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Validation of a novel radiopharmaceutical according to EU GMP requirements at Turku PET Centre

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MDP in Biomedical Sciences, Drug Discovery and Development
Master's thesis

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Abstract

Radiopharmaceuticals are essential in Positron Emission Tomography (PET) imaging that is used for diagnostic and research purposes. Radiopharmaceuticals contains a biologically interesting molecule labelled with appropriate radionuclide that allows detection with a PET device. Radiopharmaceuticals have many characteristics that make them different from other conventional pharmaceuticals. Mainly the molar amount of the substance is significantly lower, so a radiopharmaceutical has no pharmacological effect. In addition, the half-life of the radionuclide is usually short (~2-110 min) and this limits their transport for off-site use or commercialization on a large scale. Indeed, many of the radiopharmaceuticals are produced in-house for direct use.

European Medicines Agency (EMA) and national competent authorities like the Finnish Medicines Agency (FIMEA) in Finland supervice the manufacture and use of radiopharmaceuticals. In addition, the Finnish law and the Radiation and Nuclear Safety Authority (STUK) regulate the use of radioactive material.

EMAs general comprehensive guidance on Good Manufacturing Practices (GMP) from which e.g. Annex 1: “Manufacture of Sterile Medicinal Products” (updated in 2023) and Annex 3: “Manufacture of Radiopharmaceuticals” guide the production of radiopharmaceuticals. However, there is a need for more practical interpretation. Thus, the Radiopharmacy Committee of the European Association of Nuclear Medicine (EANM) has provided interpretation and opinion of experts in the field on current Good Radiopharmacy Practice (cGRPP) for small-scale preparation of radiopharmaceuticals. Also, the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) provides more interpretation.

Currently, Turku PET Centre is in the process of setting up and validating the production of a novel [^{18}F]fluoride labelled Exendin-4 based radiopharmaceutical. Exendin-4 is a Glucagon Like Protein-1 (GLP-1) receptor agonist which makes it a suitable marker to study e.g. pancreatic beta cells.

This project is carried out in Turku PET Centre Radiochemistry Laboratory and the aim is to establish the production of the novel radiopharmaceutical in compliance with EU GMP requirements. The process includes the validation of the analytical methods and the production process. The preclinical evaluation of the novel pharmaceutical is also performed before clinical studies. However, in this thesis we will be focusing on the validation of the analytical methods which objectives are to show that the developed analytical methods are appropriate for the intended use and purpose.

According to the guidelines we must confirm that the identity, concentration, quality and purity of the radiopharmaceutical are sufficiently characterized. Thus, our aim is to develop analytical methods that are specifically suited to our radiopharmaceutical and that meet GMP requirements. The methods can be qualitative or quantitative and radioHPLC is one of the relevant technics used and validated during this project. Guidelines will be studied and used to draw up and implement a Validation Plan for the analytical methods dedicated for the radiopharmaceutical.

Key words: GMP, PET, Radiopharmaceutical, validation, analytical method

1 Introduction

Positron Emission Tomography (PET) is an area of medicine that uses very small amounts of radioactive drugs also called radiopharmaceuticals, to examine and image body functions non-invasively. These radiopharmaceuticals are used in diagnostic and research purposes of cancer or metabolic diseases for instance. Radiopharmaceuticals are usually prepared in-house for immediate use, while some of them can be also purchased from external commercial vendors. There are two main reasons why they are mostly prepared in-house. Primarily due to the rapid half-life of the used radionuclides and hence the short shelf life of the product that limits the transport possibilities. Secondly, because of their specificity and limited use they are used more for research purposes. Indeed, most of the commercialized radiopharmaceuticals today, such as those for prostate cancer applications, are commercially viable because of their wide need. However, there are innovative and novel radiopharmaceuticals that are developed in-house, for example, in academic institutions such as universities or university hospitals that usually remain uncommercialized due to lack of profitability. (Hendrikse et al., 2022)

The fundamental idea behind PET is to simultaneously detect a pair of gamma rays produced by positron annihilation events from a radioactive pharmaceutical injected into a patient or study subject prior to the imaging as illustrated in Figure 1. (Rong et al., 2023). These radioactive pharmaceuticals are similar to ordinary biological molecules such as small molecules, peptides, or proteins; they are just labelled with positron emitting radionuclides. [^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG) as an example is created by replacing the OH-group of glucose to ^{18}F-fluoride. (Jiang et al., 2019) In this project we are working with ^{18}F-labelled Exendin-4 which is based on natural peptide originally obtained from Gila monster (Heloderma suspectum) (Khai et al., 2018). Turku PET center is currently finalizing its [^{18}F]exendin-4 project and will move on to clinical trials once all validations are approved. Therefore, this work will respect the confidentiality issues of the project and will not disclose all the information of the novel radiopharmaceutical. The ethical and confidentiality issues are discussed more in the discussion section of this thesis.

This thesis focuses on investigating what requirements Good Manufacturing Practice (GMP) brings to the validation process of our novel radiopharmaceutical. The aim is to build the production of our novel radiopharmaceutical in compliance with European Union (EU) GMP requirements. The validation process of a new radiopharmaceutical involves monitoring the analytical methods, which we will discuss in detail later in this thesis, as well as the

preclinical evaluation and the validation of the production process, which we will briefly describe here in the Introduction section.

1.1 PET imaging

PET is a quantitative imaging technique that enables the evaluation of both physiological and pathological processes in the living body. This technology uses radiopharmaceuticals in various applications such as to characterize organ functions, to measure various metabolic processes and to study receptor activities *in vivo*. (Alqahtani, 2023) Thus, PET can provide information about metabolic activity, chemical composition and blood flow for example. PET imaging enables dynamic real-time monitoring of radiopharmaceutical distribution and kinetics in vivo, allowing quantitative assessment of tracer uptake, receptor binding, and washout over time. Now depending on the area of the body or activity that is studied one can choose a suitable radiopharmaceutical that is distributed and accumulated in different tissues based on their individual affinities. In the images, a radiopharmaceutical appears as areas of signal intensity that reflect the distribution and accumulation of the radioactive tracer based on its biological target or metabolic activity. (Kapoor et al., 2025)

Radiopharmaceuticals can be given orally, inhaled but mostly via injection. After which it is given time to distribute throughout the body. To start the imaging the patient is slid through the central opening of the PET scanner where the detection happens. Here the position of the patient depends on the area to be scanned. The correct position of the patient is important for the imaging and in some cases, even breath-holding may be required for moments. One imaging session can last from 30 minutes to an hour. (Kapoor et al., 2025)

Today often computed tomography (CT) or magnetic resonance imaging (MRI) are taken at the same time to better localize the masses being imaged. (Kapoor et al., 2025) PET systems have advanced quickly since the first commercial ones that were introduced in 1976. Recently new PET/CT system that can image total-body at once was presented to the market. This advanced system allows high-resolution total-body imaging even in ultra-low radiopharmaceutical dose. It provides total-body dynamic scan that helps to explain multi organ normal human physiological processes as well as systemic diseases in very fast imaging speed. (Sun et al., 2024)

Like any other imaging technique PET has its own challenges. One issue is low anatomical accuracy without fusion with CT or MRI (Vaquero & Kinahan, 2015). Another thing is the high cost as it requires expensive development and production of tracers. This includes

positron-emitting isotopes that are often produced with a cyclotron in tight timing besides technically sophisticated infrastructure and devices. (Alqahtani, 2023) Although the property of quantitative results is an essential strength of PET imaging, it has some challenges. Mainly the quantitative accuracy of the measurements can be affected by both the physical effects relating to the interaction of the released annihilation photons with matter and the detectors, as well as the performance of the PET system acquisition hardware and picture reconstruction software. (Zaidi & Karakatsanis, 2017)

PET imaging has still a remarkable sensitivity at the molecular level to variations in the metabolic and biological activities. To improve it further, in order to have a multimodal imaging system that takes advantage of combining both functional and anatomical images, PET can be integrated with other techniques such as CT and MRI. (Jiang et al., 2019) In recent years, efforts are also being made to use artificial intelligence (AI) in PET imaging. In particular how machine learning and deep learning can for example improve and enhance information analyses, tumour segmentation, classification as well as survival and treatment prediction (Vazoura et al., 2024). AI has enabled and been particularly useful in whole-body PET imaging, both in terms of image reading and technological aspects (Seifert et al., 2021).

For comparison, it is worth mentioning Single-photon emission computed tomography (SPECT) which is a widely used nuclear imaging technique. SPECT uses gamma-emitting radiotracers and a revolving gamma camera to collect radiation from different angles, after which the computer reconstructs a three-dimensional image of the body's functional processes. When compared to PET imaging SPECT is more cost-effective and thus more widely available. However, SPECT has lower spatial resolution and therefore is not always suitable for precise diagnostic purposes. (Yandrapalli & Puckett, 2022)

Today PET imaging is widely used in clinical settings and drives healthcare towards better and more accurate diagnostics. All major areas of human health are represented in current clinical and research applications such as neurology, cardiology, infection, inflammation and especially oncology. Tumors are characterized by an increased consumption of glucose compared to other healthy tissues. This feature, often called as Warburg effect, is exploited in PET when using FDG. Actually, one of the most widely used radiopharmaceuticals worldwide is FDG. (Basu et al., 2014)

PET imaging primarily includes short lived positron-emitting isotopes such as cyclotron-produced ^{18}F, ^{15}O, ^{11}C or ^{13}N as well as generator-produced ^{68}Ga or ^{82}Rb. Usually, proton irradiation of natural or enriched targets are used to produce the isotopes. Naturally every

radionuclide has its own properties such as half-life that can vary from a few minutes to 110 minutes approximately for the most commonly used nuclides. Also, positron energy that affects the resolution of PET images can vary from nuclide to another. The higher the energy of the positron is, the longer the distance it travels in the body (known as time of flight, TOF) before it collides with the electron and annihilation occurs. During this distance TOF the positron deviates from its original position, so the origin of the annihilation radiation is not exactly the position of the radionuclide. When an electron and a positron combine, the two opposing charges annihilate one another, producing two gamma rays (annihilation photons) of each 511 keV that are released in opposite directions at around 180° from one another as shown in Figure 1. Those two annihilation photons are detected and registered by opposing PET detectors in the round PET scanner where it is later interpreted as an image. (Basu et al., 2014)

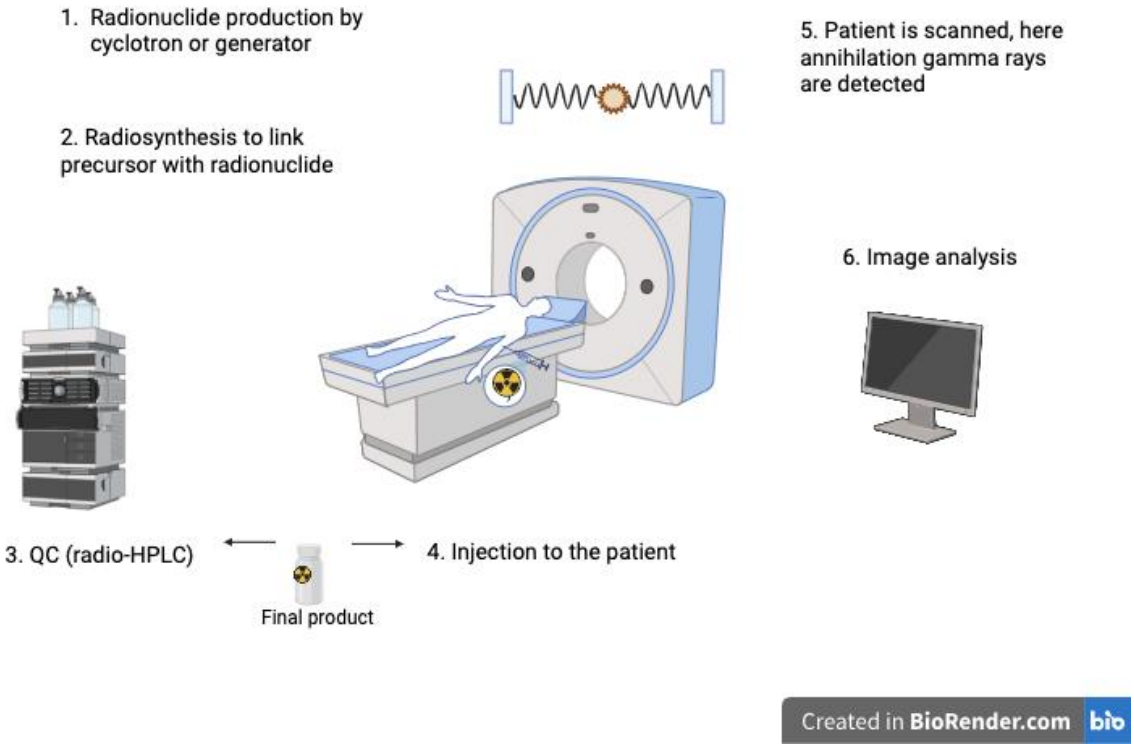

Figure 1. Principle of PET imaging. Modified from (Rong et al., 2023) Created in BioRender.com

1.2 Exendin-4

Glucagon-Like Peptide-1 (GLP-1) is a natural hormone secreted by human body, precisely by intestine, after eating. GLP-1 belongs to a group of incretins, which means it stimulates insulin secretion. It also inhibits glucagon secretion and slows down gastric emptying. In

practice, GLP-1 is the body's own signaling chemical that regulates blood sugar and appetite. It binds to the Glucagon-Like Peptide-1 Receptor (GLP-1R), which is found not only in pancreas but also in brain and lungs. Exendin-4 is a natural peptide that acts like GLP-1. Exendin-4 is a GLP-1R agonist, meaning it mimics the actions of GLP-1 but has its own characteristics that makes it beneficial. Namely, it takes much longer to degrade compared to the human GLP-1 peptide, making it more long-acting. It also binds very strongly to GLP-1R, which makes its effects more durable and effective. (Mann et al., 2010) This combination ensures a mimic that is used today as a treatment for type 2 diabetes mellitus and obesity (Deden et al., 2021). Labelled Exendin-4 has shown promise to be used as an imaging and diagnostic tool for diseases such as insulinoma and neurodegenerative diseases including Alzheimer's and Parkinson's (Shah et al., 2021; Verma et al., 2024).

Millions of people around the world suffer from neurodegenerative diseases for which there is no curative treatment available yet and a real unmet need. They are characterized by complex pathological processes such as a steady decline in selectively sensitive neurons. Today there is growing interest in the potential use of different peptides as neuroprotective treatments. Furthermore, a number of preclinical studies have documented the neuroprotective benefits of Exendin-4 as a potential therapeutic agent in the treatment and management of diseases such as Parkinson's and Alzheimer's. (Verma et al., 2024)

Insulinoma is a rare functioning neuroendocrine tumor that develops from pancreatic islet cells. Usually, the tumor secretes excess insulin which results in hyperinsulinemia that can cause hypoglycemic episodes typically in periods of fasting. The annual incidence of insulinomas ranges from 1 to 32 instances per million people, with a little female bias. The primary treatment for insulinomas is surgical resection and precise preoperative localization is essential, which is why imaging techniques play a major role in the planning of the treatment due to the small size and sometimes challenging location of the tumor. (Treiber & Igaz, 2025) Compared to other anatomical or functional imaging GLP-1R based emerging modality has demonstrated a higher sensitivity for insulinoma localization (Shah et al., 2021). Exendin-4 imaging, which is more sensitive than morfostructural imaging, provides a sensitive functional imaging tracer in both SPECT and PET applications, particularly when there is a suspicion of benign insulinomas that are not accessible by endoscopic ultrasound. Studies have been carried out mostly with [^{68}Ga]exendin-4. (Sidrak et al., 2023) This thesis presents a novel approach to PET imaging insulinoma in pancreas using [^{18}F]exendin-4.

1.2.1 [^{68}Ga]exendin

Radiopharmaceuticals that include ^{68}Ga nuclide are produced primarily by fully automated radiochemical synthesis and sourced from a commercial generator (Menon et al., 2024). Today ^{68}Ga labelled Exendin is in clinical use and studies have demonstrated its superiority in a unique prospective comparison with previous imaging modalities for preoperative localization of benign insulinomas. Especially since the product was improved by the use of the NODAGA chelator system, reducing the side effects of the earlier radiopharmaceutical. (Boss et al., 2024)

However, since the positron energy of the PET nuclides can affect the digital PET/CT scan's spatial resolution and image quality despite them having comparable count rates, can this influence the diagnostics. Now ^{68}Ga nuclide has relatively higher positron energy that causes longer tissue penetration (~3.5 mm), which reduces image resolution, especially in small lesions ≤ 13 mm in diameter. PET study pictures of ^{68}Ga labelled radiopharmaceuticals showed poorer spatial resolution and image quality than ^{18}F labelled ones. ^{18}F nuclide also allows longer imaging time compared to ^{68}Ga due to its longer half-life (~110 min. vs. ~68 min.). (Braune et al., 2022)

1.2.2 [^{18}F]exendin

Imaging pancreatic β cells with exendin-based tracers has been attempted on multiple occasions. Some may carry radiometals, such as ^{64}Cu and ^{68}Ga for PET imaging or ^{99}mTc and ^{111}In for SPECT imaging. However, in PET imaging of pancreas, a major obstacle has been the high accumulation of radiopharmaceuticals in the kidneys. (Jansen et al., 2019)

A radiopharmaceutical developed at the Turku PET Centre, namely ^{18}F-labelled [Nle^{14},Lys^{40}] exendin-4, is used in this thesis as an example for validation process. In a study done by Mikkola et al. [^{18}F]exendin-4 showed rapid renal clearance *in vivo* in rats and specific uptake in human pancreas tissue *in vitro*. Additionally, the study showed specific and sustained uptake in rat pancreatic islets. (Mikkola et al., 2016) Taking into account that ^{18}F positron energy is relatively low that leads to shorter tissue penetration (~0.6 mm) [^{18}F]exendin-4 makes a viable candidate as a radiopharmaceutical for imaging pancreas (Braune et al., 2022).

To date, there is no [^{18}F]exendin-4 based radiopharmaceutical in clinical use that target GLP-1R, although there has been increasing research into them (Kiesewetter et al., 2011). Outside the EU, in Japan 2021, was published the first phase 1 clinical trial evaluating the safety and utility of [^{18}F]exendin-4 imaging for visualising pancreatic GLP-1R. This first-in-human

study involved six healthy male volunteers who were given either a 37 MBq or 74 MBq dose of the radiopharmaceutical. The study showed promising results and more clinical trials are expected to be conducted in the EU as well. (Fujimoto et al., 2021)

1.3 Good Manufacturing Practice

At the beginning of the 1970s, there was already a need for a systematic approach that aimed to avoid surprises and mistakes as much as possible in the development and production of pharmaceuticals (Boersma, 2022). Therefore, as part of quality assurance, GMP system was created. Its definition is to have a system of procedures and practices that ensure that medicinal products are consistently manufactured and controlled according to established quality standards appropriate to their intended use, minimizing the risks inherent in pharmaceutical production. (EMA, 2008)

The European Medicines Agency (EMA) updates GMP guidelines to ensure that they are always up-to-date and fit for the intended purpose. EMA has published several Annexes that complement the GMP guidelines and ensure that the manufacture of different types of pharmaceuticals meets the requirements for safety and quality. Of which, as an example, Annex 3 Manufacture of Radiopharmaceuticals is central in this thesis. (*EudraLex - Volume 4 - European Commission*, n.d.)

The production process of pharmaceuticals is strictly guided in GMP. Initially GMP is designed for large-scale, commercial pharmaceutical manufacturing with strict documentation and batch release requirements. Since the special properties of radiopharmaceuticals must be taken into account in their production, specialized organizations were created, such as the European Association of Nuclear Medicine (EANM) (Gillings et al., 2021).

The Radiopharmacy Committee of EANM has developed Good Radiopharmacy Practice (GRPP) guidelines for support the operators in the field (Elsinga et al., 2010). Unlike traditional GMP, GRPP focuses on flexibility, rapid production timelines, and patient-specific needs. It follows always and maintains core GMP principles such as quality assurance, traceability, and validation but presents their own interpretation of practical implementation options in documentation, cleanroom classifications, and batch sizes based on local risk assessments. GRPP proposes practical and risk-based framework that is tailored to small-scale preparation of radiopharmaceuticals, typically within hospital or research environments. (Gillings et al., 2020, 2021)

The aim of GMP quality system is to ensure that a pharmaceutical, in this case a radiopharmaceutical, produced is of high quality, safe, effective and traceable. In particular, a risk management approach has elevated to an important role of the quality assurance, since radiopharmaceuticals have to take into use before all testing is complete. GMP covers many areas including personnel, facilities, equipment, working practices, documentation and deviations. (EMA, 2008) Table 1. below presents the main sections in GMP and notes the ones that are less relevant for radiopharmaceuticals due to their characteristics. Table 1. also presents example of key documents required under GMP.

Table 1. GMP sections and example of key documents required under GMP. *Less relevant for radiopharmaceuticals. (*EudraLex - Volume 4 - European Commission*, n.d.)

GMP document sections	Example of key documents required under GMP
Pharmaceutical Quality System	Site Master File
Personnel	Training Records
Premise and Equipment	Validation Master Plan (VMP)
Documentation	Standard operating procedure (SOP)
Production	Method Document (MET)
Quality Control	Contamination Control Strategy (CCS)
*Outsourced activities	Batch protocol/record
*Complaints and Product Recall	Investigational Medicinal Product Dossier
Self Inspection	Investigator's Brochure (IB)

All personnel employed should be competent and appropriately trained to their own job duties. This includes also short-term maintenance or cleaning personnel. (EMA, 2008) Guests are allowed only with accompanying trained personnel. Areas of responsibility and work tasks must be clearly defined and known. Continuous training of personnel in their own duties as well as GMP awareness is important. It is essential to document the training thus it is available on inspection visits for audits. (Boersma, 2022)

The manufacturing premises of radiopharmaceuticals must be independent, controlled and there should be limited access to only authorized personnel. The whole unit is divided into sections according to the tasks performed in them and their degree of cleanliness required. These can be, for example, incoming goods or their storage area, personnel areas or offices, working or production areas and so on. Despite the division of areas, a comprehensive and

continuously monitored and evaluated Contamination Control Strategy (CCS) must be implemented in the whole unit. CCS is board concept and should cover all aspects that may contribute to contamination risk. Premises, workstations and equipment must be designed, maintained and cleaned so that there is no risk for contamination or cross-contamination. CCS includes membrane filter integrity testing for aseptically filled radiopharmaceuticals. Currently, following to Annex 1 update, air filters are also tested before the radiopharmaceutical is being released for use. (EMA, 2008)

Clean rooms, for the manufacture of sterile products, can be divided into four clean rooms or grades A, B, C and D. When using closed automated hot-cell systems in production a grade C is required. Respectively, when aseptic processing or filling is performed it can be done in grade A environment such as laminar airflow hood. (EMA, 2008)

Documentation plays a fundamental role in GMP. Simply if it is not written it is not done or does not exist. Written protocols should be followed in the preparation, evaluation, approval, and distribution of all documentation pertaining to the manufacturing of radiopharmaceuticals. There must be clear labelling of materials and their properties such as degree of purity, shelf life, acceptance and release criteria. Similarly, for equipment there must be documentation on their name, properties, use, cleaning and maintenance with the details of the responsible persons involved. To ensure consistent operation, practical working instructions for activities must be documented in detailed standard operating procedures (SOP). Besides more technical Method Document (MET) describes how to perform a particular measurement or analysis. Overall, all work must be documented and traceable, including any deviations. Maintaining data integrity is a key element in GMP. The instructions issued must be updated at regular intervals and archived. (EMA, 2008) In Turku PET center SOPs and METs are updated at least every 3 years unless the need arises sooner. Documents are archived for at least 15 years and for some ones even for 50 years.

EMA has drawn up validation and qualification principles for the methods, processes, equipment and utilities used in the manufacture of pharmaceuticals. These should be well planned to consider the life cycle of the validated or qualified matter besides they should be performed by qualified personnel. The entity responsible for production can compile validation and qualification principles in a Validation Master Plan (VMP) or equivalent document. VMP should have the key elements presented and can include reference information if needed. VMP includes validation and qualification steps (see Figure 2.) that are part of the life cycle management of equipment and premises, whose proper documentation is

an essential part of GMP compliance. First the design qualification (DQ) to ensure that the design meets the defined requirements before purchase or acquisition. Second comes the installation qualification (IQ) where the aim is to confirm that the equipment and systems have been installed in accordance with the manufacturer's instructions. Next step is operational qualification (OQ) to ensure that equipment and systems operate under the specified operating conditions. Final step is performance qualification (PQ) where the aim is to confirm that equipment and systems operate consistently in the production environment. (EMA, 2015)

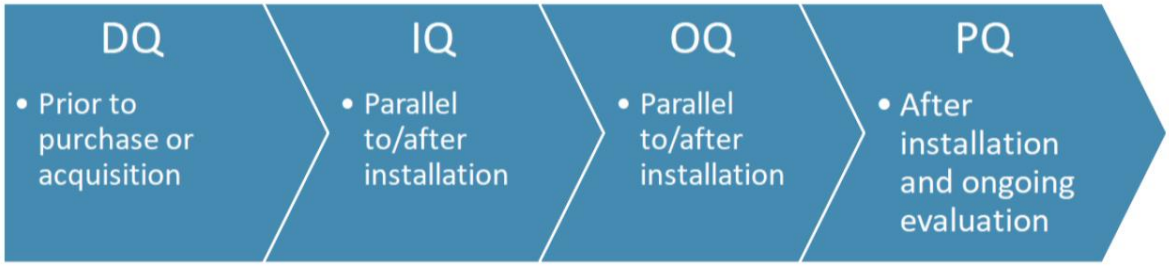

Figure 2. Validation and qualification steps according to GMP.

In terms of quality management, quality assurance (QA) and quality control (QC) are essential although they are concentrated in different areas. QC is more concerned with conventional pharmaceuticals with marketing authorization (MA) as it focuses on the testing and analyzing of the product. Thus, QC is a reactive product-oriented approach that verifies that the final product meets the required specifications. Where QA includes preventive measures to ensure that the product meets quality requirements before and during manufacture, which is more suitable to the nature of radiopharmaceuticals. Thus, QA is more of proactive process-oriented approach that ensures that quality is built into the process. (EMA, 2008)

After successful production the QA approves the batch (the radiopharmaceutical) and releases it for use by approving a written batch protocol. The QA goes through and assesses whether everything in the batch protocol has been carried out as instructed and whether the quality of the final product meets the predefined specifications. The batch protocol documents the materials used, certain parameters, for example, from the analytical method run and all the raw data. If the batch does not meet the acceptance criteria it must be rejected. When the

radiopharmaceutical is released to use it is administered directly to the patient or study object by personnel and is not distributed outside the facility. (EMA, 2008)

The European Pharmacopoeia (Ph. Eur.) is the only official and legally binding source for manufacturing and quality control standards for medicines, including some radiopharmaceuticals, in the 38 member states of the Council of Europe, which includes all EU members. It is written by expert groups and based on the specifications of approved and commonly used preparations in Ph. Eur. member states. The Ph. Eur includes both general monographs and an enormous number of individual monographs for substances for pharmaceutical use that have been approved by the Ph. Eur. Commission. (Gillings et al., 2020) Before developing a protocol for the validation it is important to check which quality references apply and whether additional quality standards are needed.

1.4 Regulation and guidance on the production of radiopharmaceuticals

Radiopharmaceuticals differ in some cases from other classical drugs, however, in principle they follow the same guidelines where applicable. Namely the main differences are the short half-life and the small amount used that avoid any pharmaceutical effect. (Gillings et al., 2021) Radiopharmaceuticals can be commercially distributed with MA as stated in European legislation, namely directive 2001/83/EC. Compared to other non-radioactive pharmaceuticals, the law is more demanding and requires MA for both ready-to-use radiopharmaceuticals placed on the market and their starting materials, such as radionuclide precursors and generators. Yet the availability of commercially distributed PET radiopharmaceuticals with MA is limited due to their unique characteristics such as very short half-life, shelf life and low financial viability. (Hendrikse et al., 2022)

Alternatively, radiopharmaceuticals can be prepared in-house for immediate own use. This is the major source of radiopharmaceuticals for routine nuclear medicine procedures that is necessary for both diagnostic and research purposes. The most obvious difference here is that in-house prepared radiopharmaceuticals without MA can only be used for private use and cannot be sold outside the establishment. Unlike the precise European legislation for commercially distributed radiopharmaceuticals with MA, in-house prepared ones have different national regulatory interpretations across Europe. (Elsinga et al., 2010)

To be able to prepare high quality radiopharmaceuticals consistently and reliably there must be comprehensively designed and correctly implemented system to ensure quality and production. EMA has drawn up Annex 1 for Manufacture of sterile medicinal products to

guide operators in the field (EMA, 2022). EMA has recently published an updated version of Annex 1 which came fully into operation in 2024. This document is the main conductor during this project. On the basis of Annex 1, EANM has proposed current GRPP for small-scale preparation of radiopharmaceuticals (Gillings et al., 2021).

In Finland, the Finnish Medicines Agency (Fimea) acts as the responsible authority by supervising and developing the pharmaceutical sector (*Fimea.Fi*, n.d.). Work towards more harmonized standards and legislation for in-house prepared radiopharmaceuticals are aimed.

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) provides references for regulatory expectations on the validation. Mainly ICH Validation of analytical procedures Q2 (R1) guides to understand and internalize the necessary requirements for the validation of analytical methods which play a central role in this thesis (ICH, 2005). This document provides a discussion of the characteristics for consideration during the validation of the analytical procedures included as part of registration applications submitted to EMA or other regulatory authorities in Japan or the United States.

All new radiopharmaceuticals should be registered in EMA's Clinical Trials Information System (CTIS) portal (EMA CTIS, n.d.). At this stage, the applicant (investigator) must submit an Investigational Medicinal Product Dossier (IMPD) to the authorities. IMPD describes the pharmaceuticals quality characteristics, manufacturing, safety and justification for use in a clinical trial. In addition, the investigator produces Investigator's Brochure (IB). This document contains all the essential information about the pharmaceutical that the investigator needs to conduct the study safely and ethically. The ICH sets out the authorities' view in Good Clinical Practice Guideline E6(R2). (ICH, 2016)

1.5 Preclinical Evaluation

EMA has instructed in the IB which non-clinical information must be included in applications for MA or clinical research (ICH, 2016). This applies to radiopharmaceuticals as well (EMA, 2018). The ICH guideline S6(R1) provide basis for preclinical safety assessment of biotechnology medicines, including radiopharmaceuticals derived from biotechnological sources (ICH, 2011). The International Atomic Energy Agency (IAEA) also provides a comprehensive interpretation and guidance for preclinical studies with radiopharmaceuticals (International Atomic Energy Agency, 2023)

Conventional drug-development regimens that rely on preclinical *in vitro* and *in vivo* research are not necessarily ideal for the clinical evaluation of radiopharmaceuticals. Therefore, target-

driven procedures that more consistently lower patient resources and decrease trial schedules may be advantageous for radiopharmaceutical drug-development processes. IMPD should specify the results that are relevant to the clinical use of the radiopharmaceutical such as toxicology, biodistribution, selectivity or specificity for example. (International Atomic Energy Agency, 2023) EANM has provided guideline for the preparation of IMPD. This aims to walk radiopharmaceutical investigators through the practicalities of preparing an IMPD. (Todde et al., 2014)

A promising preclinical studies has been carried out at the Turku PET Centre to investigate the suitability of [^{18}F]exendin-4 as GLP-1 R targeting radiopharmaceutical used in imaging pancreas. Also, its uptake to the kidneys, which has been a particular concern previously. The study used *in vivo* PET imaging and *ex vivo* organ γ-counting to evaluate the biodistribution of [^{18}F]exendin-4. The *in vivo* stability of the radiopharmaceutical was also tested. Autoradiography was used in the localisation the ^{18}F radioactivity in rat pancreas and pancreatic tissue sections from human. Immunohistochemistry labelling was used to confirm the islet tagging. Finally, the biodistribution results in rats were used to estimate the radiation doses for humans. Results showed [^{18}F]exendin-4's high selective uptake in native β cells and correspondingly low kidney radioactivity uptake that makes it a promising radiopharmaceutical for clinical imaging of pancreatic β cells. (Kirsi Mikkola, 2017; Lithovius et al., 2024)

1.6 Validation of Analytical Methods

In accordance with GMP requirements, the acting entity will prepare a validation plan for the analytical methods and a post-performance validation report for its own internal use. Validation plan describes in detail how the analysis is performed and its purpose including materials and methods needed. Validation report on the other hand includes the performance of the work, results obtained and their review. Original results such as radioHPLC chromatograms shall be attached to the validation report.

The documents are named as precisely and succinctly as possible and in accordance with the internal guidelines of the acting entity. The document can be given a distinctive identification code as well. An example name to use in Turku PET center for this analytical method validation can be “Validation plan for the identification and determination of concentration, chemical purity and radiochemical purity of ^{18}F Exendin-4 by Radio-HPLC”. The cover page shall also contain the names and signatures of the persons who prepared, controlled and approved the document with dates. Later if the accepted and signed document is updated or

changed for any reason, the identification code must be also updated by the newer version number. A version history at the end of the document shows the previous versions, the updated versions and their dates. Version history shows also what has been changed. All deviations to the validation plan shall be addressed individually in detail. The impact of the deviation on the performance and accuracy of the analytical method is determined in the validation report. (EMA, 2022)

As mentioned in the ICH guidelines, the very definition of validation is that "*Validation of an analytical procedure is the process of demonstrating that the procedure is suitable for its intended purpose*." In practice, it means that the performance, reliability and suitability of an analytical method for a particular analysis is demonstrated experimentally and in a particularly documented manner. According to ICH the main analytical procedures and validation characteristics that need to be evaluated are shown in Table 2. The validation involves the assessment of the following characteristics: Specificity, Linearity, Accuracy, Precision (Repeatability & Intermediate Precision), Detection Limit (LOD), Quantitation Limit (LOQ), Range and Robustness. (ICH, 2005)

Table 2. Validation characteristics required for different types of analytical procedures according to ICH Q2(R1).

Procedure Type	Spec.	Lin.	Acc.	Prec.	LOD	LOQ	Range	(Rob.)
Identification	✓	—	—	—	—	—	—	✓
Quant. tests for impurities	✓	✓	✓	(✓)	(✓)	✓	✓	✓
Limit test for impurities	✓	—	—	—	✓	—	—	✓
Assay (content or potency)	✓	✓	✓	(✓)	—	—	✓	✓

(Where Spec.= Specificity, Lin.= Linearity, Acc.= Accuracy, Prec.= Precision, LOD= Limit of detection, LOQ= Limit of quantitation, Rob.= Robustness, ()= may be needed in some cases)

1.6.1 Qualitative Parameters to be validated

Identity

The identily of product is determined by comparing the retention time of the Exendin-4 reference UV peak with the retention time of the radioactive peak of the [^{18}F]exendin-4 final product. When the blank solution is run there should be no UV signals disturbing the analysis

of the reference and the final product. Requirement: retention time of exendin-4 reference = [^{18}F]exendin-4 ± 0.5 minutes.

Specificity (UV-detector)

The resolution factor Rs between the reference and the precursor is calculated using Equation 1. The resolution factors are presented in the validation report. Requirement: R_S >1,5

Equation 1. To calculate resolution factor R_S

$$R_S = \frac{2(t_{R2} - t_{R1})}{w_1 + w_2} \quad (1)$$

t_R = retention time or distance from the peak of the signal along the baseline to the injection point ($t_{R2} > t_{R1}$).
w = peak-base-width (”USP width”)

Limit of detection (UV-detector)

The limit of detection (LOD) of the method is determined by running the references D1, D2, D3, D4 and D5 that are mentioned in Table 7. The results for the product and precursor are presented in the validation report. Requirement: Signal-to-noise ratio (S/N) > 3

Specificity (RA-detector)

The resolution factor Rs between [^{18}F]exendin-4 and the closest prior or after eluting impurity peak is calculated using Equation 1. The resolution factors are presented in the validation report. Requirement: Rs >1,5

Limit of detection (RA-detector)

The LOD of a radioactivity detector is determined by measuring the linear response of the detector. The radioactivity of the samples used for validation must be above the detection limit and within the linear response of the instrument. The detection limit and linear response of the radioactivity detector can be found in the instrument manual of the HPLC instrument concerned.

1.6.2 Quantitative Parameters to be validated

Specificity (UV-detector) is measured as described in 1.6.1

Repeatability, Precision (UV-detector)

The repeatability of the method is determined for three concentrations of 18F Exendin-4 in three replicates. The data to be reported are the peak area of each sample, the average peak area of triplicate and the relative standard deviation (RSD). Requirement RSD ≤ 2%

Linearity (UV-detector)

Linearity is determined separately from five different concentrations. The results are analysed by linear regression method calculated via the least squares method. The processed linearity report includes the linearity equation, the correlation coefficient (r^2) and the error sum of squares. Requirement: $r^2 > 0.98$

Accuracy (UV-detector)

The accuracy of the method is determined for three concentrations of ^{18}F Exendin-4 in three replicates. The concentrations of the samples are calculated using a calibration curve. The data is presented as recovery percentage (RP) using Equation 2. and relative standard deviation (RSD). Requirements: RP = 98 % - 102 %, RSD ≤ 2 %

Equation 2. to calculate recovery percentage (RP):

$$RP = C_{observed}/C_{actual} \times 100 \quad (2)$$

$C_{observed}$ = measured concentration, C_{actual} = prepared concentration

Range (UV-detector)

The method range is defined as the concentration range between the upper and lower levels in which the method has been shown to be repeatable, accurate and linear.

Limit of quantitation LOQ (UV-detector)

The LOQ of exendin-4 is calculated from the standard deviation (SD) of the response and the slope (S) of the regression line according to Equation 3.

Equation 3. to calculate LOQ:

$$LOQ = SD/S \times 10 \quad (3)$$

Specificity (RA-detector) is measured as described in 1.6.1. Requirement: Rs >1,5

Limit of detection LOD (RA-detector) is measured as described in 1.6.1

1.6.3 Specifications and their determination

According to ICH Q2 (R1) there are four main types of analytical procedures to be validated, shown in Table 1, which are identification tests, quantitative tests for impurities' content, limit tests for the control of impurities and quantitative tests of the active moiety. For our radiopharmaceutical active moiety is determined as a limit test as maximum 10 µg/injectable dose.

Validated parameters for [^{18}F]exendin-4 radiochemical purity determination are specificity and limit of detection. When radiochemical purity is determined as a limit test it must be at least 95%. And those for chemical purity determination are specificity, repeatability, linearity, accuracy, range and limit of quantitation. Robustness can be also determined, however it is not included in this thesis.

The unit carrying out the validation must select the most appropriate and suitable methods for the purpose to ensure the quality and safety of the products. The main analytical methods for the validation of radiopharmaceuticals are High-Performance Liquid Chromatography (HPLC), Thin-Layer Chromatography (TLC), Gas Chromatography (GC) and pH measurement. In order to be more compatible with radiopharmaceuticals, additional applications such as radioactivity detector is added to obtain radio-HPLC or radio-TLC (Kiffe et al., 2008).

Analytical method results can be expressed as precise quantitative numerical values or as qualitative inferences as limit-test values or classification (ICH, 2005). Following are presented two examples, one qualitative and one quantitative, using HPLC results. A qualitative analysis to identify the target component of an unknown sample, the retention time of the target component can be compared to the retention time of a standard sample as shown in Figure 3.

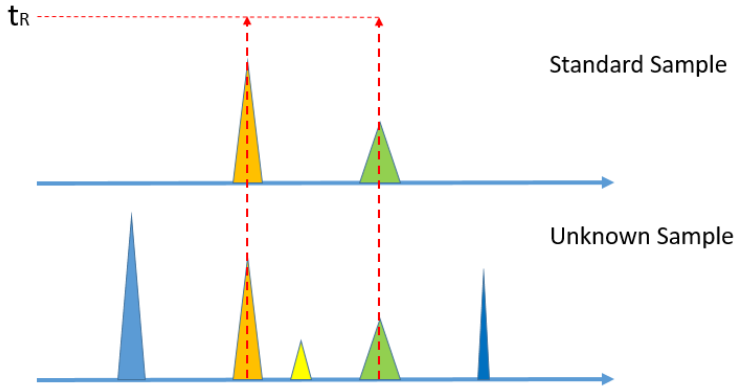

Figure 3. Qualitative identification by comparing retention times in a standard sample (upper) and an unknown sample (lower).

Quantitative analysis can be determined through two methods either by external or internal standard by using a calibration curve in both cases (Figure 4.). In the external standard method, the calibration curve is generated by plotting the concentration of the standard against the area of the peak. Here the standard calibration curve is used to extrapolate the concentration of unknown samples from their peak area. This external standard mechanism is more suitable for PET radiochemistry. On the other hand, internal standard method is created by adding a predetermined amount of internal standard to various standard concentrations and graphing the standard concentration against the ratio of the peak areas. In practice the peak area of the standard is divided by the peak area of the internal standard. Then the known fixed amount of internal standard is also mixed to the unknown samples which are quantified by extrapolating their concentration from the ratio of peak areas. In practice the peak area of the unknown sample is divided by the peak area of the internal standard. (*Qualitative and Quantitative Analysis - JASCO*, n.d.)

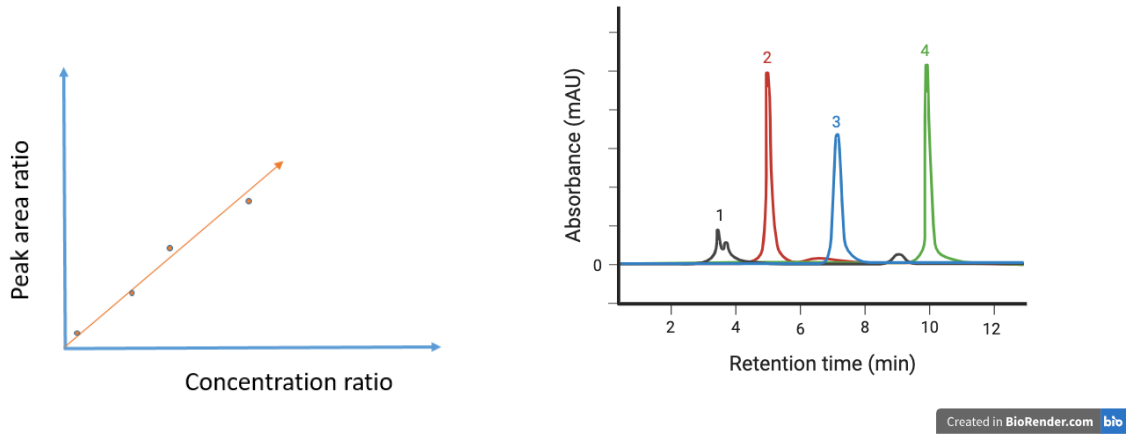

Figure 4. Quantification by internal standard method, calibration curve (left) and chromatogram (right) Created by Biorender.com.

1.6.4 Radio-HPLC

Radio-HPLC equipment is commonly used in the radiopharmaceutical production as an analytical method (Gillings et al., 2021). Therefore, in this thesis validation of radio-HPLC method is essential part of the validation process of analytical methods. Shortly, HPLC technique is used to separate liquid components based on the distribution of the sample between a mobile phase called eluent and a stationary phase in the column. Radio-HPLC is very sensitive compared to other molecular spectroscopy methods. Radioactivity detector can be linked to conventional HPLC instrument to make it more suitable for use with radiopharmaceuticals. It therefore measures radiochemical purity with a radioactivity detector and most commonly uses UV to determine concentration. The primary benefit of radio-HPLC is its simplicity; speed and precision that are crucial for radionuclides with short half-lives, such as in PET chemistry applications where a quick and precise analytical instrument is required. (Lindegren et al., 2014)

1.6.5 Radio-TLC

Compared to the HPLC, TLC is an older technology that has also been improved over time (Kiffe et al., 2008). It is a method for separating the chemical components in a mixture and determining its composition. The TLC method works so that a TLC plate is spotted with a small amount of the sample and then developed with a mobile phase. The length of the TLC plate can be beneficial for both adequate chemical separation and better possible readout resolution. However, in PET radiochemistry time is of primary importance and the method used must be as quick as possible. Here the TLC plate material and the driving solution are more relevant.

In radio-TLC, the plate is examined by radiation detector which usually moves along the plate to measure the amount of radiation produced as a function of distance. Both gamma and beta radiation can be detected using gas-based radiation detectors, which are used in most radio-TLC scanners. (Hendry, 2022) Radio-TLC can be also useful for Quality Control (QC) testing of the finished radiopharmaceutical to guarantee radiochemical purity and radiochemical identity before patient administration. The absolute advantage of radio-TLC over radio-HPLC is that the whole analyte can be visible, of course if the components can be differentiated in the first place. With radio-HPLC, it is challenging to ensure that no components are stuck in the column and thus potentially unanalyzed. Also, the method used can affect which is more viable in terms of price and speed. (Wang et al., 2019)

1.7 Validation of Production Process

Validation is a key part of GMP as well as cGRPP and includes validated synthesis methods and validated analytical methods that uses qualified synthesis and analytical equipment. In this manner, a package suitable for clinical radiopharmaceutical production can be built up. In addition to the radiochemical synthesis process, the purification and formulation methods that enable the intravenous delivery of the radiopharmaceutical to humans must be developed prior to the validation of the synthesis method. (Lahdenpohja, 2021)

Quality assurance of radiopharmaceuticals is even more crucial when compared to conventional pharmaceuticals. They need to be properly safeguarded against contamination and cross-contamination, just like any other pharmaceutical. Quality assurance in the production process of radiopharmaceuticals is even more highlighted because of their special characteristics, small production volumes and, in some cases, the requirement to release the product before testing is even completed. (EMA, 2008) To ensure that the radiopharmaceutical can be released before all tests are completed the validation shall include a comprehensive risk assessment and the preparation and quality control processes should be reliable and sufficiently robust. At least three consecutive successful preparation runs, or "Master Batches," are included in the validation, which should be carried out in compliance with a documented process. (Elsinga et al., 2010)

Our case radiopharmaceutical [^{18}F]exendin-4 is produced in an automated multistage process and the total synthesis time is 1,5 hours. ^{18}F is produced in the cyclotron by proton bombardment of ^{18}O-enriched water. (Mikkola et al., 2016) [^{18}F]exendin-4 is synthesized using a prosthetic group method in two steps. The initial stage involved synthesizing the prosthetic group, azido-PEG3-ethyl[^{18}F]fluoride, using nucleophilic fluorination reaction

from tosylate precursor and K222/[^{18}F]/K+-complex. The last stage is to use strain-promoted azide–alkyne cycloaddition reaction to create [^{18}F]F-dibenzocyclooctyne-exendin-4 ([^{18}F]exendin) from cyclooctyne-derivatized exendin-4 and azido-PEG3-ethyl[^{18}F]fluoride. Semipreparative HPLC is used to purify [^{18}F]exendin, which was then prepared in 10% ethanol in 0.1 mmol/l PBS with 0.02% ascorbic acid. At the end of synthesis the molar activity of [^{18}F]exendin is around 40 - 100 GBq/µmol. (Lithovius et al., 2024)

1.8 Aims and goals of this thesis

This thesis is involved in the development and validation of the production process of a novel radiopharmaceutical for PET imaging. The project is carried out in Turku PET Centre, radiochemistry laboratory and it focuses on reviewing what requirements GMP brings to the validation process of our novel radiopharmaceutical, especially to the development and validation the analytical methods.

We must confirm that the identity, strength, and purity of the radiopharmaceutical are sufficiently characterized. Thus, our aim is to develope and validate analytical methods that are specifically suited to our radiopharmaceutical and that meet GMP requirements. Every radiopharmaceutical must have GMP compliant and scientifically functioning analytical methods to fulfill all the radiopharmaceutical specific specifications. The aim of Turku PET Centre is to develop a GMP production process of ^{18}F-labelled exendin-4 with high molar activity and quality. The specific aim of this study is to develop and validate an analytic method as a part of the production process.

My objective in this work is to study the impact of GMP requirements on the validation process of the novel PET radiopharmaceutical. The goal is to focuse on developing the analytical methods for the novel radiopharmaceutical using two different approaches, one qualitative and one quantitative. The main objective of validating an analytical method is to show that it is appropriate for the intended use and purpose. Thus, our ultimate goal is to make the novel radiopharmaceutical available for human use. First, a risk assessment is carried out to determine what specifications are needed and then which methods meet them. Next, different approaches will be explored, either qualitatively or quantitatively. And finally, the validation plan is compiled and the validation is performed and documented according GMP requirements.

2 Results

In this thesis we focus on the validation of the analytical methods and provide direction on how to accomplish it. More specifically, first we developed a radio-HPLC analytical method that is suitable for [^{18}F]exendin-4 quality control. Suitable column and driving solution concentrations were selected. For the validation we took two different approaches to the same analytical method, one qualitative and one quantitative where the same results can be interpreted in two different ways.

2.1 Column and driving solution selection

During the selection phase, two HPLC columns were tested, one of which was selected for further validation. The tested columns were BioZen 2,6 µm Peptide XB-C18 (Product number: 00F-4768-E0) and Aeris 5 µm Peptide XB-C18 (Product number: 00F-4632-E0). Both columns use the same solid phase (XB-C18), which provides efficient peptide separation. However, the particle size of the two columns is different: one is 2 µm and the other is 5 µm. In addition, their particle structure is different: one is core-shell type and the other is completely porous. 2,6 µm column have higher resolution and is therefore more suitable for fast and accurate analyses, while 5 µm column have comparatively low resolution and are more suitable for routine analyses. BioZen 2,6 µm Peptide XB-C18 column was selected to perform the validation with.

Before carrying out the validation, an approach was taken to select the most suitable radio-HPLC method for the validation. The used flow rate, wavelength and gradient were determined beforehand. The concentration of driving solutions, eluents, suitable for ^{18}F Exendin-4 was investigated using three different combinations of options as follows:

- Test 1. Eluent A: H_2O (0,1 % TFA) and Eluent B: CH_3CN (0,1 % TFA)
- Test 2. Eluent A: H_2O (0,16 % TFA) and Eluent B: CH_3CN (0,16 % TFA)
- Test 3. Eluent A: H_2O (0,05 % TFA) and Eluent B: CH_3CN (0,05 % TFA)

Three different concentrations of Exendin-4 were used 1, 5, and 10 µg/mL. The method was tested by first running the blank, then the three different concentrations and finally the 10 µg/mL precursor. Concentrations of the eluents had a slight effect on peaks and separation of Exendin-4 from the other components of the sample. For further validation plan, the eluents used in Test 2 were selected as they allowed for a later retention time which improved the

resolution. The reference was less overlapping with the blank and precursor. In practice, the Exendin-4 spike was better shaped, narrower and higher.

2.2 Qualitative Results

2.2.1 Specificity (UV-detector)

The retention time of the reference UV peak and the retention time of the radioactive main peak of the [^{18}F]exendin-4 analytical sample was compared and thus the presence of the correct final product was determined as shown in Figure 5. There was no UV signals or impurities observed in the Blank.

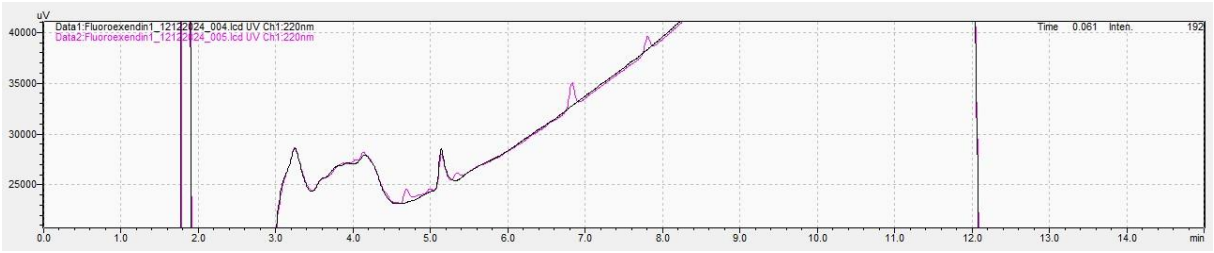

Figure 5. HPLC chromatogram of [^{18}F]exendin-4 sample and reference.

2.2.2 Limit of detection LOD (UV-detector)

Five different concentrations were used in the references. The LOD was established based on the lowest analyte concentration producing a peak distinguishable from the baseline noise (S/N ratio ≥ 3). Based on signal-to-noise evaluation, the LOD was established at 1.0 µg/mL as shown in Figure 6.

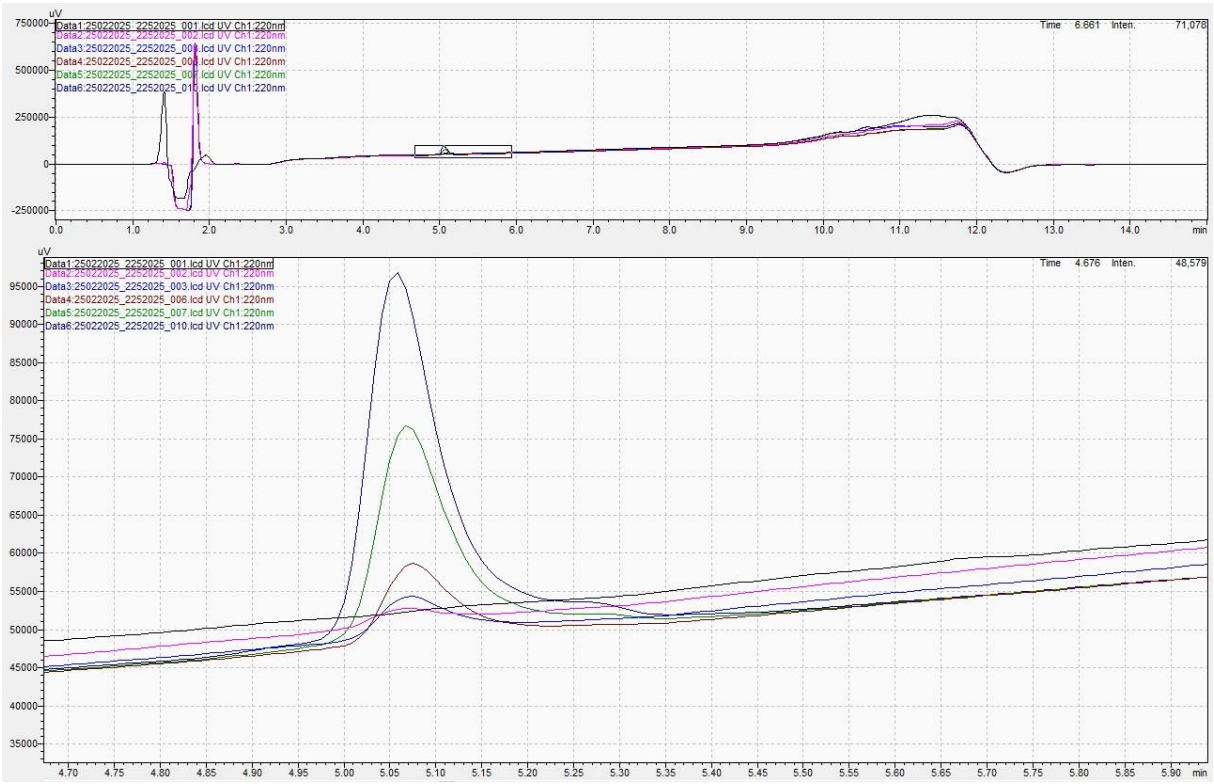

Figure 6. HPLC chromatogram of 5 different concentrations of Exendin-4 sample and Blank.

2.2.3 Specificity (RA-detector)

Specificity of [^{18}F]exendin-4 was verified by visual inspection of radio-chromatograms that showed that the main radiolabeled analyte peak was clearly separated from other potential peaks, impurities and degradation products. No signal interference was observed in the blank.

2.2.4 Limit of detection, LOD (RA-detector)

The lowest tested concentration at which the radiolabelled compound was consistently detected in all replicates was at 1.0 µg/mL. Below this level, signal visibility was inconsistent and poor. The radioactivity of the samples used for validation was above the detection limit and within the linear response of the instrument used. No interfering peaks were observed in the blank chromatogram.

2.3 Quantitative Results

2.3.1 Specificity (UV-detector)

The resolution factor (R_S) between the precursor peak and the reference peak is calculated in according to Equation1, where t_{R2}= 7.588 min, t_{R1}=5.057 min, w_1= 0.2 min. and w_2= 0.2 min.

as shown in Figure 7. Because the peaks are quite far from each other, the calculated R_S = 12.66 indicates abnormal result and it is way above the requirement $R_S > 1.5$.

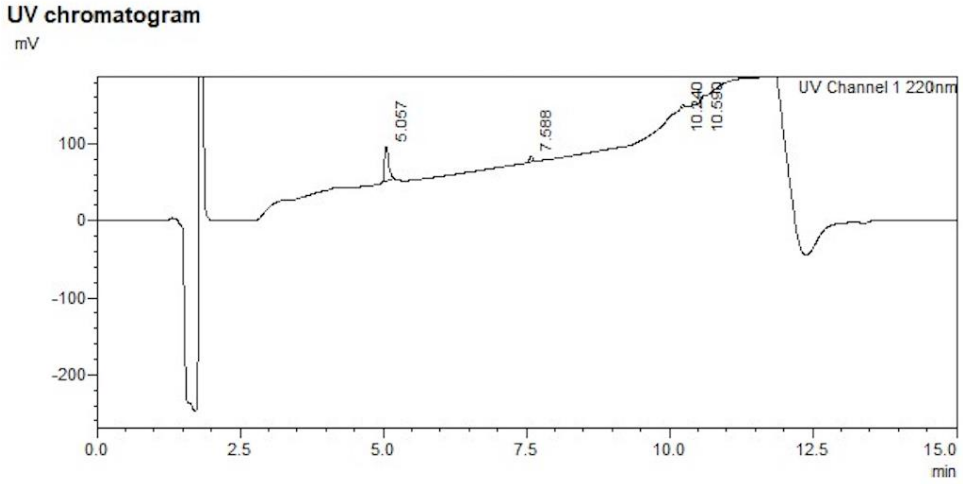

Figure 7. HPLC chromatogram of reference with 10 µg/mL.

2.3.2 Repeatability (Precision) (UV-detector)

To evaluate the repeatability (Precision) of the method we used three concentration levels (1, 5, and 10 µg/mL), each in triplicate. The average peak area of triplicate samples were used to calculate mean, standard deviation (SD), and relative standard deviation (RSD). The method scores higher than the requirement RSD ≤ 2% as shown in Table 3. in which case the result does not meet the requirement

Table 3. Repeatability values of 3 concentrations of [^{18}F]exendin-4 reference triplicate.

Concentration (µg/mL)	Peak Areas	Mean Area	SD	%RSD
10	240534, 248135, 221243	236637	13919	5.9 %
5	141511, 127426, 109360	126099	16124	12.8 %
1	19247, 13538, 11140	14642	4192	28.6 %

2.3.3 Linearity (UV-detector)

Linearity was assessed by analysing the reference at three concentration levels: 1, 5, and 10 µg/mL. Peak areas were plotted against concentration and a linear regression was performed as shown in Figure 8. The calculated correlation coefficient (r^2) = 0.9998 which is within the minimal requirement, $r^2 > 0.98$

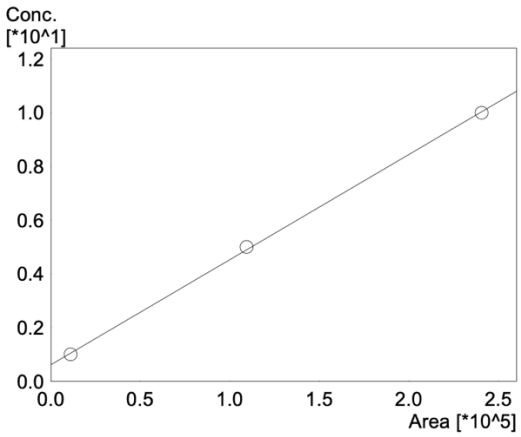

Figure 8. correlation of Exendin-4 sample concentrations 1, 5, and 10 µg/mL.

2.3.4 Accuracy (UV-detector)

Accuracy was evaluated by analysing known concentrations of the reference at three levels: 1, 5, and 10 µg/mL and each level was measured in triplicate. The concentrations were calculated based on the calibration curve and compared to the nominal values to determine percent recovery. The method shows acceptable accuracy at 5 and 10 µg/mL concentrations since they are within the requirement range of 98% - 102% as shown in Table 4.

Table 4. Repeatability values of 3 concentrations of Exendin-4 reference triplicate.

Nominal (µg/mL)	Measured (mean)	Recovery (%)
10	10.25	102.5%
5	5.39	107.8%
1	0.51	51.0%

2.3.5 Range (UV-detector)

Based on the results of linearity, accuracy, and precision, the validated quantitative range for [^{18}F]exendin-4 using UV detection was determined to be 5–10 µg/mL.

Within this range, the method demonstrates acceptable linearity ($R^2 = 0.9998$), precision (RSD ≤ 5.9%), and accuracy (recovery between 102.5% and 107.8%).

2.3.6 Limit of quantitation, LOQ (UV-detector)

The LOQ of [^{18}F]exendin-4 is calculated from the standard deviation (SD) of the response and the slope (S) of the regression line according to Equation 3.

Equation 3. to calculate LOQ:

$$LOQ = 10 \times SD/S \quad (3)$$

where S = 22814, and SD = 20. Thus, the quantitative limit of detection for [^{18}F]exendin-4 using radioactivity detection is approximately 8.8 kBq/mL.

2.3.7 Specificity (RA-detector)

Identity of [^{18}F]exendin-4 is verified by comparing its radioactivity retention time with the retention time of the reference as shown in Figure 9. The resolution factor Rs between [^{18}F]exendin-4 and the closest eluting impurity peak in the reaction mixture is calculated using equation 1. (EZChrom Elite software) Where tR2= 5,233 min, tR1=5,049 min, w1= 0,2 min and w2= 0,6 min. The calculated Rs = 0,46 is under the requirement is Rs > 1,5.

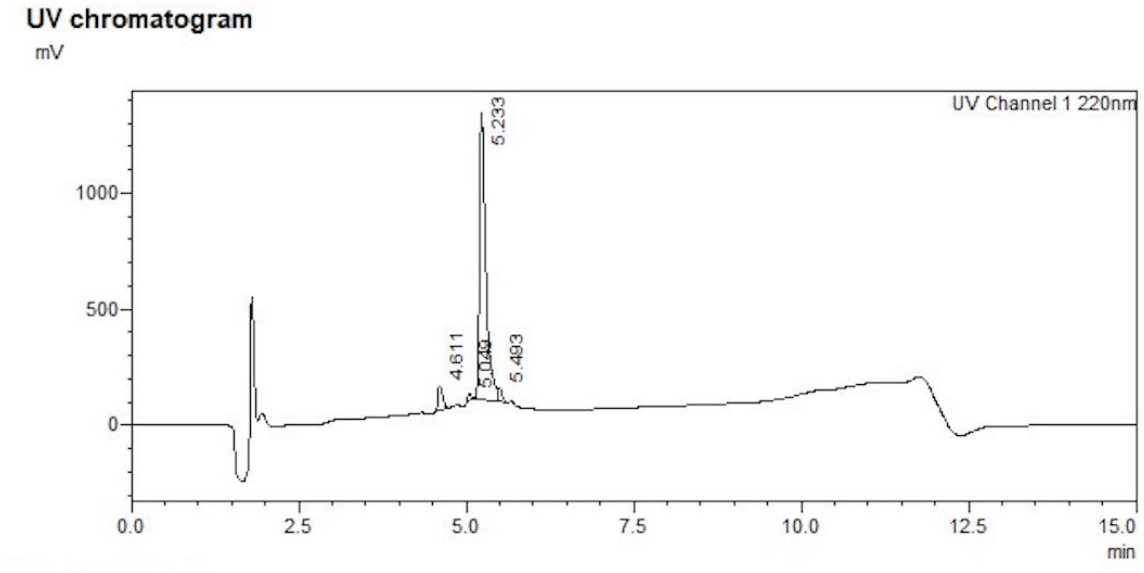

Figure 9. HPLC chromatogram of Exendin-4 sample 10 µg/mL and reference.

2.3.8 Limit of detection, LOD (RA-detector)

LOD was determined by injecting serial dilutions of [^{18}F]exendin-4 and observing the lowest activity level at which a visible radioactive peak was still detectable above baseline noise (S/N ≥ 3). A visible peak corresponding to [^{18}F]exendin-4 was observed at 0.05 MBq/mL.

2.4 Result Conclusion

Overall, the blank did not produce overlapping peaks with the samples. Meaning that the analytical method and blank (in this case the formulation solution of the final product) were

fit for purpose after a long period of development. Formulation solution keeps the final product stable within the solution. The analytical method is able to distinguish the UV signals from the formulation solution from the product signals. Determining radiochemical purity was tested both quantitatively and qualitatively. Chemical purity was more inconvenient to determine since the UV-signals were very small. This is due to the tracer amount of the product where it must be low enough not to cause any pharmacological effects to the subject being imaged. It was hard to tell quantitatively, however it could be achieved qualitatively by limit test. LOD was established at 1.0 µg/mL although the expected value in relation to the concentration of the final product was 0,5 µg/mL. Linearity was assessed quantitatively and was above the accepted correlation coefficient value of 0,98. Repeatability was well above the permitted value RSD ≤ 2% and not reliable. Accuracy was within the accepted values of recovery 98% - 102% only at concentrations 5 and 10 µg/mL, although it was expected to be met at concentration 1 µg/mL as well. On this basis, the permissible range of the method was set respectively to 5 - 10 µg/mL and was not sufficient to quantify the concentration of our final product qualitatively.

Below in Table 5. are presented summary of [^{18}F]exendin-4 radio-HPLC method quantitative validation results. For comparison, a summary of the qualitative validation results of the same radio-HPLC method are presented below in Table 6.

Table 5. [^{18}F]exendin-4 radio-HPLC quantitative analytical method validation result summary.

Test parameter	Acceptance criteria	Result
Specificity (UV)	Resolution between peaks Rs > 1,5 (^{18}F Exendin-4 vs. nearest impurity peak)	Rs = 12,66
Specificity (RA)	Resolution between peaks Rs > 1,5 (^{18}F Exendin-4 vs. reference)	Rs = 0,46
Repeatability (UV)	RSD ≤ 2% (1, 5, and 10 µg/mL each in triplicate)	1µg/mL: RSD= 28,6% 5µg/mL: RSD= 12.8% 10µg/mL: RSD= 5,9%
Linearity (UV)	Correlation coefficient r^2 > 0.98 (1, 5, and 10 µg/mL used)	r^2 = 0.9998
Accuracy (UV)	Recovery 98% - 102% (1, 5, and 10 µg/mL each in triplicate)	1µg/mL: 51,0% 5µg/mL: 107.8% 10µg/mL: 102,5%

Range (UV)	Based on linearity, accuracy, and precision	5–10 µg/mL
LOQ (UV)	Signal/Noise ratio ≥ 10	8.8 kBq/mL
LOD (RA)	Signal/Noise ratio ≥ 3	0.05 MBq/mL

Table 6. [^{18}F]exendin-4 radio-HPLC qualitative analytical method validation result summary.

Test parameter	**Acceptance criteria**	**Result**
Specificity (UV)	Visual check, retention time of ^{18}F Exendin-4 sample and reference	Similar retention time, no signal in Blank
LOD (UV)	S/N ratio ≥ 3, based on lowest distinguishable peak	1.0 µg/mL
Specificity (RA)	Visual comparison check on peaks	Analyte peak separated from others, no peaks in blank
LOD (RA)	S/N ratio ≥ 3, based on lowest distinguishable peak	1.0 µg/mL

3 Discussion

The aim of this work was to get familiarised to requirements and guidelines for the in-house production of PET radiopharmaceuticals. In additon, the aim of the laboratory work included in this thesis was to develop a radioHPLC method for the analysis of a novel radiopharmaceutical and finally perform the validation of the developed method according to GMP requirements. Operating in a GMP environment is required for the in-house prepared radiopharmaceuticals to be approved for use. The novel radiopharmaceutical is expected to make value to PET imaging and drug research especially in areas such as diabetes and pancreatic beta-cell imaging. Particularly to quantify beta-cell mass in the pancreas (Kirsi Mikkola, 2017). It will be helpful in the diagnosis and therefore planning of the treatment of insulinoma lesions, especially the small ones that are less than 13 mm in diameter (Braune et al., 2022).

In order to guarantee the safety and effectiveness of radiopharmaceuticals and to make it easier for patients to access novel and extremely effective diagnostic and therapeutic techniques, industry players such as EANM continues to engage in a variety of initiatives pertaining to guidelines and education. They strive for more harmonized policy and guidelines in the future mainly for the in-house prepared radiopharmaceuticals in Europe. (Hendrikse et al., 2022)

The guidance provided by authorities and leading organizations in the field plays a key role in this thesis. As far as can be described one of the important materials used in this thesis are these guidelines. The student had to familiarize herself with, understand and accordingly use in carrying out the work and making decisions. It is important to understand the characteristics of radiopharmaceuticals and the exceptions that apply to them. Worth noting is that working with such extensive and heavy official documents created its own difficulties.

3.1 Challenges During Validation

Since most of the work was done with very small volumes and concentrations, the working and pipetting techniques had a significant impact on the results. It was noted that significantly better and more accurate results were observed when the pipette tip was rinsed or slightly splashed into the solvent solution, ensuring that all the material was transferred and washed out. Also, some of the smaller pipette tips had a protective filter in them that was not noticed at first. When the work was repeated, it was noticed that the small filters were affecting the

results, and the tips were replaced with ones that did not have the filter. It is also important to select the used materials and equipment to be suitable for handling peptides.

The characteristic features of the peptide such as high lipophilicity and high molar activity of the final product pose challenges for handling, weighing and pipetting. When the substance was weighed it was slightly sticky and fluffy. Due to high molar activity, the molar amounts of the peptide were low which even increased the above mentioned challenges. When dissolving into ultrapure water it needed proper time and mixing to dissolve. Also, when a frozen sample was brought into use, special care had to be taken to ensure that it was well defrosted and dissolved.

3.2 Interpretation of the Validation Outcomes

The purpose of the work was to demonstrate in a documented manner that the radio-HPLC analytical method used in the quality control of [^{18}F]exendin-4 consistently gives correct and accurate results with a high degree of confidence. The aim can either be to achieve precise quantitative values or only qualitative results, for example as a limit tests. Meaning we can determine our radiopharmaceuticals chemical and radiochemical purity or also determine its precise concentration. The validation of the analytical method is performed in accordance with GMP requirements and the written instructions of the operating environment, for us, the National Turku PET Centre.

The final radio-HPLC run showed lower peaks compared to previous ones done in planning phase. This may have been influenced by the pipetting errors and techniques mentioned above as well as the challenging characteristics of [^{18}F]exendin-4 substance as a peptide. Low signal has a significant impact on the validity of the results. It was challenging to distinguish peaks and calculate the parameters of the validation especially the quantitative ones.

4 Materials and methods

All the instruments, devices and equipment used, such as radio-HPLC, were already qualified during the first installation of the devices. The detailed performance of the work, such as the batch code of the synthesis, the preparation of reference solutions, the equipment and materials used and the analytical conditions were recorded on a signed paper document. Here it is always important that everything is recorded in clear and unambiguous manner in accordance with Annex 1 (EMA, 2022). The chromatograms were saved in known folder (data file) with a unique name and date. The chromatograms were printed and the chemical composition of the sample, the concentration, the initials of the analyst and the signals contained in them were uniquely named (date, time, system name and analysis file name are printed automatically).

Initially a validation method was created for the radio-HPLC instrument and a folder where to collect the results. The column to be used was installed to the system. To avoid air bubbles, the instrument was set to run the autopurge program that lasts approximately for 13 minutes. Next, the pump of the instrument was turned on and the column was allowed to equilibrate for approximately 20-30 minutes. Finally, the apparatus was set to run the samples in the following order: blank, reference samples from D1 to D5 and finally the mixture of reference and precursor. The settings were programmed to run references D2, D4 and D5 in triplicate.

4.1 Materials

Used equipment:

- HPLC system: HPLC system 10 (or similar IQ/OQ HPLC -device)
- Column: bioZen 2,6 µm Peptide XB-C18
- Column temperature: Room temperature
- Product number: 00F-4768-E0
- Part No.: 21

Used chemicals:

- H_2O Ion exchange, ultrapure
- Trifluoroacetic acid (TFA) HPLC grade

- Acetonitrile (CH_3CN) HPLC grade
- NaCl (fys. 0.9 % w/v) Ph. Eur. i.v.
- Ethanol (EtOH) Ph.Eur. (> 99,7 til-%)
- Precursor GMP grade
- Reference HPLC grade
- [^{18}F]exendin-4 HPLC grade

Solutions:

Eluent A, H_2O (0.16 % TFA): 1000 ml graduated flask was filled approximately to half with ultrapure water. 1.6 ml of trifluoroacetic acid (TFA) was pipetted into the volumetric flask. The flask was then filled to the mark with ultrapure water. The solution was mixed and transferred to the reservoir bottle. An appropriate label was attached to the reservoir bottle. Gas was removed using an ultrasonic aspirator.

Eluent B, CH_3CN (0.16 % TFA): 1000 ml graduated flask was filled approximately to half with acetonitrile (HPLC grade). 1.6 ml of trifluoroacetic acid (TFA) was pipetted into the volumetric flask. The flask was then filled to the mark with acetonitrile. The solution was mixed and transferred to the reservoir bottle. An appropriate label was attached to the reservoir bottle. Gas was removed using an ultrasonic aspirator.

Blank / formulation solution was prepared by measuring 13 ml of infusion buffer solution (0.1M phosph. +tween 80 5mg/ml) and adding 1 ml of EtOH.

Precursor stock solution was prepared by weighting 1.00 mg of precursor NLE 14,Cys40(Mal-dPEG(4)-DBCO)Exendine-4x and dissolving it into 1 ml of ultrapure water. The aimed concentration is 1 mg/ml.

Stock solution of the reference was prepared by weighting 1.00 mg of the reference NLE14,Cys40(MaldPEG(4)DBCO- AzidePEG4F) Exendine-4 and dissolving it into 1 ml of ultrapure water.

1 mg/ml concentrated precursor as well as stock solution was prepared beforehand and kept in the freezer. Solutions were defrosted at room temperature and mixed well before use.

Stock solution of the mixture was prepared by combining 200 µl of precursor and 200 µl of stock solution into a 1:1 ratio.

Reference and precursor validation references (Dilutions D1, D2, D3, D4 and D5) of the stock solution of the mixture was prepared by using the stock solution and the formulation solution (blank solution) according to Table 7.

Table 7. Preparation of validation references.

Solution	Stock solution of the mixture (EXENDIN-4 precursor and EXENDIN-4) [µl]	Blank [µl]	Volume of the final solution [µl]	EXENDIN-4 precursor strength [µg/ml]	EXENDIN-4 strength [µg/ml]
D1	25	475	500	10	0.5
D2	50	450	500	10	1.0
D3	100	400	500	10	2.0
D4	250	250	500	10	5.0
D5	500	0	500	10	10.0

4.2 Method

The validation uses the developed [^{18}F]exendin-4 radio-HPLC analytical method for concentration determinations, radioactive purity determination and identification of the correct final product. The parameters of the radioactivity detector are preset and can be found in the radioactivity detector hardware folder.

- Flow rate: 1,0 ml/min.
- Wavelength: 220 nm
- Driving eluents: A: H_2O (0.16 % TFA) and B: CH_3CN (0.16 % TFA)
- The ratio of driving the eluents is set according to Table 8.

Table 8. Gradient of the eluents A and B.

Time (min.)	Eluent A (%)	Eluent B (%)
0.01	75	25
1.00	75	25
2.5	60	40
7.5	40	60

9.0	20	80
10.0	20	80
10.5	75	25
11.0	75	25
15.0	75	25

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Abbreviations list

AI	Artificial intelligence
AU	Absorbance unit
CCS	Contamination Control Strategy
cGRPP	current Good Radiopharmacy Practice
CH3CN	Acetonitrile
DQ	Design Qualification
EANM	European Association of Nuclear Medicine
EU	European Union
EMA	European Medicines Agency
EtOH	Ethanol
F	Fluorine
Ga	Gallium
GLP-1	Glucagon-Like Peptide-1
GLP-1R	Glucagon-Like Peptide-1 Receptor
GMP	Good Manufacturing Practice
IB	Investigator's Brochure
ICH	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IMPD	Investigational Medicinal Product Dossier
IQ	Installation Qualification
MA	Marketing authorization
MET	Method Document
OQ	Operational Qualification

PQ	Performance Qualification
PET	Positron Emission Tomography
Ph. Eur.	The European Pharmacopoeia
QA	Quality Assurance
QC	Quality Control
RA	Radioactivity
radio-HPLC	radio High Performance Liquid Chromatography
radio-TLC	radio Thin Layer Chromatography
SOP	Standard operating procedures
SPECT	Single-photon emission computed tomography
TFA	Trifluoroacetic acid
VMP	Validation master plan

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