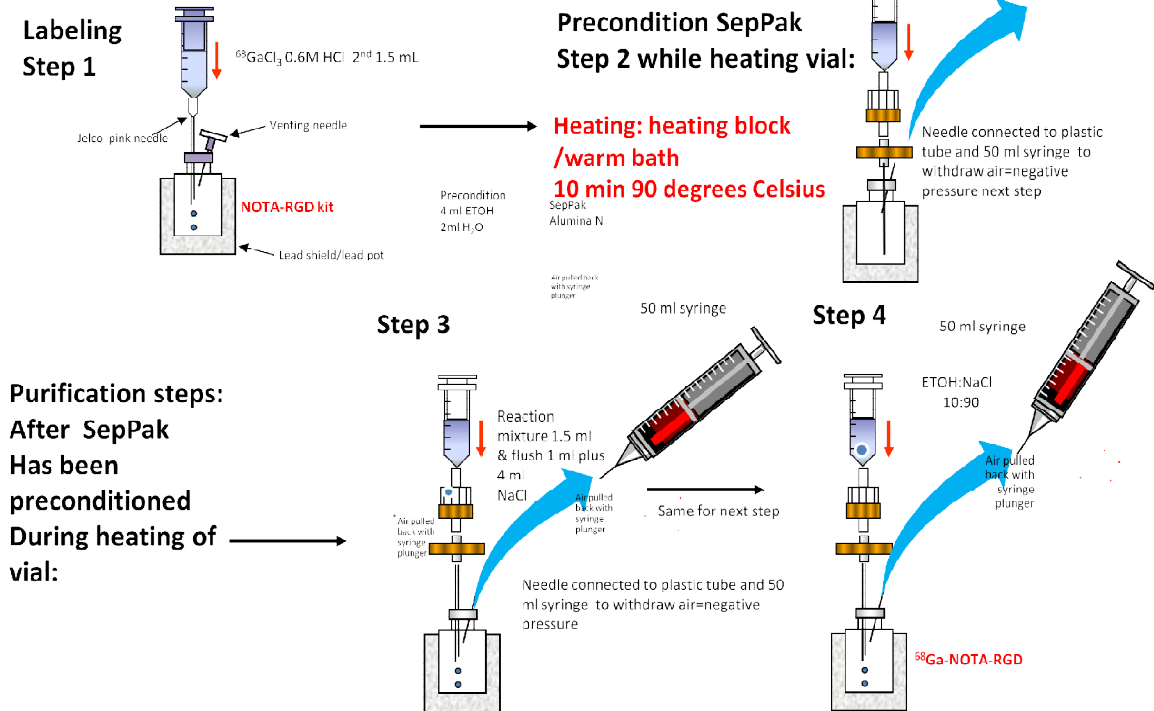


Figure 3-9: Radiolabeling of the inhouse prepared NOTA-RGD kit (SOP Addendum B).

Radiolabeling was performed according to step one. The $^{68}\text{Ge}/^{68}\text{Ga}$ generator was eluted using 0.6 M HCl (10 ml). The first 1.5 ml of eluted solution was disregarded. The second 1.5 ml elution was added to the NOTA-RGD kit vial by using the plastic part of the Jelco needle, 22 Gauge. The kit vial was vortexed for 30 seconds resulting in a clear liquid. The kit vial was boiled for ten min at 90 °C in a boiler or when not in use by other staff of Steve Biko Hospital, heated in a heating block for ten min also at 90 °C. The kit vial was left to cool for 5 min. The pH was tested and documented. The radioactivity was measured, therefore counted in the Geiger-Muller counter.

Purification was then done in step 2 to 4 (Figure 3-9). Purification materials was prepared before the radiolabeling and included SepPak Alumina N (500 cc), ETOH, Suprapure water, 2 sterile vials, decrimper, pipette, 2 x 2 ml syringes, 1x 5ml syringe (use for step 2 saline rinsing), 10 ml EtOH: Saline :10:90 (10 ml ensures enough for a second labelling), forceps (medium size) for vial transfer when activity needed to be

measured, 50 ml syringe with plastic connecting tube (same as nebulizer connecting tube), two needles: 25 Gauge for connecting to connecting tube and 50 ml syringe, 23 gauge for connecting to SepPak, saline $^{68}\text{Ge}/^{68}\text{Ga}$ generator (iThemba labs: IDB Holland).

The SepPak was connected to the needle inserted into the one sterile empty vial.

The 50 ml syringe was attached to the 25 gauge needle and also connected to the same vial. The syringe plunger was used by pulling it backwards to withdraw air and precondition the SepPak with 4 ml ethanol (EtOH) and 2 ml water. The ethanol and water was disregarded and the vial kept for the following step (step 3 in Figure 3-9) The reaction mixture (1.5 ml) was transferred into the SepPak with a pipette or syringe (without the needle). The empty vial was rinsed with 1 ml saline and also transferred into the SepPak. It was left to run through the column again by pulling plunger on 50 ml syringe while drops and not a stream of liquid was visible, therefore the speed of withdrawing air was also important. Disconnecting the syringe from the tube at any time resulted in the stop of flow of liquid therefore ensuring while more liquid added to the SepPak that the column did not run dry as yet. Another 4 ml of saline was used to rinse the solution from the SepPak column and the column was still kept wet.

Step 4 (Figure 3-9) was the important step in which a new sterile vial was used and connected to the SepPak, connection tube and as well as 50 ml syringe to this vial. Three ml of the 10 % ethanol: saline (EtOH: NaCl) solution was transferred to the SepPak until the column ran dry. The liquid collected in this last collection vial was the product therefore ^{68}Ga -NOTA-RGD. ITCL was performed as previously described by using Citric Acid as well as Sodium Carbonate as flow mediums.

3.1.4 Radiolabeling and Purification: additional info

$^{68}\text{Ge}/^{68}\text{Ga}$ -generator elution and pre-purification (done by overseas for additional data and publication with this study's in-house prepared kits).

^{68}Ga Gallium(III)chloride (^{68}Ga) was routinely eluted from a SnO_2 -based $^{68}\text{Ge}/^{68}\text{Ga}$ generator (1.85 GBq, IDB Holland, The Netherlands) by fractionated elution with moderate acidic condition (0.6 N HCl) assembling the ^{68}Ga activity in 2-3 ml eluate (reference method). For a set of experimental radiolabeling (N = 4) the ^{68}Ga activity was eluted from a TiO_2 -based $^{68}\text{Ge}/^{68}\text{Ga}$ generator (1.1 GBq, Eckert & Ziegler, Germany)

using lower acidic condition (0.1 N HCl); yielding 7.5 ml eluate which was subsequently mixed with 30% HCL to yield a molarities > 5.5 M. A strong anionic Dowex resin (100-200mesh, Fluka Analytics) was used to purify the activity solution from potentially co-eluted metals and ^{68}Ge . The resin-bound ^{68}Ga was desorbed with ultrapure water (Fluka Analytics) and assembled in 0.8 ml acidic solution (+SAX method). Alternatively, a fractionated elution similar to the reference method was performed on the TiO_2 -based $^{68}\text{Ge}/^{68}\text{Ga}$ generator (-SAX method).

3.1.5 Purification of the Radiolabelled Product: Solid phase extraction/SPE:

In this study purification solutions were prepared prior to radiolabeling of ^{68}Ga -NOTA-RGD; Ethanol (EtOH), Suprapure water and EtOH: Saline in 20:80 and 10:90 was prepared. SepPak connected to a sterile vial and a connecting tube was also prepared (Figure 3-10).

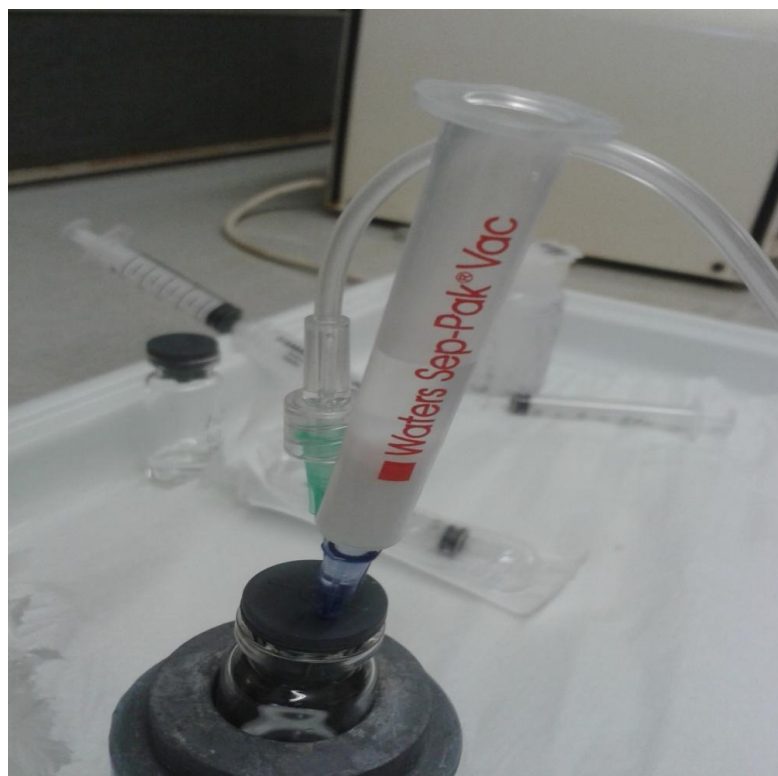


Figure 3-10: Purification SepPak.

^{68}Ga -NOTA-RGD was purified from unreacted ^{68}Ga species by reversed-phase chromatography. The C18 cartridge (500 mg 3 cc vac) was be pre-conditioned with 4ml

ethanol and equilibrated with 2ml sterile pyrogen free water (suprapure, deionised Millipore 18.2M Ω). A sterile 2 ml syringe was used and sometimes a pipette to transfer the reaction mixture onto the C18 Sep-Pak cartridge. It was essential to use non-metal in this step for example no metal needles were used in this process due to metal influence in the labelling process when radioactive metals used in labelling. The outflow was collected in a waste vial one. A sterile syringe was used and sometimes a pipette rather when available to rinse the reaction vial with 1 ml saline. This then was collected in waste vial number two. This was rinsed with another 4 ml saline. Then experiments was done with rinsing concentrations of 3 ml of ethanol/saline (10:90) and 2ml ethanol/saline (20:80) to wash out the product from the C18 Sep-Pak cartridge into a sterile vial. The activity of the product, empty vial and cartridge was measured. Originally the last step was rinsing with 2 ml ethanol/saline (20:80) due to great purity however this changed when a clinical dose concentration and correct pH was needed for the preclinical imaging of three vervet monkeys. It was required for preclinical imaging that the ^{68}Ga -NOTA-RGD solutions were sterile-filtered through a 0.22 μm membrane using a Millex, low protein-binding filter (Millipore).Preclinical

Vervet monkeys to be scanned were treated as clinical patients for example everything regarding sterility, pH, minimum radioactive dose per weight injected and injecting a pure product was applied. Therefore the correct clinical conditions were mimicked.

3.1.6 Quality Control

Instant thin layer chromatography (ITLC) analysis

Labelling efficiency was determined as described by Jeong (2008) and Breeman et al., (2005). Briefly, ITLC-SG paper was used as stationary phase and spiked at the bottom with 3-4 μl of the crude or pure ^{68}Ga -peptide solution, followed by 4-5 min exposure to the mobile phase (0.1 M citrate, pH 4.5). For testing colloid forming a mixture of 1 M ammonium acetate/methanol 1:1 (v/v) was used. The activity distribution was analyzed on an ITLC scanner (VSC-201, Veenstra Ind., Oldenzaal, Netherlands) using a gamma radiation detector (Scionix 25B25/1.5-E2, Bunnik, Netherlands) by obtaining chromatograms. If peak identification was possible, an “area under the curve” analysis allowed subsequent calculation of percentage peak recovery (Genie2000 software, Veenstra Ind., Oldenzaal, Netherlands) to calculate the percentage labelling efficiency.

High performance liquid chromatography (HPLC) analysis

Radiochemical purity was determined by high performance liquid chromatography (HPLC) analysis using a Agilent 1200 series instrument coupled to 6100 Quadruple mass spectrometry detector, (Agilent Technologies Inc., Wilmington DE, USA), diode array detector (DAD) and radioactive detector (Gina Star, Raytest, Straubenhardt, Germany). A Phenomenex Luna C18 column was used (250 x 4.6 mm, 5 μ m) for studies at iThemba LABS, Cape Town and Agilent Zorbax SB column (250x 4.6 mm, 5 μ m) at the rest of the studies performed at Necsa, Pretoria. The mobile phase consists of (A) 0.1% trifluoroacetic acid (TFA) in water and (B) 0.1% TFA in acetonitrile; gradient elution 0-2min 5%B and 2-32min 65%B and 32-35 min 5 % (this was added to equilibrate the column faster for the next injection). Column temperature and flow rate were set on 40° C and 1.0 ml/min respectively. Unbound ^{68}Ga was eluted at 2.3-4 min; ^{68}Ga -NOTA-RGD was eluted 11.4-12.5 min, respectively. The retention times of the crude and SPE-purified compounds were corresponding to each other.

Quality control was performed by High pressure liquid chromatography instant thin layer chromatography (ITLC) was also done in comparison to HPLC when possible. Resource wise, Steve Biko Hospital would need to do quality control in the clinical environment. Even though HPLC is the preferred method due to its sensitivity in detecting radiochemical purity as well as impure peaks. It was also interesting to compare the HPLC results with the results that National University (SNU) obtained by labelling also a RGD kit with their 0.1 M ^{68}Ge / ^{68}Ga Generator.

3.1.7 Kit shelf-life

The kit shelf life as well as labelled product's shelf life is of significance for patient management and planning in a Nuclear Medicine department. The half-life of the radioisotope plays a role in the labelling shelf life. It is suggested that the labelled shelf life should be of for example Technetium-99m (SPECT compound) compounds should be between 0.5 and 18 hour (Saha, 2010). When looking at positron emission labelled radiopharmaceuticals, one would anyway want the quickest possible patient administration due to very short half-lives and therefore such calculations would not necessarily play a major role.

The kit stability and shelf life integrity was investigated for good radiopharmacy practice purpose and optimum labelling efficiency as well as purity needed for clinical purposes.

In this study, the NOTA-RGD kit integrity and labelling efficiency was investigated over a period of 205 days post kit manufacturing. Kits containing 60 µg of RGD was investigated. According to IAEA documentation specific SPECT, Technetium-99m cold kits have a shelf life from months to years, depending on the compound (IAEA, 2008)

3.2 Preclinical

3.2.1 Ethical approval

Xenografted study; Ethical approval was obtained from the North-West University Ethical Committee for Mice Xenografts with approval project number: NWU-00042-12-A5. This included inoculation of a $\alpha_v\beta_3$ cell line A549 in nude mice as well as comparing uptake of the radiotracer in healthy versus tumour bearing nude mice.

Vervet monkey study: The study was approved by the Animal use and care committee University of Pretoria (Annexure C): S4560/09 as in Figure 3-11: Ethical approval from Animal use and care committee (AUCC), Addendum D.below. The initial application included requesting approval for ^{68}Ga as well as Copper-64 (^{64}Cu) labelled with UBI which was changed to requesting labelling with RGD due to UBI labelling giving problems at the time. Three primates were imaged on the PET-CT scanner at Steve Biko Hospital. The approval request was specific due to lack of small animal PET-CT scanners (so called micro-PET) in South Africa. The reason that monkeys were the preferred animals for this study is because they have the size of small children. Small children are scanned on the Human PET-CT scanner and a small amount of radiotracer is needed for the advanced PET crystals to detect this. The monkeys that were scanned for this study, ranged from 5, 8 -7, 6 kg (Mean weight is 6, 8 kg) and mean injected dose of radiotracer only 2, 56 mCi. This is only 0, 37 mCi per kg of the patient/monkey.



S4560/09

Replace S4556/09 and S4282/08

Figure 3-11: Ethical approval from Animal use and care committee (AUCC), Addendum D.

3.2.2 Xenografts: Mice

The experiments with xenografts were conducted according to the South African code of practice for the care and use of animals and carried out by the Preclinical Drug development Platform (PCDDP) of the North West University. Mice ex vivo data were obtained. Two mice were tumour bearing after implantation of the A549 cell line. RGD has been proven to be uptaken in $\alpha_v\beta_3$ integrin expression cells as discussed previously; therefore internalisation studies were not needed. It was therefore decided to do very limited mice xenografts study to indicate the in house prepared ^{68}Ga -NOTA-RGD performs as expected.

The organs of two healthy and two tumour mice organs were harvested 2 hours after intravenous injection in mouse tail with ^{68}Ga -NOTA-RGD. Several organs as documented below were harvested. Only two mice data have been used for comparison.

The organ containing tubes were counted in the Geiger-Muller counter. All tubes were also weighed according to 'empty' and 'full' when organs were included after harvested.

Harvested organs / tissues

- Brain
- Thyroid
- Heart
- Lung
- Stomach
- Intestines: large and small separate
- Liver
- Kidneys Right and left together
- Bladder/Urine
- Muscle
- Femur
- Tail
- Blood
- Corpse
- Testis
- Pancreas
- Tumour reference

3.2.3 Scanning on Clinical PET.CT scanner and SUV

3.2.3.1 Patient background: Vervet Monkeys

Biodistribution in healthy vervet monkeys

The present study was undertaken with NOTA-RGD to validate its potential PET/CT. The purpose is to evaluate the normal distribution of ^{68}Ga -NOTA-RGD in primates. ^{68}Ga -NOTA-RGD has showed in studies that it has a very fast blood clearance.

From former experience we know that the animals handle the administration radiopharmaceuticals and subsequent scanning well (Ebenhan et al., 2012). The half-life of the radionuclides to be used is short which limits the contamination risk by excretion. During these experiments the biodistribution needs to be recorded in larger animals to have sufficient resolution on a human PET-CT scanner. These experiments will be non-terminal vervet monkey trials.

Healthy vervet monkeys were scanned after ^{68}Ga -NOTA-RGD administration to reveal generic tracer biodistribution and to obtain pharmacokinetic parameters in vivo. High uptake levels in urinary bladder and kidneys dominated the ^{68}Ga -NOTA-RGD biodistribution, however, it's acceptable for a PET agent, because it can be easily kept under a hazardous radiation level and will seldom disturb image analysis.

The obtained tracer activity was sufficient for administration of 14 MBq /kg in large vervet monkeys up to 7.50 kg to evaluate the in vivo biodistribution and also achieve prior information about the in vivo stability. Results showed rapid blood clearance within 60 min post-injection and gradual soft tissue clearance via renal excretion and transient liver uptake; no activity was found in potential target organs such as lungs, brain or abdomen.

Animals, chemicals, and material

The experiments with primates were conducted according to the South African code of practice for the care and use of animals approved carried out by the Biomedical Research Centre of the Pretoria University. The animals were obtained from its colony at Onderstepoort and checked for general health to void occult infection. Pharmaceutical grade chemicals including 30% hydrochloric acid (HCl) Suprapure grade were purchased from Merck. Cartridges for solid-phase extraction (SPE) were

obtained from Varian, Phenomenex and Waters Corporation. Instant thin-layer chromatography-silica gel (ITLC-SG) was purchased from PALL Life Science. Sterile Millex GV filters were obtained from Millipore. High-performance liquid chromatography grade water was produced in-house using a Simplicity 185 Millipore system.

3.2.3.2 Scintigraphy and pharmacokinetics

The monkeys were treated like human patients and handled with respect and professional ethics at all times.

Three vervet monkeys (*Cercopethicus aetiops*) according to ethical application and approval was investigated for normal biodistribution of the radiopharmaceutical ⁶⁸Ga-NOTA-RGD. The monkeys travelled from Onderstepoort to Steve Biko Hospital, assisted, supported and handled by veterinary staff including a veterinarian. They were housed according to ethical approval in accordance with South African National Standards (SANS) and at all times handled by the veterinarian as well as scanned in his presence (behind the lead glass at the PET-CT scanner). The animals were sedated. They were weighed and during scanning protocol, urine and blood samples taken. The animals were scanned as below on different dates to be able to give full attention and support to one monkey only. The anaesthetic was administered by the veterinarian only through an infusion pump. The endotracheal tube inserted into the trachea. An ECG was connected and the animal at all times monitored. Some of the tubes are visualised on the scintigraphic images obtained on the scanner and above explains the foreign objects on the images. The Jelco needle that was placed in the femoral artery's purpose was to connect this to a device monitoring the monkey's arterial blood pressure. The purpose was secondly to obtain blood samples through the arterial catheter.

The monkey's radiopharmaceutical as well as weight information is as below:

Table 3-2: Three Monkeys Preclinical PET-CT scan info

Scanned patient	Monkey one	Monkey two	Monkey three
Date of PET/CT	24 August 2012	27 August 2012	31 October 2012
Scan			
Patient Weight	7,6 kg	7 kg	5,8 kg

Injected Dose	4,100 mCi	1,498 mCi	2,100 mCi
Scan 30 minutes post injection	–	–	Scanned
Scan 60 minutes post injection	Scanned	Scanned	Scanned
Scan 90 minutes post injection	Scanned	Scanned	Scanned

Mean injected dose of monkeys is 2, 56 mCi. Mean weight of three monkeys is 6, 8 kg.

A clinical PET-CT camera (Siemens Biograph True Point, 40 slice CT) was used for image acquisition. Images were acquired in three-dimensional mode and reconstructed with and without attenuation correction (CT-based) using ordered subset expectation maximization to yield axial, sagittal, and coronal slices (CT parameters: 120 kV, 100 mAs, slice thickness 5 mm, 0.8 mm pitch, matrix size 512 x 512) to yield axial, sagittal and coronal slices. All images were first evaluated qualitatively followed by semi-quantification analysis using Siemens e. Soft software on MI Processing workstation (version 2.0.21; ABX GmbH). For the biodistribution experiments, three-dimensional volume of interests (VOIs) were drawn manually over urinary bladder, brain, bone (shoulder), muscles (triceps/quadriceps), kidneys, heart, liver and spleen. The final values were expressed as organ standardized uptake value ($SUV = [\text{activity per ml tissue}] / [\text{injected activity per body weight}, \text{g/ml}]$). Additional images for illustration were processed with courtesy of Siemens International (Erlangen, Germany) on a Multimodality Work Place using Software version VE52A with Fused Vision 3D. For the patient case study, if abnormal uptake was present, a region of interest was manually drawn around any lung lesions or any organ of interest (the size was selected to correspond to the pathology on CT) and expressed as SUV. We used the maximum SUV (SUV_{max}) in this study, which represents the pixel with the highest tracer uptake in the lesion. All VOI's done independently. Volume rendering techniques were done by Siemens International. VOIS and SUV is further explained below.

3.2.3.3 Data processing: scintigraphy, blood and urine samples and SUV

VOIS: organs / tissues to obtain SUV

A volume of interest (VOI) has been drawn as listed below and as example in Figure 3-12, around the monkey's organs to obtain SUV values as discussed in this previous section. Volumes of interest were drawn over the liver, lung, heart, small intestine, large intestine, spleen, kidneys and a background area to determine the uptake in each organ. These values together with the information gained from the blood and urine samples, have been used to draw time-activity curves to determine the biodistribution of the radiopharmaceutical.

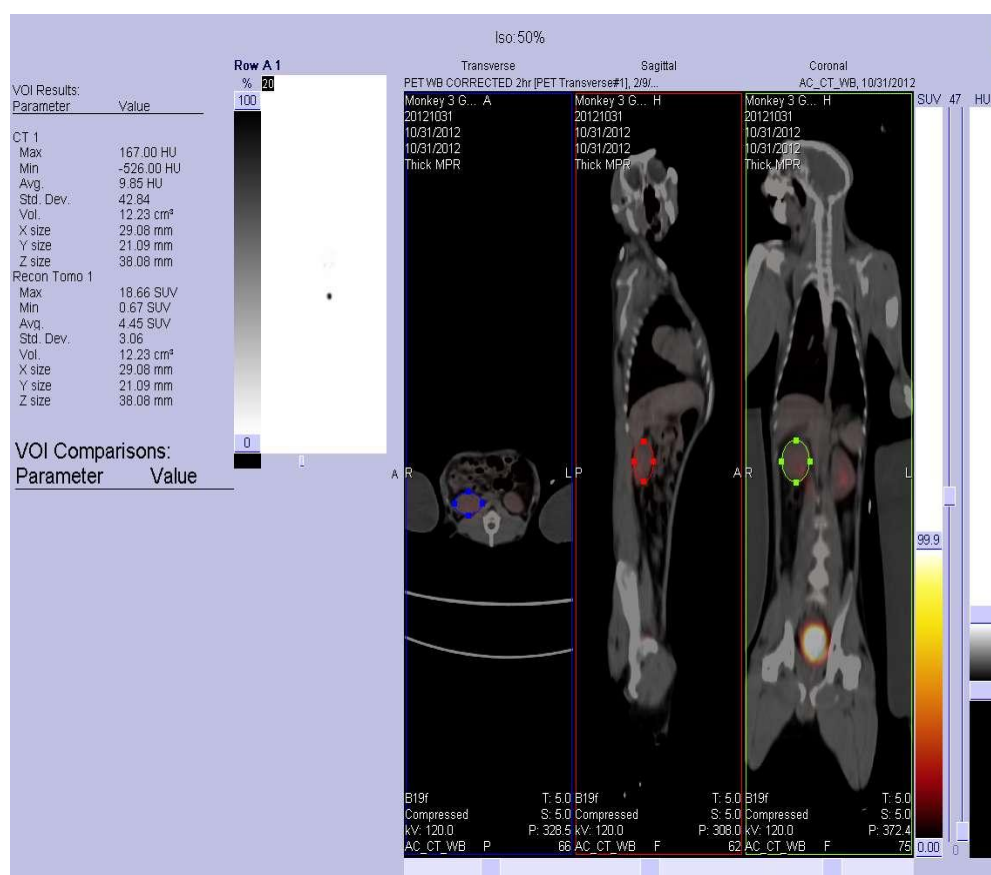


Figure 3-12: Volumes of interest(VOIs) drawn around the Monkey's organs for SUV calculation.

VOIs have been drawn over the following organs:

- Brain

- Heart
- Left hind
- Kidney Right
- Kidney Left
- Shoulder Left
- Liver
- Triceps Right
- Bladder
- Spleen

SUV as explained previously as automatically calibrated in a PET-CT scanner nowadays regarding the system's quality control with its suggested radioactive calibration sources and calibrations are performed to ensure perfect system operation as well as other info that different PET-CT scanners need for example patient weight and the amount of injected radiopharmaceutical dose is available. VOIs are drawn around the patient organs and SUVs are then calculated automatically by the system. SUVs (Standardized uptake values) are as explained by Kinahan and Fletcher (2010) as related to kBq/ml therefore concentration and calculated as below:

$$\frac{SUV=r(a'/w)}{[1]}$$

In which r refers to radioactivity and a' refers to the radiopharmaceutical amount. As stated before, patient weight needs to be available in order for software of the PET-CT to calculate SUV. The w in this formula refers to patient weight. The above authors further explain that it does not matter how much activity is injected, the distribution throughout the body would still be 1 g/ml. SUV significance comes in handy when oncology patients need evaluation of Tumours for example before, during or after chemotherapy. This will depend on when a patient could be scanned for example when funds would be available. In South Africa therefore the scans are not always performed in the above chronologic order. SUV is used for reporting purposes by Nuclear Medicine

Physicians or Radiologists in a PET-CT environment. As discussed above in this study SUVmax have been used for monkey organ comparison in this study. This is in relation with Kinahan and Fletcher's findings (2010) for example that SUV_{mean} does not have the same reproducibility as SUV_{max} . All results are discussed in the next chapter.

Chapter 4: Results and discussion

“Negative results are just what I want. They’re just as valuable to me as positive results. I can never find the thing that does the job best until I find the ones that don’t.”...Thomas Edison

4.1 Imported cold kits background results

⁶⁸Ga-Radiolabeling of **NOTA-SCN-Bz-RGD** and NOTA-RGD (inhouse prepared):

At Seoul National University analysis of the prepared results by ITLC using sodium carbonate as well as citric acid as flow mediums (Figure 4-1) showed ITLC reaction mixtures citric acid and sodium carbonate. Citric acid refers to labelling efficiency and sodium carbonate to the radiochemical purity with regards to colloid formation. Figure 4.1b shows the HPLC results which refer to good product radiochemical purity and a very small peak of possibly free ⁶⁸Ga. while showed good product radiochemical purity. A very small peak of free ⁶⁸Ga was observed. However during the formulation of the inhouse prepared kit in this study, below two percent of free ⁶⁸Ga was also observed on HPLC. It was possible though to get rid of free ⁶⁸Ga that was observed on the HPLC results by conducting a purification method.

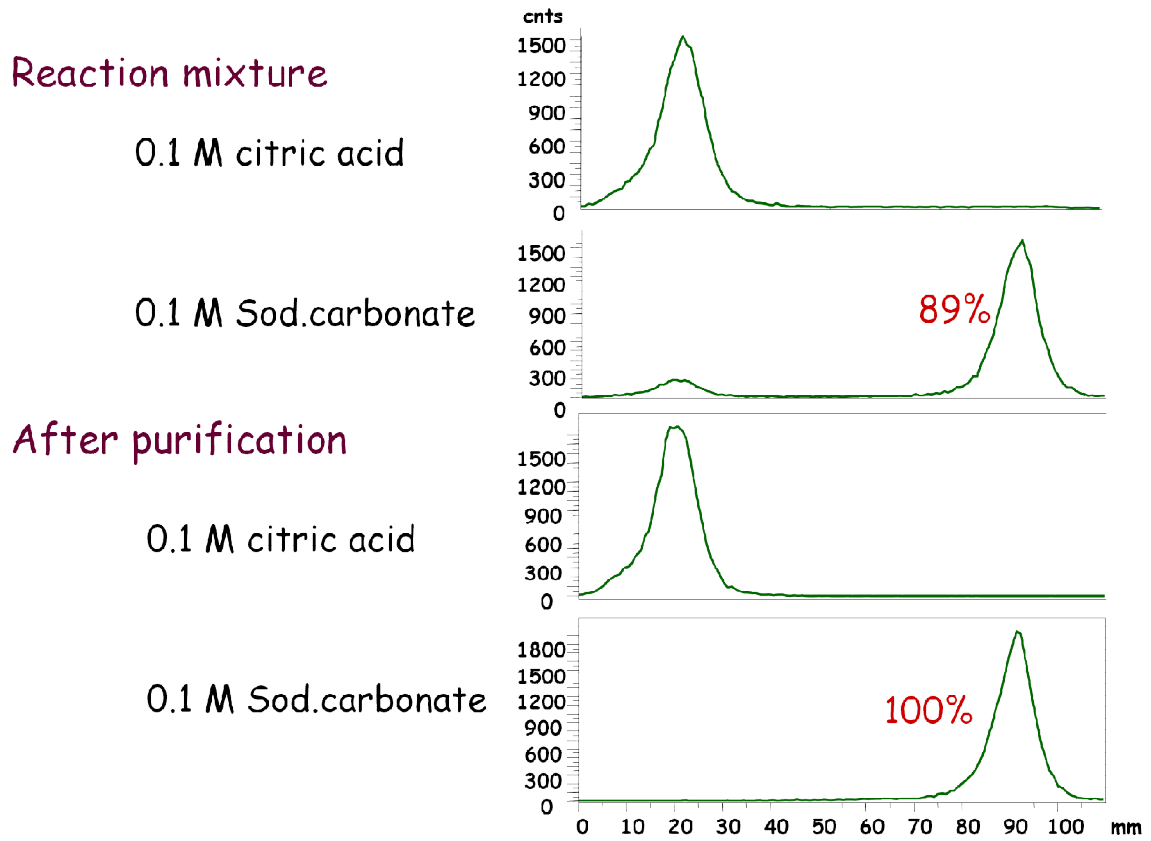


Figure 4-1: ITLC analytical results of radiolabelled $^{68}\text{Ga-NOTA-SCN-Bz-RGD}$ (Joeng, 2009).

HPLC purification

^{68}Ga -NOTA-RGD labeling
S.A.: 0.5~ 4.7 Ci/ μmol

column : Waters XTerra® prep.
(RP18, 10 μm 10 mm x 250 mm)
solvent : A Ethanol
B 0.01% TFA in D.W
0% ~100% A in B 30min

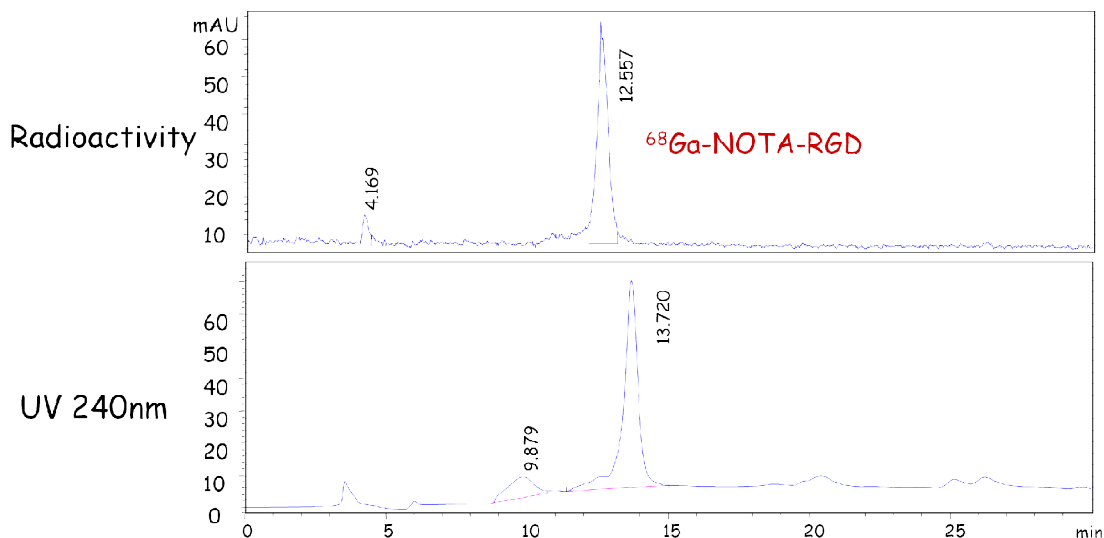


Figure 4-2: HPLC results of ^{68}Ga -NOTA-SCN-Bz-RGD (Joeng, 2009).

4.1.1 ITLC and results SNU imported cold kits

It was extremely difficult to get any labelling with the first batch using the imported kit. Therefore we focused on obtaining the optimum pH by varying the pH to the ^{68}Ga eluate. This also did not improve labelling. Since no stability tests as well as stability test on travelling outside a fridge, for the imported kits were obtained and no information regarding the shelf-life was available it could be that the kits expired and therefore the unsuccessful results. All imported kits were marked 'for experimental use only'.

The second batch also only gave intermittent results; labelling was achieved before the purification step but the SepPak purification step retained the biggest percentage of the product which suggested that this was not suitable for preclinical imaging or for human scintigraphic.

The imported tests that we tested gave the following results: Based on ITLC analysis the NOTA-SCN-Bz-RGD kits from SNU showed of a $55 \pm 10\%$ labelling efficiency (N = 11), however, during SPE purification a complete adsorption of the activity to the

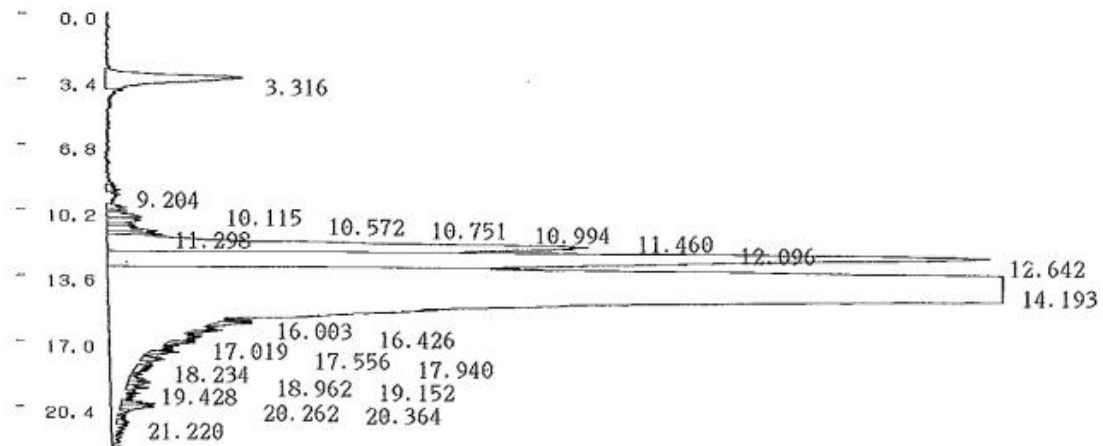
cartridge matrix (SepPak Alumina N, Waters) occurred. HPLC analysis showed multiple peaks indicating decomposition. An overall high colloid forming tendency was observed, amounting to $24 \pm 13\%$. Colloid formation was tested specifically with ITLC using the sodium carbonate reaction mixture as discussed in chapter 3. Varying the labelling parameters (molarity or type of buffering solution, molarity or volume of ^{68}Ga -solution, pH, incubation duration or temperature) did not positively affect the purification step. The mean pH was 5.4 after radiolabeling. The HCl molarity was always 0.6 M. The elution buffer was also changed to investigate if labelling efficiency would increase. At the start of the study sodium hydroxide was used which was later changed to sodium acetate (NaOAc). This however had no impact on the imported kit labelling efficiency but was key to the success of the in-house prepared kits.

4.1.2 Purification:

4.1.2.1 HPLC at iThemba LABS

HPLC results of the first in-house prepared kits formulated at iThemba LABS and dried in a desiccator is hereby documented. The rest of the kits were formulated at Radiochemistry NECSA. The first inhouse kits prepared from RGD from Futurechem were formulated and dried in a desiccator and showed great labelling efficiency of almost 90% as well as purity of almost 90% were obtained. Examples are below in Figure 4-2 and Figure 4-3 of HPLC done on the same methods as discussed in Chapter 3, however different column as discussed was used than the column used at NeCSA.

Initially the results were obtained after SepPak and desorbing radiolabelled product with 20% ethanol and 80% saline solution. As discussed this was then changed for clinical purposes and lower volume for preclinical scanning. (Initial purity was higher with 20% ethanol but the yield resulted in an amount of activity too low for example never more than 50% for scanning purposes.)



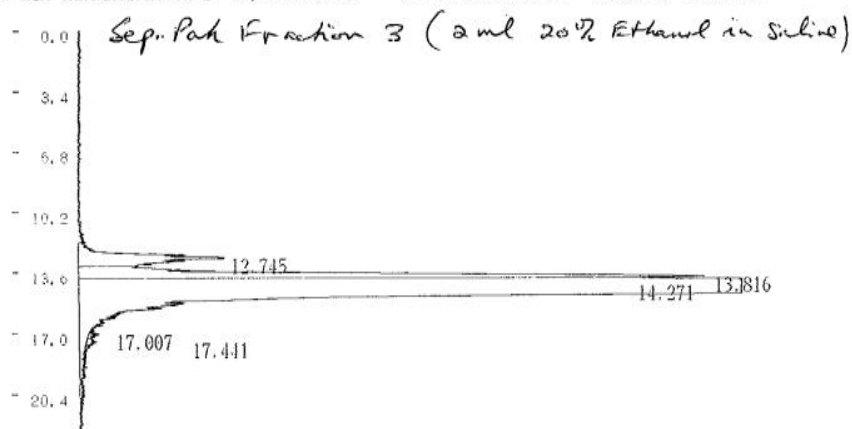
** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
2	16	3.316	539501	19652	V		0.8714	
	49	9.204	29492	1849	V		0.0476	
	54	10.115	35150	2714	V		0.0568	
	56	10.572	58198	4913	V		0.094	
	57	10.751	69096	4541	V		0.1116	
	58	10.994	28633	3774	V		0.0462	
	59	11.298	86196	7087	V		0.1392	
	60	11.46	57941	7624	V		0.0936	
	61	12.096	2046825	67845	V		3.3059	
	62	12.642	4398954	125778	V		7.1049	
	63	14.193	54177156	1349606	SVE		87.5028	
	65	16.003	44960	4394	TV		0.0726	
	67	16.426	20837	3773	TV		0.0337	
	72	17.019	29896	2936	TV		0.0483	
	75	17.556	30776	4076	TV		0.0497	
	76	17.94	31582	3228	T		0.051	
	78	18.234	37852	1832	TV		0.0611	
	80	18.962	22104	1996	T		0.0357	
	81	19.152	29493	3139	TV		0.0476	
	82	19.428	22420	3097	TV		0.0362	
	87	20.262	39958	4412	TV		0.0645	
	88	20.364	55961	4259	TV		0.0904	
	92	21.22	21779	1760	TV		0.0352	

Figure 4-3: HPLC at iThemba labs testing the first inhouse prepared kits.

Figure 4-3 contains a small peak at 3,316 min which could most likely be an impurity/free ⁶⁸Ga of 0, 87 % radiochemical purity. The two peaks though at 12,096 min and 14,193 min are both RGD product peaks contributing to 7,10 and 87,50 % radiochemical purity respectively.

Peak PROC/CALC ERROR CH=2, 11: Insufficient SLICES/PEAKS Defined
 C-RSA CHROMATOPAC CH=2 Report No.=78 DATA=1:CHRM2.C00 12/04/26 11:27:16



** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
2	64	12.745	891816	27614	V		3.0253	
	65	13.816	2157281	119817	V		7.3181	
	66	14.271	26376882	812977	SV		89.4782	
	73	17.007	20185	1801	T		0.0685	
	74	17.441	32385	1891	TV		0.1099	
TOTAL			29478544	964100			100	

Figure 4-4: HPLC at iThemba labs testing the first inhouse prepared kits: specific using the 20:80 percent ethanol saline to desorb the radiolabelled product from the SepPak cartridge.

In Figure 4-4, the peak at 12,745 minutes has a 7,31 % radiochemical purity while the peak at 14,271 minutes has 89, 47 % radiochemical purity. These two peaks are the product peaks which sometimes differed in intensity for example two conformant peaks. The labelling efficiency was 99, 50%. All product peaks were always visible between 10.5 and 14 min.

Purification (solid phase extraction)

K30 refers to 30 µg of RGD in the cold kit and K60 to 60 µg of RGD in the cold kit. Post radiolabeling, SPE matrices were evaluated for optimal purification of ⁶⁸Ga-NOTA-RGD from free radiogallium and ⁶⁸Ga-colloids (Table 4-1). Both SPE types show high total desorption levels with an alcoholic saline solution, amounting to a total percentage of adsorbed activity of 89 ± 3% and 76 ± 5% for Sep-Pak C18 500 mg 3cc vac and SPE cartridge Sep-Pak light C18 100 mg, respectively. Significant higher amounts of activity was desorbed with 10% alcoholic saline solution ($P= 0.049$), hence, a significant lower

SPE bound activity ($P= 0.038$) occurred for Sep-Pak C18 500 mg 3cc vac over the online C18 light cartridge. For preclinical application the SPE elution with ethanol/saline 20/80 (v/v) protocol was avoided to achieve the minimum solvent levels for ethanol in the final solution to be injected. The purified ^{68}Ga -NOTA-RGD was found to be stable in the injection solution up to the 60 min tested. The product radioactivity achieved was 204 ± 44 MBq ($n = 10$); (even with this small scale production it would be sufficient to inject a patient with 185 MBq within 15-20 min after radiolabeling, full scale production would ideally amount to 812 ± 84 MBq (calculated from $N = 3$ experiments). The radiochemical purity (RCP) of ^{68}Ga -NOTA-RGD determined by HPLC was $99.2 \pm 0.54\%$ ($n = 11$), samples showed traces of ^{68}Ge ($\leq 0.0004\%$) and cationic metal impurities of 0.5 to 9 ppm, only. The calculated percentage of ethanol was ≤ 2.0 -2.5% in the final solution of the radiopharmaceutical product. K60 (60 μg RGD kit) ^{68}Ga -NOTA-RGD kits labelled with an average specific activity of 6.1 GBq/ μmol (Range: 2.8-7.9 GBq/ μmol), the full-scale specific activity based on the herein reported kit performance is calculated as 18.1 GBq/ μmol .

Table 4-1: Purification

Performance of SPE Purification on K60 ^{68}Ga -NOTA-RGD Kit Radiolabeling

SPE Sep Pak Type	C18 500mg 3cc vac column #	C18 light (100mg) online cartridge #
Adsorbed Activity (AA) (MBq)	250 \pm 64	119 \pm 10
Total SPE Elution (% AA)	89 \pm 3	76 \pm 5
Elution Fraction: 10 / 90 (v/v) Ethanol /	77 \pm 4*	63 \pm 5
Saline		
20 / 80 (v/v) % Ethanol / Saline (v/v)	12 \pm 1	13 \pm 1
Retained Activity (% Range AA)	4-13*	15-37

#) Analysis of K60 kits with ≤ 90 days shelf lives are summarized. Values are expressed as mean (\pm SEM; $N = 4$). * = statistically significant difference (*Student's t-test* $P \leq 0.05$).

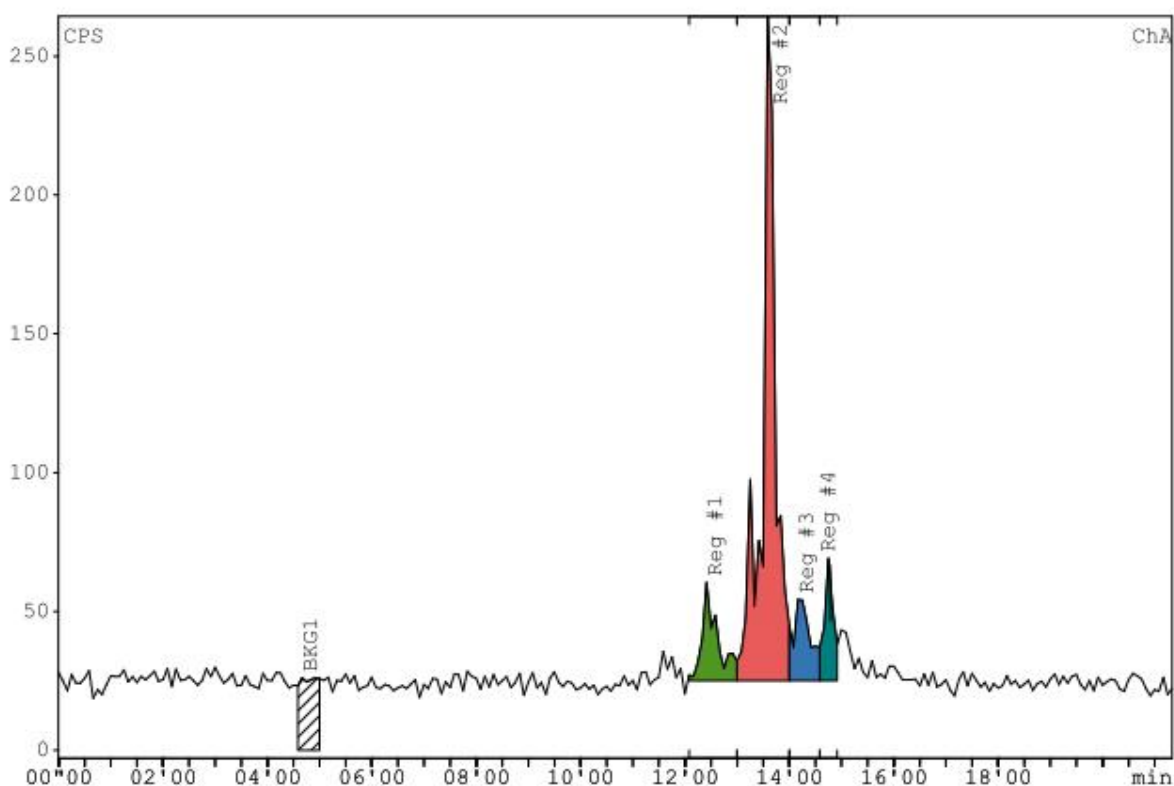


Figure 4-7: HPLC of product after purification.

4.1.3 ^{68}Ga eluate purification and NOTA-RGD radiolabeling

NOTA-RGD was successfully labelled with ^{68}Ga eluted from SnO_2 - and a TiO_2 -based generator (Table 4-2). Both generators provided a similar yield of radiogallium and the complete labelling procedure including quality control was achieved in 34 and 38 min (reference and –SAX , fractionated elution applied) or 43 min with implementing anion exchange pre-purification (+SAX) of the ^{68}Ga -eluate solution. Results indicate both a substantial reduction of colloid formation and a slight increase in the percentage labelling efficiency (%LE) for ^{68}Ga -NOTA-RGD utilizing pre-purified generator eluate.

4.1.3.1 Pre purification additional info (performed by co-worker at the University of in Leuven, Belgium)

Table 4-2: Influence of Generator Eluate Pre-Purification on ⁶⁸Ga-NOTA-RGD radiolabeling

⁶⁸Ga-NOTA-RGD Radiolabeling				
Elution method*		FE (Reference)	FE, + SAX	FE, - SAX
Generator type		iThemba 50 mCi	Eckert & Ziegler 30 mCi	
Solid matrix material		SnO ₂	TiO ₂	
Mobile phase		0.6 M HCl	0.1 M HCl	
Generator shelf life span (d)		96 - 140	124 - 134	101 - 108
Yield ⁶⁸Ga (%)		94 ± 2 (n=7)	81 ± 4 (n = 11)	90 ± 1 (n = 7)
⁶⁸Ga-elution discharge (%)	bound	-	8.6 ± 4.0	-
	liquid	5.9 ± 1.9	8.5 ± 2.9	10.2 ± 1.3
ITLC	Crude labelling (%LE)	71 ± 6	81 ± 6	74 ± 6
	RCP (%)	99.0 ± 0.50	99.2 ± 0.33	99.4 ± 0.25
	Colloids (%) loss	8.0 ± 2.0	4.5 ± 2.2	8.5 ± 3.8
Sep Pak (%) loss		13 ± 2	12 ± 2	13 ± 3
Duration (min)		38 ± 2	43 ± 2	34 ± 4

*) Elution method: fractionated elution (FE) combined with (+SAX) and without (-SAX) pre-purification followed by standard labelling procedure were applied. Values are expressed as mean (± SD; N ≥ 3)

4.1.3.2 Mass determination of NOTA-RGD for radiolabeling

Manual radiolabeling was done prior to the kit formulation. At first about 10 µg as NOTA-RGD was the same amount as in the SNU donated cold kit was giving a 31± 3 % LE (N = 4). For improvement, kits containing 30 µg (K30; 16.8 µM) or 60 µg (K60; 33.8 µM) were compared with manual radiolabeling procedures adding 30 µg (NK30; 16.5 µM) or 60 µg (NK60; 33.1 µM) to the buffered generator eluate solution. Levels of

colloid formation were less for both the kits K30 ($P = 0.073$) and K60 ($P = 0.081$), compared to manual ^{68}Ga -NOTA-RGD labelling (NK30 and NK60; Figure 4-8 A). Significant higher radiolabeling (% LE) was achieved using the K30 ($P= 0.01$) or K60 ($P= 0.00017$) kits over manual radiolabeling (NK30 and NK60; Figure 4-8 B). Neither the amount of colloid forming ($P = 0.225$) nor the %LE ($P = 0.139$) differed between K30 ($N = 4$) and K60 ($N = 7$) kits labelling.

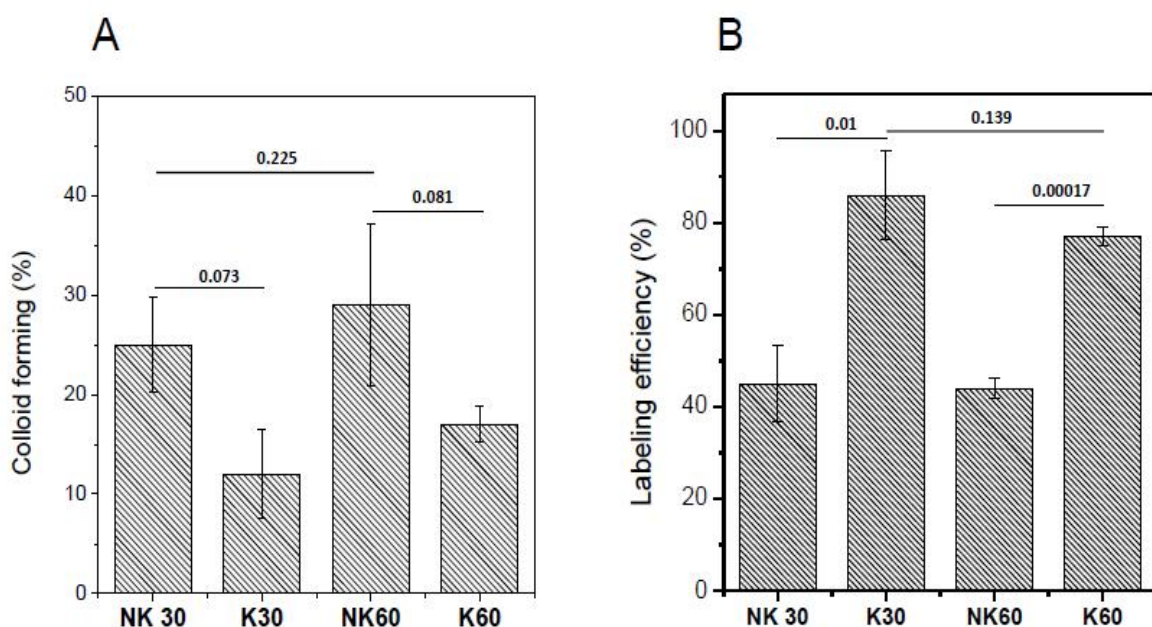


Figure 4-8: NOTA RGD kit performance: K30 and K60.

4.1.4 Kit shelf life integrity and K60 performance

A linear decrease was observed when kit integrity was analysed, comparing radiolabeling efficiency (%LE) with kit stability over days. ^{68}Ga -labeling of NOTA-RGD kits (K60) with the shelf span up to 205 days post manufacturing ($y = -0.3286x + 100$; ($r^2 = 0,892$) $N= 19$). Results indicated that $> 75\%$ LE and $> 50\%$ LE radiolabeling can be achieved within the first 76 d and 152 d, respectively (Figure 4-8: NOTA RGD kit performance: K30 and K60. Figure 4-8). A kit vial labelled 205 days post manufacturing still show 19.6% labelling efficiency, however HPLC/ITLC analysis detected $>52\%$ compound losses (SepPak and colloid forming) and radiolabelled by-products presuming kit decomposition. The limited data on K30 kits show reduced integrity (93% at 31 d and 66% at 90 d) compared to K60 kits. Figure 4-8 shows the impact of the

NOTA-RGD kit integrity on the percentage radiolabeling efficiency. Samples were radiolabelled and analyzed with HPLC and/or ITLC. Results from N=19 individual experiments are expressed as percentage of added ^{68}Ga -activity.

4.1.5 $^{68}\text{Ge}/^{68}\text{Ga}$ generator data

Figure 4-9 represents a generator that was 150 days old when this graph was created. By the time of this graph created the eluted ^{68}Ga was documented over 7.5 months. This generator was eluted by fractions of 0.5 ml. This is as proposed by IDB Holland/iThemba LABS user manual as documented earlier. This generator was eluted by using 0.6 M HCl. Dat comparison is made with N = 3 elutions within one to two weeks around the stated manufacturing time. This graph shows the gallium-68 peak performance obtained in a specific volume that assisted us in knowing which ml to focus on using and prevent us from measuring all the 0, 5 ml fractions in a clinical environment.

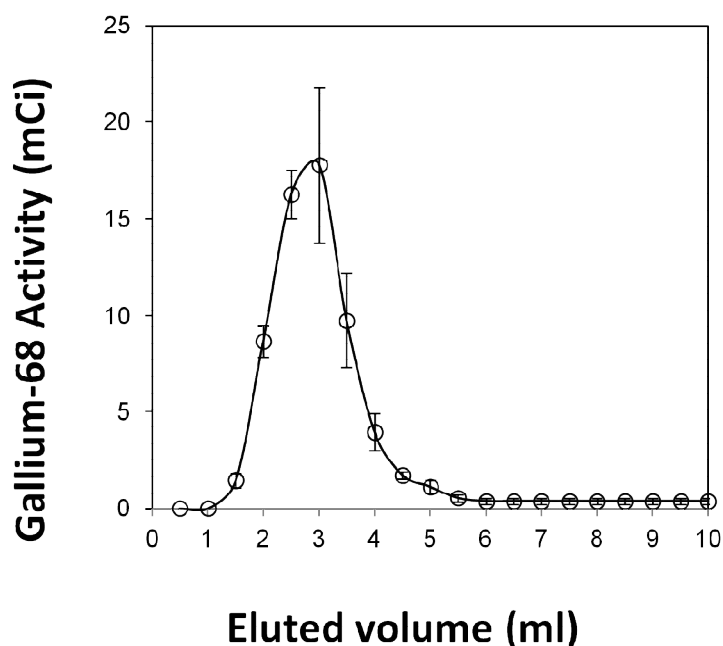


Figure 4-9: Impact of the NOTA-RGD kit integrity on the percentage radiolabeling efficiency.

It is also essential in clinical application to know the best time to milk (elute) the generator for ^{68}Ga use in order to obtain the best activity and labelling efficiency. A rapid activity increase has been obtained with the iThemba LABS generator (Figure 4-10) indicating that the best time to milk the generator is 4-5 hours after the previous elution and this could be determined by fractionated elutions of 0.5 ml.

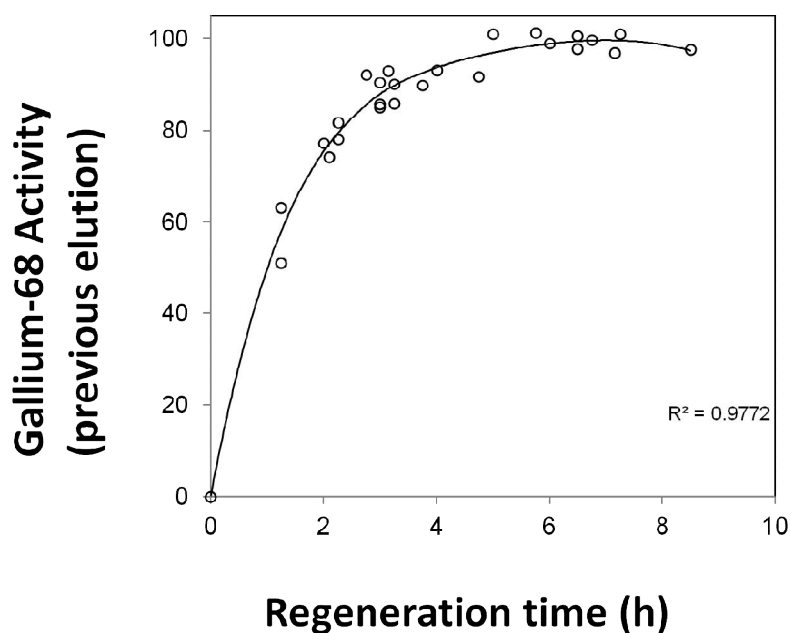


Figure 4-10: Regeneration time from the parent decay.

4.2 Xenografts: Mice

Figure 4-11 represents the percentage of injected dose per gram of organ of the mice xenografts. In this limited (confirmation) study only two xenografted mice and two healthy mice were used. Both tumours of the two tumour mice did not have good

condition tumours i.e. the tumours were too big and blood supply was limited However one tumour bearing mouse (inoculated with the A549 cell line) gave a 6.2:1 tumour to muscle ratio and a 5.1:1 tumour to lung ratio. In Figure 4-11 the average of 2 animals was reported As far as we are aware this was the first xenografted study performed with ^{68}Ga -NOTA-RGD produced from a 0.6 M generator.

Unfortunately due to very low counts (due to the rapid decay of Ga-68), very small organs (uncertainty in weight) and only 2 animals, the standard deviation (SD) in this case is very high. Especially high SD for bladder and urine was recorded due to very small weight of the organs. Nonetheless an average tumour to muscle ratio of 3.2:1 was recorded while the tumour to lung ratio was 2.6:1. A tumour to healthy tissue ratio of 3:1 is essential to proof positive uptake of a tracer by the tumour.

It was clear that increased activity through excretion organs for example kidney and urine/bladder was seen. It is also evident that there is no target organ in which the ^{68}Ga -NOTA-RGD shows increased uptake. The tumour however does take up the ^{68}Ga -NOTA-RGD which was expected.

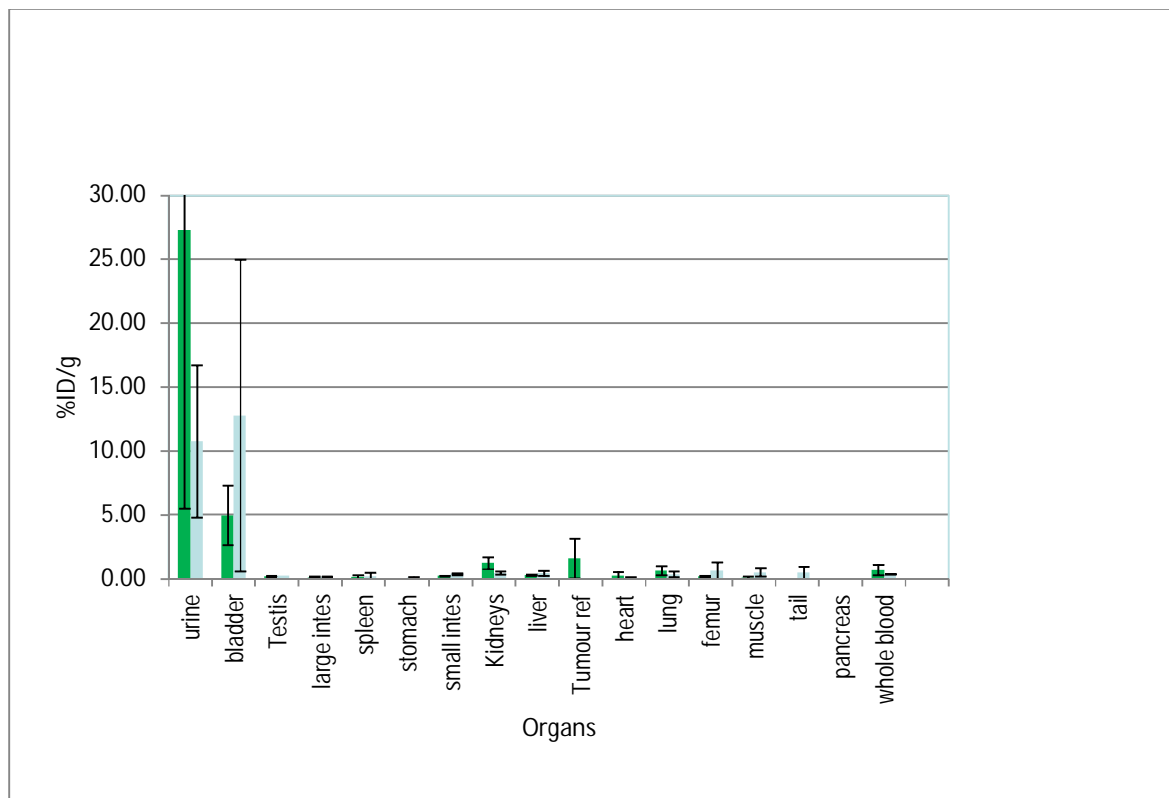


Figure 4-11: Percentage Injected dose per gram in Xenografted mice. The first (green) series is the tumour mice and the second (light blue) the healthy mice.

4.3 Preclinical Imaging: Pharmacokinetics, biodistribution, and Image gallery

Preclinical imaging has been performed and volume rendering techniques applied to show detailed preclinical images as the one below in Figure 4-12.

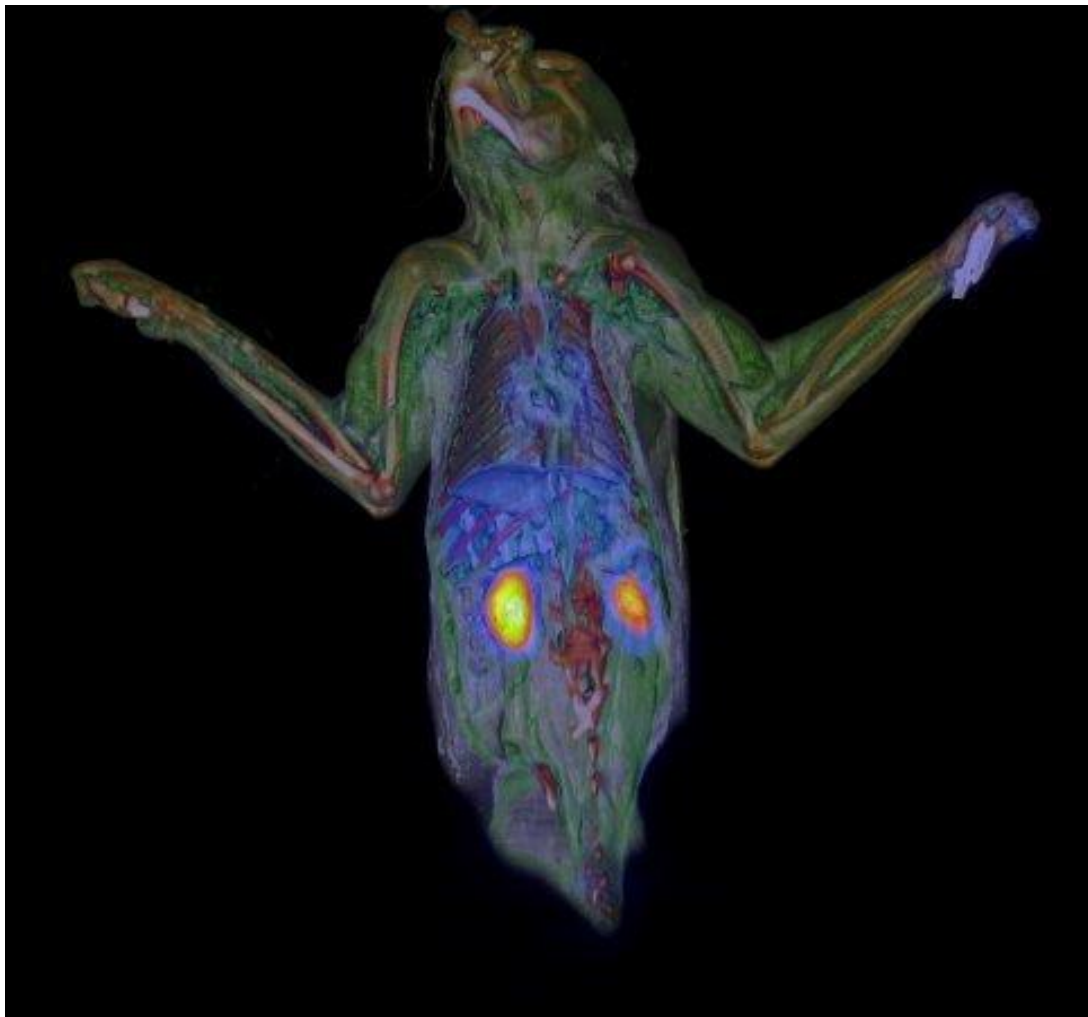


Figure 4-12: Volume rendering monkey image of biodistribution of radiotracer (Performed by Viola Satzinger from Siemens Erlangen).



Figure 4-13: Radiochemical purity and yield of three vervet monkeys.

The radiochemical purity (RCP) and labelling efficiency of the three different radiopharmaceuticals that was prepared for scanning the vervet monkeys were compared. The labelling efficiency mean was 51.21%. The RCP mean however was almost 100% ,99.50% which was crucial for preparing protocol and SOP's that would ensure this purity before this study goes clinical. The labelling procedure for the last monkey gave the highest labelling efficiency of 64%. This monkey was scanned during the first stages of a new generator.

SUVs were obtained as on the example of Figure 4-14 below and as previously discussed, specific SUV_{max} was used. ($SUV = [\text{activity per ml tissue}] / [\text{injected activity per body weight}], \text{g/ml}$).



Figure 4-14: Max SUV used for patient pathology and clinical example urine.[VOIs processing]

Volume rendering processing shows excretion through kidneys more clear below in Figure 4-15.

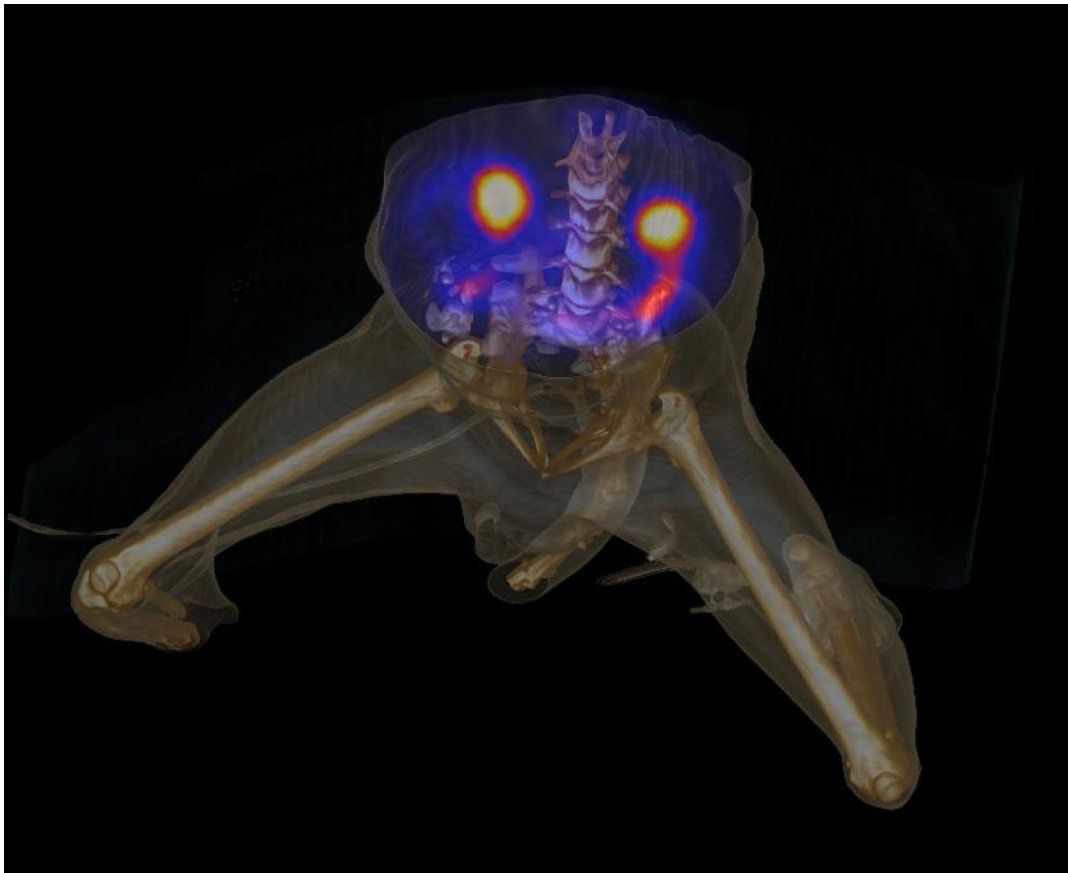


Figure 4-15: Pelvic volume rendering of one vervet monkey to visualize radiotracer uptake in kidneys.

Figure 4-16 shows no uptake in bones: Bone images and spinal detail are seen from the CT scan. Again the two colourful blue, orange and yellow images are the two kidneys through which the ^{68}Ga -NOTA-RGD is excreted.

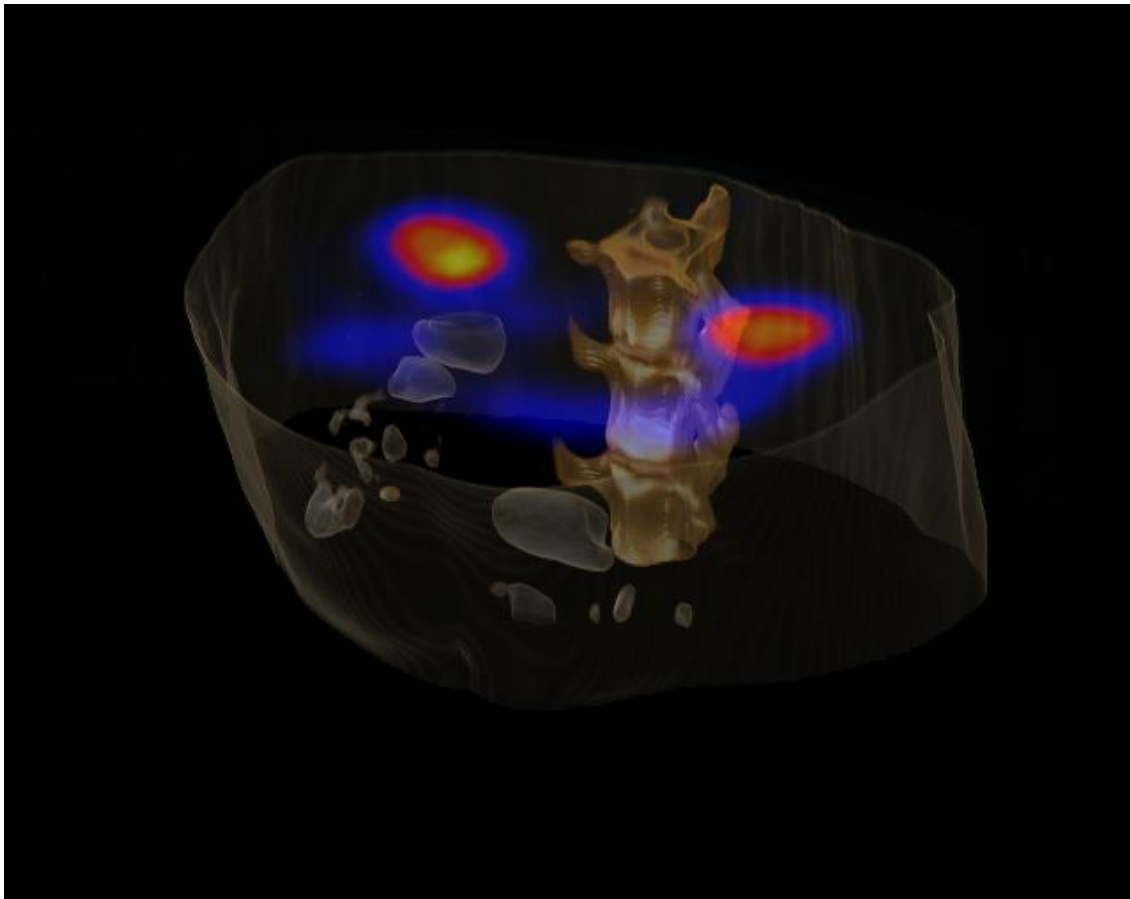


Figure 4-16: Volume rendering image of monkey of radiotracer in kidneys and imaged of spine from CT.

All organ or tissues applicable to this tracer uptake (except kidneys) showed peak tracer concentration at 30 min declining more or less gradually until 120 min. The present study included the monitoring of the activity levels in blood and urine samples and subsequent calculation of a rapid release represented by a biological half-life of 5 min for ^{68}Ga -NOTA-RGD, however, 54% of the injected activity dose was recovered in total urine, 120 min post injection. Based on our findings we have reason to believe that uptake, distribution and excretion of NOTA-RGD is very favourable to monitor lung cancer. It is noted, that the biodistribution showed no hepatobiliar excretion and may therefore postulated to may detect tumours located in pancreas, abdomen and even prostate (as previously described).

Table 4-3: Organ and Tissue Concentration of ⁶⁸Ga-NOTA-RGD in Healthy Vervet Monkeys;

Organ/ Tissue	SUV			
	30 min p.i. [#]	60 min p.i. [*]	90 min p.i. [#]	120 min p.i. ^{\$}
Heart	1.01	0.89 ± 0.32	0.62	0.35 ± 0.12
Liver	1.24	1.71 ± 0.70	1.91	0.49 ± 0.08
Spleen	1.22	1.60 ± 0.49	1.39	1.10 ± 0.60
Bladder, urinary tract	24.7	31.2 ± 14.5	24.1	23.5 ± 3.4
Kidney	3.53	3.19 ± 0.88	2.78	1.94 ± 0.84
Quadriceps muscle	0.19	0.24 ± 0.04	0.22	0.22 ± 0.04
Triceps muscle	0.18	0.23 ± 0.05	0.22	0.22 ± 0.03
Bone (shoulder)	0.36	0.43 ± 0.12	0.38	0.39 ± 0.09
Brain	0.04	0.04 ± 0.02	0.05	0.07 ± 0.01

Values expressed as mean SUV (±SEM) if applicable.

*) N = 3 animals; \$) N = 2 animals, #) N = single animal

Figure 4-17 and d shows Concentration of ⁶⁸Ga-NOTA-RGD in blood and urine samples from healthy vervet monkeys. Values in blood (Figure 4-17) assembled as a time-activity curve following an exponential decline ($y=0.751e^{-0.151x}$; $R^2= 0.971$) after Savitzky-Golay data smoothing algorithm applied. Measurements in urine (Figure 4-18) show a linear incline ($y=0.125x$; $R^2=0.967$). Results are expressed as mean ± SD of N ≤3 animals and expressed as percentage of the injected ⁶⁸Ga-activity per bodyweight (%ID/g).

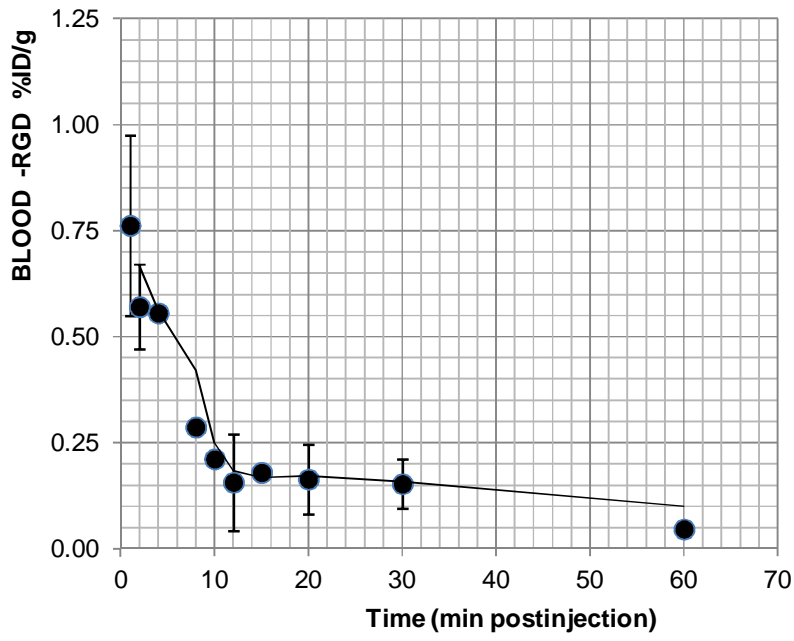


Figure 4-17: Time/activity curve of ⁶⁸Ga-NOTA-RGD in monkey's blood.

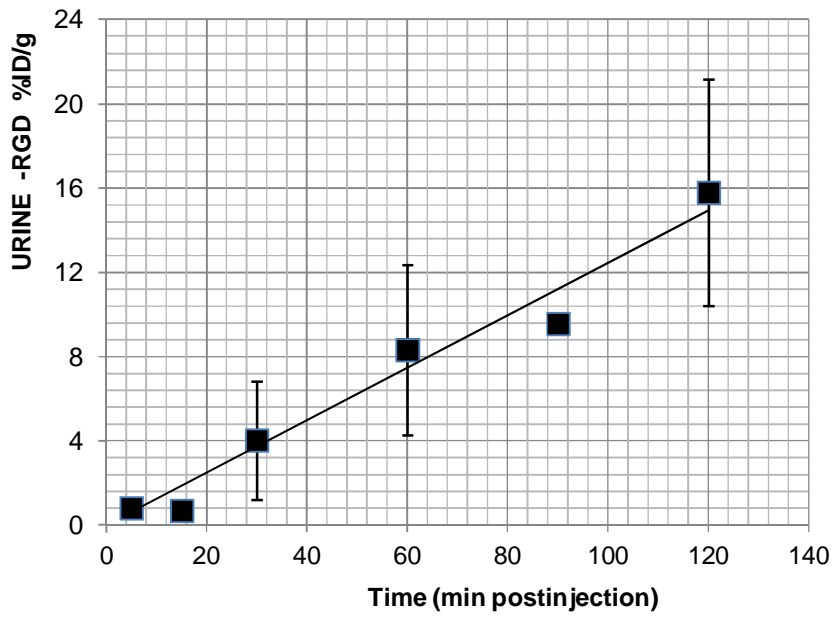


Figure 4-18: Time/activity curve of ⁶⁸Ga-NOTA-RGD in monkey's urine.

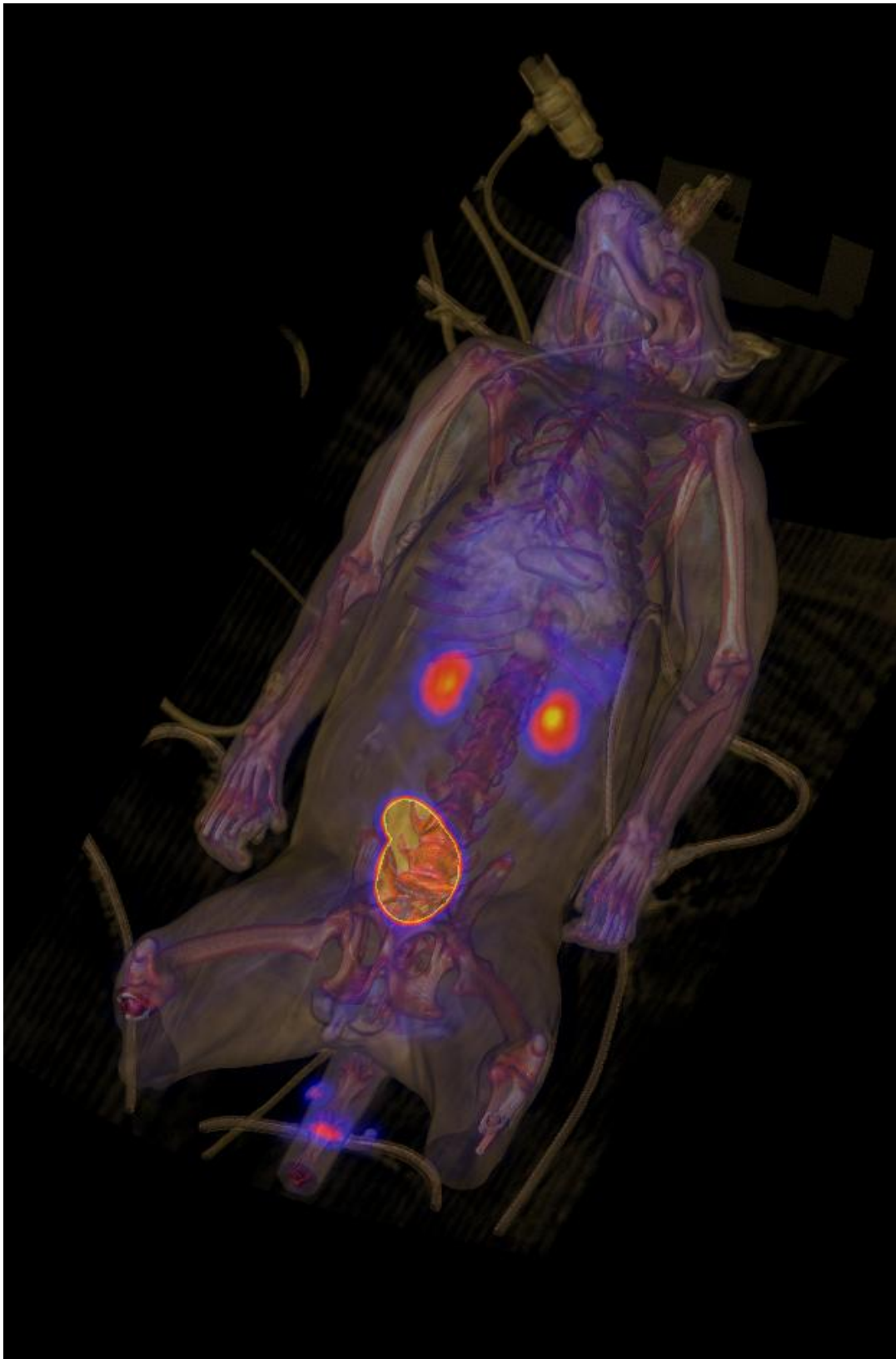


Figure 4-19: Volume rendering of monkey from head to pelvis showing endotracheal tube, kidneys and urine in bladder.

4.4 ⁶⁸Ga-NOTA-RGD Concentrations in Blood and Urine of healthy vervet Monkeys

The time dependent concentration of ⁶⁸Ga-NOTA-RGD measured from individually taken blood samples (N = 10) was further optimized by mathematical data smoothing using Savitzky-Golay algorithm (Origin Pro 8, OriginLab Corporation, Northampton). The results of the fluid analysis of ⁶⁸Ga-NOTA-RGD confirmed that the peak blood activity concentration was obtained 1 min after injection, amounting to 0.75 ± 0.07 % ID/g (Figure4.1.1.1.c). The blood-bound activity rapidly declined ($y=0.751e^{-0.151x}$ $R^2 = 0.971$) as represented by a pharmacological half-life of only 5 min and an elimination rate of 9 %ID/h. Urine samples showed steadily accumulating activity levels of ⁶⁸Ga-NOTA-RGD following a linear incline ($y=0.125x$; $R^2=0.967$; N = 6) peaking at 16 ± 5 %ID/g at 120 min (Figure4.1.1.1.d). The accumulation rate was calculated 7.5 % ID/h with a total urine recovery reaching 54 ± 4 %ID (N = 3) after 120 min.

The obtained tracer activity was sufficient for administration of 14 MBq /kg in large vervet monkeys up to 7.5 kg to evaluate the *in vivo* biodistribution and also achieve prior information about the *in vivo* stability. Results showed rapid blood clearance within 60 min post-injection and gradual soft tissue clearance via renal excretion and transient liver uptake; no activity was found in potential target organs such as lungs, brain or abdomen in is in relation to what is needed in a clinical radiopharmaceutical.

Additionally, all organ or tissues (except kidneys) showed peak tracer concentration at 30 min declining more or less gradually until 120 min. The present study included the monitoring of the activity levels in blood and urine samples and subsequent calculation of a rapid release represented by a biological half-life of 5 min for ⁶⁸Ga-NOTA-RGD, however, 54% of the injected activity dose was recovered in total urine, 120 min post injection. Based on our findings we have reason to believe that uptake, distribution and excretion of NOTA-RGD is very favourable to monitor lung cancer. It is noted, that the biodistribution showed no hepatobiliar excretion and may therefore be postulated to may detect tumours located in pancreas, abdomen and even prostate (as earlier discussed).

Chapter 5: Conclusions, future work and recommendations

Cyclotron produced radioisotopes are currently used for imaging lung cancer, although it cannot yet differentiate, for example in a single pulmonary lesion, between a tumour and tuberculosis. Consequently, imaging can only be done close to cyclotrons, limiting the availability of imaging as well as the availability of various cyclotron radiotracers. Therefore there was a need for the formulation of an in-house prepared kit for labelling NOTA-RGD with ^{68}Ga eluted from a $^{68}\text{Ge}/^{68}\text{Ga}$ generator for imaging integrin $\alpha_v\beta_3$ expression in lung cancer.

We set out to formulate an in-house prepared cold kit after the imported NOTA-SCN-Bz-RGD cold kits failed to show consistent radiolabeling with ^{68}Ga eluted from the $^{68}\text{Ge}/^{68}\text{Ga}$ iThemba LABS generator, distributed by IDB Holland bv. We have completed formulation of cold kits, radiolabeling and purification standard operating procedures (SOPs) successfully in productions of 30 and 60 μg batches. SOPs for kit formulation and radiolabeling with purification can be found in the attached annexures A and B respectively.

The synthesis involved raw peptide material from Futurechem, Korea. Seoul National University's radiolabeling steps with regards to radiolabeling, heating of the product as well as purification were followed, although different kit formulation and purification methods were implemented. HPLC results were compared with ITLC done at Necsa in order for correct ITLC results to be known when working in a clinical environment where only ITLC is available.

HPLC results were compared with ITLC results done at Necsa in order for correct procedure and results to be found for implementation at Steve Biko Hospital.

This study succeeded in meeting the initial needs as stated below:

A new NOTA-RGD cold kit was formulated for labelling with gallium-68 eluted from a 0.6 M generator and a SOP was written for kit formulation (Annexure A). A radiolabeling and purification method was formulated to label and then clean all free gallium-68 and other impurities from the radiolabelled tracer and a SOP was written (Annexure B). Mice xenografts were performed and the biodistribution between healthy and tumour bearing mice compared. The ^{68}Ga -NOTA-RGD that was labelled with the in-house prepared

cold kit showed uptake in the tumour of the tumour bearing mice that was inoculated with A 459 cells. The radiotracer also showed a higher uptake in the tumour than in the muscle and showed fast blood clearance and excretion through the kidneys and then the bladder. Three vervet monkeys were scanned and the biodistribution of the ^{68}Ga -labelled radiotracer examined. Normal radiotracer uptake and also fast blood clearance and excretion through the kidneys and bladder were obtained with no adverse reactions, which make this a promising radiopharmaceutical. The advantage is that cheaper, faster than cyclotron produced radiotracers, can be produced, which are more readily available as a positron emitter for imaging lung cancer and integrin $\alpha_v\beta_3$ expression tumours.

Therefore this new in-house cold kit for labelling with a 0.6 M $^{68}\text{Ge}/^{68}\text{Ga}$ generator has been tested from molecule to mouse to monkey and in the near future will be tested in man.

This study may in future have a major impact on guidelines and management of TB infected patients, with regard to targeted imaging and therapy. Clinical trials are still needed for this purpose. Furthermore it may facilitate the dissemination of the ^{68}Ga -labelled radiotracers approach worldwide especially in developing countries as an alternative to cyclotron produced PET tracers.

The future might include testing tracers like this on both PET-CT and PET-MRI which could have an advantage of combining high MRI spatial resolution and PET's high soft tissue contrast to obtain physiological information. Due to lack of resources, it would be an option to contact the raw product supplier, Futurechem, regarding future possibilities of qualifying a cGMP kit. Integrin expression comparison with PET.CT and MR or scanning with PET.MR may be utilized in the future.

*“The modern $^{68}\text{Ge}/^{68}\text{Ga}$ generators have proved to be a milestone for non-invasive state-of-the-art PET/CT imaging. **After that there was no looking back for Ga-68 imaging**”, (Shukla and Mittal, 2013).*

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Annexure A

Manufacturing work instruction for NOTA-cyclic RGDyK Kit production

DOC No: RC-NOTA RGD-WIN-11002

Revision: 0

Date: January 2014

Document Approval:

	Name	Signature	Date
Prepared By:	J Wagener/(I Schoeman)		
Checked By:			
Approved By:	J R Zeevaart		

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1. QA Records	2. JR Zeevaart
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Manufacturing work instruction for NOTA-cyclic RGDyK Kit production

DOC No: RC-NOTA RGD-WIN-11002

Revision: 0

Date: 2014-01-14

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1 Purpose

This document defines and specifies the manufacturing procedures applicable to the pharmaceutical product NOTA-cyclic-RGDyk(Futurechem).

2 Scope

This procedure describes the synthesis of NOTA-cyclic-RGDyk(Futurechem) manufactured kits by Radiochemistry, Necsa.

3 Abbreviations and definitions

NOTA-RGD	NOTA-cyclic-RGDyk
RPO	Radiation Protection Officer
PPE	Personal Protective Equipment
RSP	Responsible Pharmacist

4 Reference

The following documents had a direct influence in the process and activities defined in this document:

- 1.COA Futurechem manufacturers of NOTA-RGD
2. Jeong J.M, Hong M. K,Chang Y.S, Lee Y, Young J.K, Cheon G.J, Lee D.S, Chung J, Lee, M.L, Preparation of a promising Angiogenesis PET Imaging Agent: ⁶⁸Ga-Labeled c(RGDyK)-Isothiocyanabenzyl-1,4,7-Triazaclononane-1,4,7-Triatetic Acid and Feasibility Studies in Mice. The Journal of Nuclear Medicine, 2008, 49(5):830-836.
3. Jeong J.M et al, Formulation and Preparation of ⁶⁸Ga-NOTA-RGD for Angiogenesis Imaging. Seoul National University:(powerpoint).

5 Responsibilities

1. Responsible Scientist is responsible for:
 - a) carrying out the synthesis as detailed in this instruction and for completion of the Batch record (**RC-NOTA RGD-WIN-11002**)
 - b) overall performance of the QC procedures, including the label checking.
2. Responsible Pharmacist is responsible for:
 - a) checking of QC results;
 - b) final release of product.
3. QC staff are responsible for:
 - a) QC testing of the product according to the appropriate work instructions.

6 Safety

6.1 Prerequisites

- i) Each scientist assigned to manufacture this product must be suitably trained to perform the synthesis and be informed of relevant regulations.

6.2 PPE

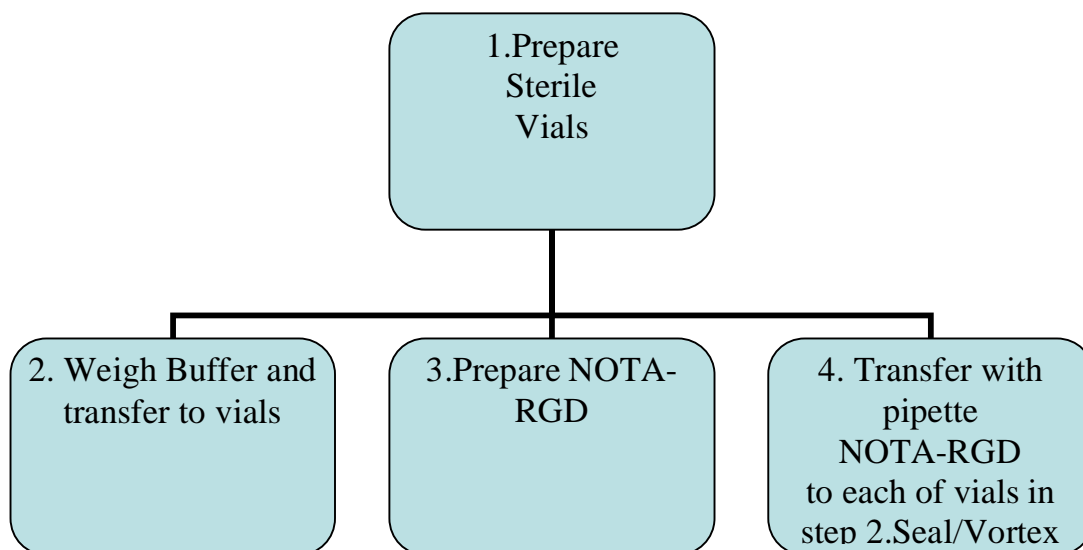
- i) Wear a clean room suit, powder free gloves, and overshoes during synthesis.

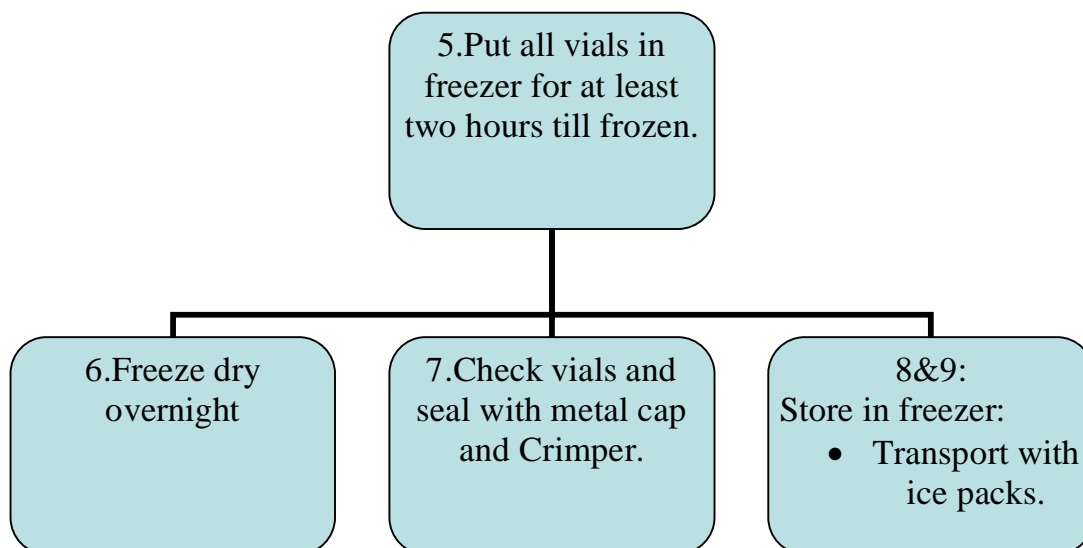
6.3 Precautions

- i) New materials entering the clean room must be cleaned and then wiped with 70% alcohol before and after pass-through.

7 Method

7.1 Flow chart for kit production of RGD-NOTA: According to SOP step numbers:





Equipment and materials

Instruments:

Operate all equipment as described in manuals or SOP

- a) Sterile vials.(10 ml rubber seal)
- b) Crimper for cap sealing.
- c) Balance(for weighing buffer)
- d) Spatula:small(for use while weighing buffer)
- e) Filtration apparatus (filter paper to work on during buffer measurements)
- f) Vortex
- g) 100-1 000µl pipette and tips
- h) 10-100µl pipette and tips
- i) Labeling device to print etiquettes
- j) Forceps(to put rubber seal caps back on sterile vials)
- k) Freezer
- l) Freeze drier

Chemicals/Raw materials:

- m) NOTA-RGD Powder/Aliquots(if previously prepared)
- n) Suprapure water
- o) Buffer:Sodium acetate tryhidrate

- p) 10-100 μ l pipette and tips

7.2 Preparation of NOTA-RGD Kits

- 1 Prepare e.g. 10 sterile vials on filtration paper in laminar flow cabinet.(Unsealed rubber)
- 2 Use the small spatula and weigh on the sensitive Balance for each vial 147 mg Buffer(Sodium Acetate Tryhidrate).NB each time use forceps to put rubber seal cap back on each vial.
- 3 Prepare the RGD-NOTA:Use 100 μ l/100 μ g suprapure water/NOTA-RGD:
Therefore use the 100-1 000 μ l pipette to transfer 1 000 μ l suprapure water to 1000 μ g NOTA-RGD and vortex(500 μ l to the current 500 μ l aliquots).
NB: Visually: make sure clear transparent like water and no more white powder visible. Therefore CLEAR.
- 4 Transfer NOTA-RGD to each of the buffer containing sterile vials:
Use the 10-100 μ l pipette and transfer 60 μ l(therefore 60 μ g) to each of the 10 prepared vials and seal only with rubber cap.
NB: Little liquid with a lot of salt but VORTEX till this forms a thicker' liquid and the salt has a clearer dissolved appearance if not fully dissolved. Make sure all is on bottom of the vial still.
- 5 Freezer: Put vials in freezer for about two hours. Check that solvent is solid and frozen before putting in the freeze drier.
- 6 Freeze dry overnight (rubber caps positioned also for freeze dryer so not yet fully sealed with metal cap).
- 7 Check that all vials freeze dried and then seal with Crimper and metal cap.
- 8 Store in freezer.
- 9 Transport with ice pack.

8 Records

All comments concerning this preparation must be noted on the Batch Record.

All records are kept by Radiochemistry on BPMS system according to Procedure Control of Records.

No.	Record	Responsibility	Duration	Location
1	Batch processing record (RC-NOTA RGD-WIN-11002)	Responsible Scientist	5 years	RC-BPMS system
2	Quality control specification and analysis sheet ((RC-NOTA RGD-WIN-11002)	QC Officer	5 years	RC-BPMS system

Annexure B

**Manufacturing work instruction for
NOTA-cyclic RGDyK Kit labelling
with Gallium-68 eluted
from ⁶⁸Ge/⁶⁸Ga generator
and purification**

DOC No: RC-NOTA RGD-WIN-11002

Revision: 0

Date: January 2014

Document Approval:

	Name	Signature	Date
Prepared By:	J Wagener/(I Schoeman)		
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Manufacturing work instruction for NOTA-cyclic RGDyK Kit labeling with Gallium-68 eluted from ⁶⁸Ge/⁶⁸Ga generator and purification

DOC No: RC-NOTA RGD-WIN-11002

Revision: 0

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8.2 Preparation of ⁶⁸ Ga-NOTA-RGD Kits and purification	7
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1 Purpose

This document defines and specifies the labeling and purification procedures applicable to the pharmaceutical product NOTA-cyclic-RGDyK(Futurechem) for labeling with Gallium 68 eluted from the $^{68}\text{Ge}/^{68}\text{Ga}$ generator.

2 Scope

This procedure describes the labeling of NOTA-cyclic-RGDyK(Futurechem) manufactured kits by Radiochemistry, Necsa and Gallium-68 therefore ^{68}Ga -NOTA-RGD as well as the purification method.

3 Abbreviations and definitions

NOTA-RGD	NOTA-cyclic-RGDyK
RPO	Radiation Protection Officer
PPE	Personal Protective Equipment
RSP	Responsible Pharmacist
^{68}Ga	Gallium-68

4 Reference

The following documents had a direct influence in the process and activities defined in this document:

1. COA Futurechem manufacturers of NOTA-RGD
2. Jeong J.M, Hong M. K, Chang Y.S, Lee Y, Young J.K, Cheon G.J, Lee D.S, Chung J, Lee, M.L, Preparation of a promising Angiogenesis PET Imaging Agent: ^{68}Ga -Labeled c(RGDyK)-Isothiocyanabenzyl-1,4,7-Triazaclononane-1,4,7-Triatetic Acid and Feasibility Studies in Mice. The Journal of Nuclear Medicine, 2008, 49(5):830-836.
3. Jeong J.M et al, Formulation and Preparation of ^{68}Ga -NOTA-RGD for Angiogenesis Imaging. Seoul National University: (powerpoint).
- 2.? As jy nog references benodig van tesis

5 Responsibilities

1. Responsible Scientist is responsible for:
 - a) carrying out the synthesis as detailed in this instruction and for completion of the Batch record (**RC-NOTA RGD-WIN-11002**)
 - b) overall performance of the QC procedures, including the label checking.
2. Responsible Pharmacist is responsible for:

- a) checking of QC results;
 - b) final release of product.
3. QC staff are responsible for:
- a) QC testing of the product according to the appropriate work instructions.

6 Safety

6.1 Prerequisites

- i) Each scientist assigned to manufacture this product must be suitably trained to perform the synthesis and be informed of relevant regulations.
- ii) Request for irradiation and transport records must be checked to ensure all the required information has been supplied.
- iii) Reagents used in this synthesis should be prepared beforehand and stored appropriately.
- iv) The Labeling laboratory used in the production process must be clean and free of any items associated with any previous work procedures.
- v) All precautions and safety instructions have been read and understood.

6.2 PPE

- i) Wear a clean room suit, powder free gloves, and overshoes during synthesis.
- ii) Use electronic personal dosimeter to monitor possible radiation exposure after production.

6.3 Precautions

- i) New materials entering the clean room must be cleaned and then wiped with 70% alcohol before and after pass-through.
- ii) All production activities must be performed behind lead shielding.
- iii) RPO shall monitor radioactive starting material before entering the facility and product or any items before exiting the facility for radioactive contamination.

6.4 Housekeeping

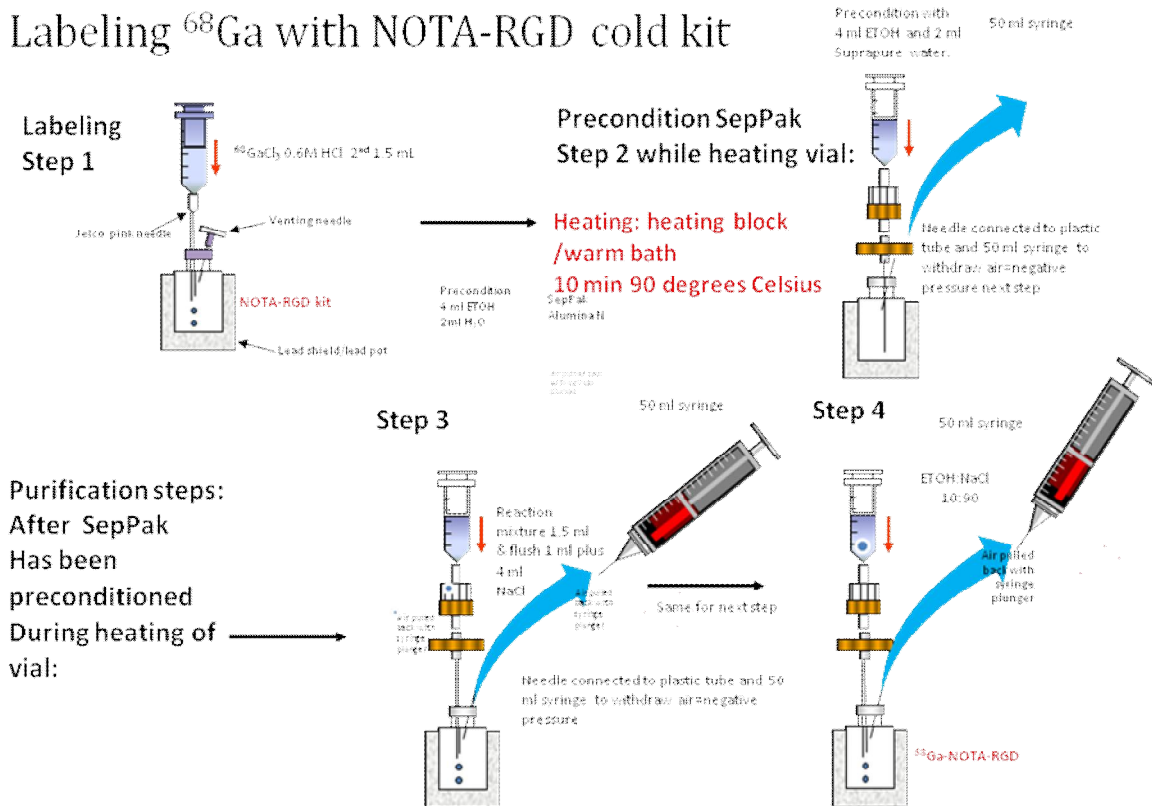
- i) Remove all items used during synthesis after RPO monitoring.

7 Method

7.1 Flow chart for NOTA-RGD kit with Gallium-68 and Purification:

Labeling ^{68}Ga with NOTA-RGD cold kit

Labeling ^{68}Ga with NOTA-RGD cold kit



Equipment and materials

Instruments and raw materials:

Operate all equipment as described in manuals or SOP

Step 1:

Radiolabelling:

- a) $^{86}\text{Ge}/^{68}\text{Ga}$ generator elute.
- b) Disregard the first 1.5 ml.
- c) Add second second 1.5 ml to the NOTA-RGK Kit vial by using the plastic of the jelco needle 22 G.
- d) Vortex 30 seconds. Liquid should be clear.
- e) Boil at 90 Degrees Celsius for 10 minutes.
- f) Test pH. Measure radio activity.
- g) Do Purification step 2-4.

Purification Step 2-4

- a) SepPak Alumina N (500 cc)
- b) ETOH
- c) Suprapure water
- d) 2 sterile vials
- e) Decrimper
- f) Pippet/2 x 2 ml syringes
- g) 1x 5ml syringe (use for step 2 saline rinsing)
- h) 10 ml EtOH:Saline :10:90 (10 ml ensures enough for a second labeling)
- i) Forceps medium for vial transfer when need to measure
- j) 50 ml syringe with plastic connecting tube (same as nebulizer connecting tube)
- k) Two needles: 25 Gauge for connecting to connecting tube and 50 ml syringe
- l) 23 gauge for connecting to sepPak
- m) Saline
- n) $^{68}\text{Ge}/^{68}\text{Ga}$ generator (iThemba labs: IDB Holland)

7.2 Preparation of ^{68}Ga -NOTA-RGD Kits and purification

Step 2:

Precondition the sepPak that is connected to needle inserted in the one sterile empty vial. Attach the 50 ml syringe to the 25 gauge needle and also connect to the same vial. Pull syringe plunger to withdraw air and precondition the sepPak with 4 ml EtOH and 2 ml water.

Discard the water and use the same vial and keep connected to sepPak and 50 ml syringe.

Step 3:

Now transfer the reaction mixture (1.5 ml) onto the sepPak with a pipette or syringe (without the needle).

Then rinse the empty vial with 1 ml saline and also transfer onto sepPak and let it run through the column again by pulling plunger on 50 ml syringe.

Stop transfer if need by disconnecting syringe from tube. This should not yet run dry!

Now also rinse another 4 ml saline and still keep the column wet.

Step 4:

NB! Now use a new sterile empty vial (the second vial) and connect the sepPak as well as 50 ml syringe to this vial.

Transfer 3 ml of the 10 % EtOH in EtOH:Saline solution and let this run dry.

Do ITLC by using Citric Acid as well as Sodium Carbonate as reaction mixtures.

8 Records

All comments concerning this preparation must be noted on the Batch Record.

All records are kept by Radiochemistry on BPMS system according to Procedure Control of Records.

No.	Record	Responsibility	Duration	Location
1	Batch processing record (RC-NOTA RGD-WIN-11002)	Responsible Scientist	5 years	RC-BPMS system
2	Quality control specification and analysis sheet ((RC-NOTA RGD-WIN-11002)	QC Officer	5 years	RC-BPMS system

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Ref: H001-09

27 January 2009

ANIMAL USE AND CARE COMMITTEE

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Dr J Rijn Zeevaart
NECSA
(zeevaart@necsa.co.za)

Dear Dr Zeevaart

H001-09: UBI labeled with Ga-68 and Cu-64 as infection imaging agent. Biodistribution evaluation in the normal vervet monkey model using PET-CT (O Knoesen)

The above protocol was approved by the Animal Use and Care Committee at its meeting held on 26 January 2009.

Best regards


Elmarie Mostert
AUCC Contact Person

Copy: Dr O Knoesen
Dr R Auer

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1908 - 2008



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ANIMAL USE AND CARE COMMITTEE (AUCC)

APPLICATION FOR AMENDMENT

Submission Date		For Administrative Purposes	Project No.	
AUCC approval Date			Signature (only on approval)	

TITLE	H001-09; UBI labeled with Ga-68 and Cu-64 as infection imaging agent. Biodistribution evaluation in the normal vervet monkey model using PET-CT		
AMENDMENT	Including of another peptide (RGD) in the mention protocol		
RESEARCHER	Dr JR Zeevaart	SIGNATURE	

This application form is for obtaining prior approval by the AUCC for deviations from the approved application for any desirable or necessary significant changes that may need to be made in the methods and procedures used which may affect the welfare of the animal subjects.

A departure from the approved application that was necessitated by an urgently required response due to a clinical situation to an individual animal is not considered a significant change as long as it remains a once-off event. *A report shall however be submitted to the AUCC for noting.*

Significant changes: any act or omission that will increase the impact a procedure or method will have, or is expected to have, on the welfare of animals. This includes, but is not limited to, any increase in animal numbers, any increase in the severity of procedures, any increase in the duration of holding and usage time of the animals, converting terminal surgery to survival surgery, etc. Furthermore, any changes that may decrease the statistical power of an experiment are also considered significant changes.

1. Explanation and justification

(Explain in detail why the proposed amendment is required, what events led up to the decision and justify the amendment against the principles of Replacement, Reduction, and Refinement.)

The approved protocol (H001-09) makes provision to test Ga-68 or Cu-64 labelled UBI on three monkeys with a repeat after 6 weeks. UBI is a peptide that is selective for infection. The synthesis of this peptide has however been problematic and the labelling yields in the laboratory have not been adequate. The synthesis project has been restarted but it is not expected to provide a peptide ready for injection soon. In the meantime another peptide has been acquired that can also be labelled with Ga-68 or Cu-64. It is the RGD based peptide. Radiolabeled Arg-Gly-Asp (RGD) and bombesin (BBN) peptide analogs have been extensively investigated for the imaging of tumor integrin alpha(v)beta(3) and gastrin-releasing peptide receptor (GRPR) expression, respectively. Increased expression of $\alpha v\beta 3/\alpha v\beta 5$ integrin is involved in angiogenesis and the inflammatory process in atherosclerotic plaques. The novel ^{68}Ga -RGD peptide binds with high affinity to $\alpha v\beta 3/\alpha v\beta 5$ integrin. The ^{68}Ga -RGD peptide has been extensively investigated (in various animal models as well as humans) in atherosclerotic plaques. The peptide is available in kits which facilitates the labelling process and ensures the quality of the labelling. Kits also ensure that inter institute differences in results are limited. The intended study in SA with RGD forms part of a wider international testing which aims to test its clinical application in humans. Animal test are required before an application for human clinical trials is made.

Partly due to the problems experienced with the UBI Dr Knoesen is no longer involved in the project and has other responsibilities at NTP. Dr Thomas Ebenhan will take over this responsibility. He is a postdoc from Germany and has worked at Novartis in Switzerland running their radioactive animal testing programme and has intimate knowledge of both the technical and regulatory issues involved with testing of radiolabelled compounds in animals.

S4560/09

Replce S4556/09 and S4282/08

2. **Proposed amendment**

(State in detail the proposed amendment. Refer to the appropriate sections in the previous protocol. Should the amendment affect any section of the original application that requires signatures of the relevant authorised persons, such consent and signatures shall be provided. In case of extensive amendments, consider submitting a revised proposal.)

The approved protocol (H001-09) makes provision to test Ga-68 or Cu-64 labelled UBI on three monkeys with a repeat after 6 weeks, therefore six animal studies in total. Herewith it is requested that 3 monkeys tests will be reserved to test Ga-68 labelled RGD and three for Ga-68 or Cu-64 labelled UBI and that no repeat tests will be possible.
The principle investigator will change from Dr O Knoesen to Dr T Ebenhan.