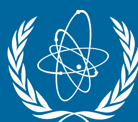
IAEA RADIOISOTOPES AND RADIOPHARMACEUTICALS SERIES No. 8

Guidance for Preclinical Studies with Radiopharmaceuticals



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GUIDANCE FOR PRECLINICAL STUDIES WITH RADIOPHARMACEUTICALS

INTERNATIONAL ATOMIC ENERGY AGENCY
VIENNA, 2023

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FOREWORD

Preclinical evaluation is an integral part of the development of any drug, including radiopharmaceuticals. Over the years, progress in the related disciplines of biology and chemistry has resulted in a variety of molecules being available for the development of a new generation of radiopharmaceuticals, which aim to deliver the radioisotope to a specific target at a cellular or molecular level. This creates a demand for in-depth evaluation of radiolabelled molecules at preclinical stages. Simple biodistribution studies in laboratory animals provided necessary information for earlier generation radiopharmaceuticals used for organ function imaging. Today, additional studies involving different in vitro techniques are required to ascertain the biological properties of radiolabelled molecules in order to obtain approval for testing in laboratory animals. The availability of specific animal disease models and technical developments in the imaging of small animals provide valuable information for the evaluation of new drugs or radiolabelled molecules prior to clinical trials.

Preclinical evaluation of some radiopharmaceuticals has been addressed by IAEA coordinated research projects related to radiopharmaceutical development, and some of the associated methods have been reported in related IAEA publications. This publication aims to address the demand for comprehensive information covering all aspects of preclinical testing and is the outcome of two consultants' meetings, where experts discussed various aspects related to in vitro and in vivo preclinical evaluation of radiopharmaceuticals. This publication is expected to be useful not only to researchers engaged in radiopharmaceutical development, but also to Member States planning to set up or upgrade facilities for radiopharmaceuticals research.

The IAEA officer responsible for this publication was A. Korde of the Division of Physical and Chemical Sciences.

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1. INTRODUCTION

1.1. BACKGROUND

Radiopharmaceuticals are radiolabelled formulations used for diagnostic, therapeutic and disease monitoring purposes in nuclear medicine practice, as well as research tools for the pharmaceutical industries [1, 2]. Diagnostic radiopharmaceuticals are used for organ function imaging and to trace processes such as regional blood flow or hypoxia. They can also be used to quantify the metabolism or target engagement of a specific drug, as well as to quantify a specific receptor system in vivo [3]. Therapeutic radiopharmaceuticals are used to treat diseases — most commonly cancer. In this case, the ionizing radiation of the conjugated radionuclide induces cell death, and the carrier molecule functions more as a vehicle to target the right cells [4].

Since the application of ^{131}I for diagnosing and treating thyroid disorders over 80 years ago, these techniques have been nurtured over multiple generations of development. In the present era, radiopharmaceuticals are a multibillion dollar global market, running the gamut from simple labelled compounds to sophisticated molecular machines. They have the ability to actively localize the area of the disease and to provide in-depth clinical profiles that aid in identifying effective personalized treatment strategies or deliver precisely targeted therapy with minimized collateral damage. They may also serve as quantifiable markers of biological function to assess the efficacy and safety of other novel pharmaceutical formulations. Thus, both nuclear medicine, with its increasingly sophisticated list of applications, and mainline drug development benefit from the creation of new radioactive compounds to address specific needs.

Even though radiopharmaceuticals account for only a fraction of pharmaceutical products, the process of generating new radiopharmaceuticals is subject to many logistical and regulatory requirements, as any other general drug development. This is especially true for therapeutic radiopharmaceuticals, which exert a pharmacological effect. Hence, before any radiopharmaceutical is cleared for use in humans, it needs to undergo rigorous testing to provide in-depth characterization of its behaviour, both physicochemical and biological, to assess its safety and suitability for the intended clinical application. This is collectively described as ‘preclinical development’. These studies include, for example, stability and affinity measurements, or determination of the radiopharmaceutical’s target engagement. Other parameters that are evaluated during preclinical studies include the drug’s biodistribution profile, identification of metabolic pathways and metabolites, and estimation of radiation doses that could be delivered during clinical trials. Additionally, even though most radiopharmaceuticals are

applied only in tracer doses in terms of drug content, which usually have no or very limited pharmacological effect, currently toxicology testing is required for all radiopharmaceuticals undergoing clinical translation. In this respect, it is of vital importance that preclinical testing follows standardized protocols that allow a direct comparison of results obtained in different laboratories and meet the current regulatory requirements for subsequent clinical trials.

1.2. OBJECTIVES

The primary objective of this publication is to provide a baseline guide for preclinical evaluation of radiopharmaceuticals, as a general review of the requirements of a facility where such work is conducted, as well as insight into the various scientific activities that constitute this process. It also aims to provide the general principles and baseline preclinical study protocols to characterize the safety, efficacy and quality of research on radiopharmaceuticals under development. The guidance provided here represents expert opinion but does not constitute recommendations made on the basis of a consensus of Member States.

1.3. SCOPE

The scope of this publication includes a series of recommendations aimed at providing useful reference for developing facilities to design preclinical study workflows for different radiopharmaceuticals. The principles and protocols discussed herein provide guidelines for biological assessment of candidate compounds that are consistent with the principles of good laboratory practices and generate valid preclinical scientific data towards approval for clinical translation.

The information provided in this publication will be useful to professionals engaged in the development and deployment of radiopharmaceuticals for the benefit of a large number of patients.

1.4. STRUCTURE

This publication gives an orderly overview of the various stages of the preclinical evaluation process, with an effort to supply in-depth detail in the relevant areas. Section 1 outlines the objectives, scope and overall structure of the publication. Section 2 discusses the aspects of radiopharmaceutical design that are related to preclinical evaluation. Section 3 details the basic requirements and relevant procedures of assessing candidate molecules in vitro in cell, tissue or

serum matrices outside living systems to provide an effective screening platform. An effective in vitro assessment testing regimen helps to refine the selection of compounds that need to be taken up for testing in animal models, addressing ethical and cost concerns. The principles and baseline procedures for in vivo and ex vivo experiments in animal models are discussed in Section 4. This includes topics such as general considerations for experiments involving animals, ex vivo radioactivity distribution study and radiometabolite analysis, as well as in vivo biodistribution studies and scintigraphic imaging procedures for radiotracers using single photon emission computed tomography (SPECT) and/or positron emission tomography (PET) systems.

The studies required for preclinical toxicity assessment to estimate the risk–benefit profile of a candidate radiopharmaceutical are discussed in Section 5. Additionally, Section 5 highlights existing guidelines and recommendations related to the development of therapeutic radiopharmaceuticals. Clinical translation of a radiopharmaceutical is also reliant on the dosimetric data obtained in preclinical studies. Section 6 provides insight into pharmacokinetic modelling and medical internal radiation dose (MIRD) principles of calculations and discusses various aspects related to the extrapolation of animal dosimetric data obtained from imaging and activity distribution studies to human studies. Section 7 highlights the frequently undervalued necessity of proper collection and reliable storage of objective, scientifically robust data that could be submitted to the regulatory authorities for subsequent clinical trials, providing several suggestions to users in these aspects.

Considerations related to the setting up of an effective preclinical evaluation facility, including designing the facility, essential equipment and staff training, and necessary parameters pertaining to safe handling of radiation and animals are discussed in Section 8. Quality control (QC) and quality assurance (QA) practices are essential for generating reliable preclinical data. An in-depth practical guide to implementation of these measures is provided in Section 9.

Lastly, Section 10 is essential to this guide, giving detailed methodologies for several of the techniques discussed. It also includes useful example study protocols for some of the evaluations commonly performed that are relevant to preclinical development and research applications of radiopharmaceuticals.

2. GENERAL CONSIDERATIONS OF RADIOPHARMACEUTICAL DEVELOPMENT: PRECLINICAL ASPECTS

For a given radiopharmaceutical, the physical characteristics of the radionuclide (type of emission, energy, half-life) determine its potential diagnostic, theranostic or therapeutic application, while the chemical and/or biological behaviour of the carrier molecule govern its affinity, selectivity and generally its pharmacological profile in a given application [2]. Inorganic ions, small organic molecules, peptides, proteins and even particles and polymers can serve as carriers for radionuclides in radiopharmaceuticals [5]. As with any other drug, alterations made in the carrier molecule can affect parameters such as target binding, distribution or metabolism which are important factors for the success of a radiopharmaceutical and its intended use. From a general point of view, every radiopharmaceutical should accumulate specifically within the target region and possess low binding to any other tissue [6]. The design process for any radiopharmaceutical needs to ensure adherence to the following properties for nuclear medicine purposes [1]:

(a) Rapid uptake and sufficient period of retention in the region of interest;
(b) Minimal uptake or quick washout from non-target regions;
(c) Minimal non-selective retention in the target region;
(d) Adequate in vitro and in vivo stability, with minimal presence of redistributing radio metabolites;
(e) Minimal unintended toxic or pharmacologic effects;
(f) Viable economics and logistics for preparation and administration.

2.1. RADIOPHARMACEUTICAL DESIGN AND DEVELOPMENT

The development of a new radiopharmaceutical is a multidisciplinary process that involves contributions from different fields of research including nuclear chemistry, synthetic chemistry, pharmaceutics, molecular biology and drug pharmacology. In general, radiopharmaceutical development involves the following phases:

(a) Identification of a biological target with diagnostic or therapeutic relevance (target identification and validation);

(b) Identification of a good lead molecule that shows suitable characteristics — such as affinity, selectivity or lipophilicity — to develop a radiopharmaceutical (lead identification);
(c) Optimization of the chemical structure with respect to properties such as binding affinity, offtarget binding, metabolism and labelling capabilities with a radioisotope that has nuclear decay properties matching the biological half-life of the radiopharmaceutical (lead optimization);
(d) Development and optimization of a radiolabelling procedure that provides sufficient radioactive yield, radiochemical purity and molar activity for preclinical studies (labelling procedure development);
(e) Preclinical assessment (see Section 2.2 regarding tracer evaluation);
(f) Upscaling of production and ensuring good manufacturing practice (production);
(g) Clinical translation (clinical phase).

In general, the process to develop a novel radiopharmaceutical is complex, consisting of a regimented sequence of interconnected protocols that requires specialized infrastructure, individual expertise and coordinated teamwork. Target selection is one of the most critical steps. Besides relevance of the target for a certain disease, it should allow for clear demarcation between diseased and healthy organs/tissues either as a unique marker or in degree of expression. From the perspective of diagnostics, clear cut-off values (yes/no situations) are preferable, and the target should show a level of tracer uptake that will yield adequate information within the limitations of resolution and quantitation of the chosen imaging modality. In the case of radiopharmaceuticals for therapy, it is crucial — from the point of view of safety and efficacy — that the chosen target can be reached selectively. This is possible, for example, when a target is selectively expressed in the region of the disease and absent or only minimally present in non-diseased tissue.

The lead molecule identification is the next critical step. Often, lead molecules are identified through standard high throughput screening programmes. Alternatively, candidates may arise from reported biomolecules or synthetic chemical structures known to possess affinity or selectivity for the target. An example for such a process is the development of radiopharmaceuticals targeting amyloid β: these structures originate from the previously known amyloid dye thioflavin T.

In comparison to ‘standard’ drugs, radiopharmaceuticals need much higher ‘target to background’ ratios to increase contrast and/or reduce irradiation of

healthy tissue. The three main factors that determine the possibility of a high target to background ratio are the following:

(i) High affinity and selectivity of the radiopharmaceutical for its target, considering the density of the target of interest in the tissue environment;
(ii) Negligible binding to other non-specific cell components — in other words, the radiopharmaceutical should possess a low non-displaceable binding component (e.g. binding to phospholipids);
(iii) Rapid clearance from the rest of the body.

The non-displaceable binding component and clearance from the body is partly influenced by the lipophilicity. Metabolism is another important parameter that influences the target to background ratio. To minimize complications in radiometabolite analyses originating from different uptake in various tissues compared with the parent tracer, it is preferable to use lead molecules that either do not get metabolized in vivo or whose metabolic products are quickly eliminated. This property can also be influenced by the radiolabelling position within the molecule, as reviewed in Ref. [7]. In the case of therapeutic radiopharmaceuticals, the retention time at the site of action is an important factor influencing the therapeutic efficacy.

A broad range of radionuclides are available to develop radiopharmaceuticals for diagnostic and therapeutic applications. These radionuclides are produced primarily in a cyclotron or a nuclear reactor. Access to certain medically important radionuclides is also possible via generator systems. A list of radionuclides used in nuclear medicine is shown in Table 1. β^- or α emitting radionuclides are currently used within the clinic for therapy, whereas β^+ or γ emitters are used for imaging [8, 9]. Most of these radionuclides have been extensively studied in terms of their chemical properties to help design appropriate molecular carriers for a required application. The design may involve linking the radionuclide to the carrier ligand directly or indirectly, by means of a bifunctional chelating agent that conserves the pharmacological properties of the carrier molecule.

TABLE 1. SELECTED RADIONUCLIDES USED IN RADIOPHARMACEUTICALS

Radionuclide	Decay mode	Half-life	Common route of production	Most common carrier molecules
Carbon-11	β^+	20.4 min	Cyclotron	Small molecules
Fluorine-18	β^+	1.83 h	Cyclotron	Small molecules, peptides

TABLE 1. SELECTED RADIONUCLIDES USED IN RADIOPHARMACEUTICALS (cont.)

Radionuclide	Decay mode	Half-life	Common route of production	Most common carrier molecules
Nitrogen-13	β^+	9.97 min	Cyclotron	Simple chemical products (NH_3), small molecules
Oxygen-15	β^+	2.04 min	Cyclotron	Simple chemical products (H_2O, CO_2, CO, O_2, etc.)
Copper-64	β^+	12 h	Cyclotron	Peptides, antibodies
Zirconium-89	β^+	78.4 h	Cyclotron	Peptides, antibodies, cells
Iodine-124	β^+	100.2 h	Cyclotron	Small molecules, peptides
Gallium-68	β^+	68 min	Generator or cyclotron	Small molecules, peptides
Iodine-123	γ	13.22 h	Cyclotron	Small molecules, peptides
Technetium-99m	γ	6.01 h	Generator or cyclotron	Small molecules, peptides
Indium-111	γ	67.32 h	Cyclotron	Peptides, antibodies
Lutetium-177	β^-	6.64 d	Nuclear reactor	Small molecules, peptides, antibodies
Yttrium-90	β^-	2.67 d	Nuclear reactor	Small molecules, peptides, antibodies
Iodine-131	β^-	8.02 d	Nuclear reactor	Radionuclide in ionic form, small molecules, peptides, antibodies
Actinum-225	α	9.92 d	Nuclear reactor, cyclotron	Small molecules, peptides, antibodies
Astatine-211	α	7.2 h	Cyclotron	Small molecules, peptides
Radium-223	α	11.43 d	Nuclear reactor	Radionuclide in ionic form
Bismuth-213	α	45.6 min	Linear accelerator, nuclear reactor, generator	Small molecules, peptides, antibodies

2.1.1. Preclinical evaluation of novel radiopharmaceuticals

Preliminary in vitro preclinical evaluation studies of a novel radiopharmaceutical are carried out to evaluate whether the radiopharmaceutical under development can be used in vivo. Many pharmacokinetic properties, such as target to background ratios or target retention, can only be evaluated in vivo. These properties, however, are essential to estimate whether the radiopharmaceutical meets the requirements for further clinical translation. Figure 1 depicts the typical processes of preclinical evaluation of radiolabelled candidates towards their selection for clinical translation.

Firstly, it should be demonstrated that the radiopharmaceutical accumulates in the region expressing the target of interest and that the levels of radioactivity in the target region reflect the expression or the activity of the target. This can be achieved, for example, by blocking the target with an unlabelled drug, using transgenic animal models (including target knockouts) or correlating the signal to the expression or the activity of the target determined by in vitro or ex vivo methods [10]. Radiometabolism studies are needed to determine to what degree metabolites interfere with the diagnosis or therapy in terms of decreasing the target to background signal or increasing the radiation burden to healthy tissue. Ideally, metabolite studies are carried out in plasma as well as in vivo, and include in vitro studies on isolated human hepatocytes. The latter should be

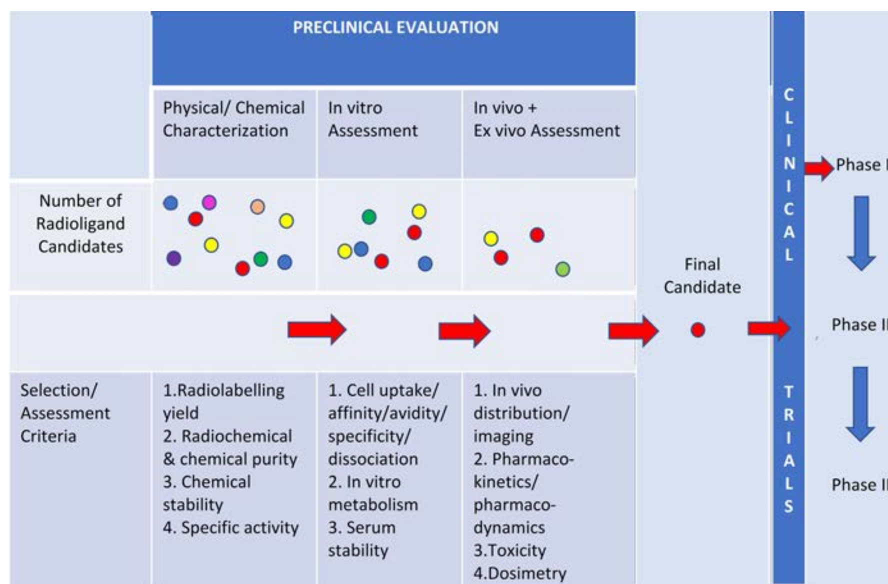

FIG. 1. Process flow for preclinical evaluation of radiolabelled molecules.

carried out to determine early whether there are species differences concerning metabolism between the preclinical model and humans. Biodistribution studies are important to calculate organ distribution and whole body dosimetry and can be performed via imaging or ex vivo experiments. Competition and inhibition studies are performed for radiopharmaceuticals retained via a specific target binding mechanism in animals treated with saturating doses of an unlabelled drug specific for the target of interest. It is important to mention that the specific activity can influence the biodistribution of the radiopharmaceutical. In general, candidates with high specific activity are preferred to avoid self-blocking effects and thus reduce target binding. The specific activity of a radiopharmaceutical is defined as the amount of radioactivity per unit mass of the compound, including all isotopes. Consequently, this value usually declines with time as the decay proceeds and reduces the radioactivity, whereas the molecular mass — mainly determined by non-decaying derivatives — stays approximately the same. This is especially critical for ^{11}C labelled radiopharmaceuticals produced from $[^{11}C]CO_2$, since isotopic dilution mainly stemming from ^{12}C cannot be avoided [11]. It is also important for preclinical studies to minimize the precursor amount administered in vivo, since the precursor may have a similar biodistribution profile and may be able to block the target. Precursors are usually separated from the radiopharmaceutical by chromatographic or extraction methods such as high performance liquid chromatography (HPLC) and solid phase extraction. However, in some cases separation from the precursor is difficult. This is typical for large precursor molecules such as nanomedicines. Therefore, it is often essential that only minimal amounts of such precursors be used in the labelling process.

Similar to other pharmaceuticals, therapeutic radiopharmaceuticals should be evaluated preclinically with both in vitro testing and animal studies to gather sufficient data on the agent's potential behaviour in human patients with respect to safety and efficacy. As with diagnostic radiopharmaceuticals, the pharmacology, toxicology and dosimetry should be evaluated in a series of controlled experiments. What makes preclinical evaluation of therapeutic pharmaceuticals unique is the fact that biodistribution and dosimetry play a much more significant role, since these results can be used to assess radiation induced toxicity (often the only toxicity caused by this class of agents) and, at the same time, give information on efficacy when dose escalation studies are performed. Therefore, biodistribution and dosimetry studies have to be rigorously designed and executed to mimic the intended use of the agent in the clinic as much as possible.

2.1.2. Pharmacology

Primary pharmacology evaluation, either in the form of animal testing or in vitro testing, should be conducted to adequately elucidate the molecular mechanism of action. Additionally, a separate safety pharmacology evaluation (i.e. the agent's effects on vital organs) is not necessary since these parameters are normally evaluated either during the ligand induced toxicology study, the biodistribution study, or both.

2.1.3. Imaging/biodistribution

The biodistribution and dosimetry studies should be conducted in animals in order to assess the distribution of radiopharmaceuticals to various organs and the associated radiation dose to the respective organs. Generally, a single animal species is adequate and both sexes are evaluated unless the planned indication is for a single sex (e.g. prostate cancer in men or ovarian cancer in women). The design of the biodistribution study should accurately reflect the planned use of the radiopharmaceutical during clinical evaluation with respect to dose, route of administration, concomitant medication and organs expected to be affected — especially the liver, kidneys and bone marrow. The dosimetry evaluation should include generation of the integrated time of the activity curves for every organ for a sufficient time post-administration. In cases where the radionuclide has naturally occurring daughter decays, these daughters should be incorporated in dosimetry and biodistribution studies. The obtained animal biodistribution data can be used to provide an estimate of the dosimetry in human patients. However, human dosimetry should be confirmed in human patients once the clinical study is initiated. Additionally, it is recommended to incorporate evaluation of clinical vital signs, body weight, haematology and serum chemistry into the biodistribution and dosimetry studies in order to evaluate the results. Those data could contribute when performing an assessment of radiation induced toxicity, in addition to conversion of the obtained organ dosimetry values into estimated organ radiation induced toxicity.

2.1.4. Toxicology

When evaluating the toxicology of therapeutic radiopharmaceuticals, both the radionuclide induced toxicity and the ligand induced toxicity should be assessed. Once the biodistribution and dosimetry evaluation is completed, the obtained data can be used to evaluate the radiation induced toxicity. This can be done through conversion of the measured organ dosimetry to organ toxicity estimates using methodologies described in the scientific literature and general

scientific knowledge on the relationship between organ specific dosimetry and the respective organ toxicities. The clinical vital signs and organ evaluation data obtained during the biodistribution study may also be used in addition to dosimetric calculations.

Vector induced toxicity should be evaluated using the cold pharmaceutical, similarly to the evaluation of ligand induced toxicity for diagnostic radioactive pharmaceuticals and non-radioactive pharmaceuticals. The study design, with respect to the number of administrations and the dose, should accurately reflect the planned use in the clinic. The same single species that was used in the dosimetry and biodistribution studies should also be used in evaluating ligand induced radiotoxicity. Sometimes, the first in human (FIH) study may involve planned escalation of the total mass of the agent in order to determine the optimal mass dose in human patients. In those cases, the mass of the cold pharmaceutical used in the ligand induced toxicology assessment should be representative of the highest possible mass planned to be used in a clinical setting. Generally, a dose at least 100 times higher than the human mass dose, adjusted to the animal body weight, should be used for toxicity evaluation in animals.

For therapeutic radiopharmaceuticals, no additional geno-toxicology, reproductive toxicology, or carcinogenic testing is normally required, since β and α radiation is known to be inherently genotoxic, causing damage to DNA.

2.2. APPLICATIONS FOR DRUG DEVELOPMENT

Drug development is a lengthy, expensive and laborious process. Preclinical studies using a well established radiopharmaceutical or radiolabelled drug candidate are helpful at various stages of development. In vitro assays such as receptor radioligand binding assays are commonly used for screening and selection of proper lead molecules with desired affinity and selectivity; whereas in vivo pharmacokinetics (PK) studies in animals offer various advantages owing to the associated radioactive markers. Such studies accelerate the evaluation process and decrease the costs involved in drug development. The following sections describe some of the ways in which preclinical studies are applied for drug development.

2.2.1. Preclinical studies using well established radiopharmaceuticals

The vast majority of preclinical studies are carried out with well established radiopharmaceuticals and can be used to: (a) validate an animal disease model, (b) evaluate the effect of a drug or (c) determine a drug’s receptor occupancy as part of drug development programmes. Moreover, established

radiopharmaceuticals can be used to better understand a certain disease. The advantage of molecular imaging compared with extensive biodistribution studies is the higher translatability value for early phase 0/I clinical trials, as well as the ability to use the same animal in longitudinal studies. An exemplary list of well established radiopharmaceuticals that can be used as biomarkers of disease progression or treatment monitoring is shown in Table 2.

TABLE 2. APPLICATION OF WELL ESTABLISHED RADIOPHARMACEUTICALS

Radiopharmaceutical	Biological processes	Field	Application
[^{18}F]FDG	Glycolysis	Oncology	Tumour staging, therapy monitoring
[^{18}F]FDG	Glycolysis	Inflammatory diseases	Arthritis
[^{18}F]FDG	Glycolysis	Cardiology	Assessment of myocardial viability
[^{18}F]FDG	Glycolysis	Endocrinology	Diabetes mellitus
[^{18}F]FDG	Glycolysis	Neurology	Brain disorders
[^{18}F]FLT	Thymidine kinase activity	Oncology	Brain tumour monitoring
[^{18}F]FAZA	Hypoxia	Oncology	Tumour monitoring
[^{18}F]FET	Amino acid transport	Oncology	Brain tumour viability monitoring
[^{18}F]FDOPA	Dopaminergic neurons	Neurology	Dopaminergic neuron plasticity
[^{11}C]raclopride	D_2 dopamine receptors	Neurology, psychiatry	Receptor occupancy Dopamine release Intrastriatal neuron plasticity
[^{11}C]PIB/[^{18}F] amyloid RF	Misfolded amyloid	Neurology	Amyloid deposition
[^{11}C]/[^{18}F]choline	Choline kinases	Oncology	Prostate cancer monitoring
[^{123}I]ioflupane	Dopamine transporter	Neurology	Parkinson’s disease

TABLE 2. APPLICATION OF WELL ESTABLISHED RADIOPHARMACEUTICALS (cont.)

Radiopharmaceutical	Biological processes	Field	Application
[^{177}Lu]PSMA-617/ [^{68}Ga]/[^{18}F]PSMA	Prostate specific membrane antigen	Oncology	Prostate cancer treatment and imaging
[^{177}Lu]lutathera/ [^{68}Ga]DOTATOC/ [^{68}Ga]DOTATATE	Somatostatin receptor	Oncology	Neuroendocrine tumours treatment and imaging
[^{15}O]H_2O	Blood flow	Cardiology	Myocardial blood flow
[^{13}N]NH_3	Blood flow	Cardiology	Myocardial blood flow
[^{223}Ra]$RaCl_2$	Bone turnover	Oncology	Metastatic bone cancer
[^{131}I]NaI	Iodine absorption	Oncology	Thyroid cancer

During the preclinical development of novel therapeutic strategies, imaging with SPECT/PET radiopharmaceuticals can be used to assess in vivo response to the regimen, helping to reduce the number of study animals required. This is particularly useful when the same techniques are applied in actual clinical practice to monitor a patient's response to treatment. In oncology, efficacy studies may be performed using PET imaging, for example with [^{18}F]FDG if the drug under development targets a glycolytic tumour, while other radiopharmaceuticals such as [^{11}C]/[^{18}F]choline or [^{68}Ga]/[^{18}F]PSMA are suitable to image non-glycolytic prostate cancers. For drugs treating differentiated neuroendocrine tumours, [^{68}Ga]DOTATOC, [^{68}Ga]DOTATATE or [^{18}F]FDOPA may be used. Moreover, [^{18}F]FDG may permit assessment of the effect that a tested drug has on brain inflammation or to monitor central nervous system disorders. [^{11}C]PIB or ^{18}F labelled amyloid targeting radiopharmaceuticals may be used to evaluate the efficacy of drugs acting on amyloid deposition, whereas [^{123}I]ioflupane or PET analogues are useful markers to monitor a pharmaceutical's effect on dopaminergic nerve ending plasticity or viability.

2.2.2. Preclinical studies using a radiolabelled drug candidate

Drug molecules under evaluation can be labelled with suitable radionuclides. Both the chemical structure and the biological half-life of the drug candidate require consideration while choosing the suitable radionuclides.

Short lived PET radionuclides such as ^{11}C and ^{18}F are commonly used to label small organic molecules, whereas radiometal diagnostic nuclides with a reasonably longer half-life, such as ^{64}Cu, ^{177}Lu, ^{111}In and ^{89}Zr, are the choice for biologics based candidates, including polypeptides and monoclonal antibodies. Preclinical studies with radiolabelled drugs under development aids in evaluating various pharmacological parameters such as the precise localization of the intended target or its PK. Another application of PET in drug development is the in vivo measurement of drug receptor interaction and the calculation of receptor occupancy by a drug at a given dosage. Occupancy studies may be performed to verify the mechanism of drug action or there may be in vivo PK/pharmacodynamics modelling to determine the kinetics of drug occupancy in the target organ. Occupancy studies require that the drug under evaluation bind to a target that can be studied with an existing validated radiopharmaceutical. Examples include [^{11}C]raclopide for dopamine D_2/D_3 receptors or [^{11}C]DASB for serotonin uptake sites [12].

2.3. DIFFERENCES AMONG SPECIES

Translating radiopharmaceuticals from animals to humans is not always straightforward, since differences among species often limit the translatability [13]. Obviously, it is important to choose an animal species that expresses the target without major structural alterations and at similar abundance. Metabolism differences between species have to be considered with respect to kinetics as well as metabolism pathways [14, 15]. As a general guide, larger species have a slower metabolism. Importantly, rodents show higher efflux transporter activity than pigs, monkeys and humans do [16]. As such, unsatisfying results from rodent studies should be carefully checked in this respect.

2.4. QUALITY CRITERIA OF RADIOPHARMACEUTICALS FOR PRECLINICAL RESEARCH

QC of a radiopharmaceutical concerns the radiochemical identity and purity, radionuclide identity and specific activity of the radiopharmaceutical, as well as of all possible contaminations. This is essential to be able to correctly analyse data in preclinical studies and exclude impurities that influence the results of the study. In contrast to clinical production, a good manufacturing practice grade radiopharmaceutical is not usually needed for preclinical studies. However, it is important to estimate or determine the content of the excipients in the initial formulation, since these excipients may influence the outcome of the study.

3. IN VITRO TESTING

In vitro studies conduct a given procedure in a controlled environment outside a living organism. Here, in vitro testing is defined as preclinical experiments involving cultured cells or fractionated cell extracts (isolated nuclei, membranes, etc.), plasma or tissue samples (whole or dispersed/homogenized) derived from humans or other animals. In contrast to ex vivo studies, radioactivity is not already present in the samples at the time of collection. However, samples will be exposed to radiopharmaceuticals during experiments to study specific mechanisms.

3.1. STUDY DESIGN

The main purpose of in vitro testing is to eliminate or reduce the need for animal testing by allowing for judicious selection of lead compounds using specific tests. It helps to minimize efforts by eliminating non-functional ligands from advancing in the pipeline and to identify potentially hazardous or toxic materials before they are tested in animal models.

3.1.1. Binding studies

Binding studies evaluate the binding characteristics of a specific ligand, such as target affinity/selectivity or target binding kinetics. A brief overview of some key characteristics, especially with respect to their utility in predicting the ligand's in vivo performance as a radiopharmaceutical, is given below.

(a) For a reversibly binding ligand, the affinity can be estimated directly in a saturation assay or indirectly in a competition assay and is expressed as the dissociation constant (K_D) in the case of a saturation assay or as the inhibition constant (K_i) for a competition assay. Lower K_D or K_i values mean higher affinity. As a rule of thumb, the ratio between in vitro estimates of the expected target density (known as the B_{max}) and the ligand affinity (either K_D or K_i) should be >5. Radiopharmaceuticals currently in use mostly satisfy this rule.

(b) Affinity measurements obtained in vitro can assist in the selection of the radiopharmaceutical candidate that should be further developed. Not many targets have densities above 10 nM; therefore, the K_D or K_i values of a radiopharmaceutical should be approximately 1 nM or lower. A radiopharmaceutical's affinity can also be used to select which dose should be administered to achieve the desired target occupancy.

(c) It is essential to assess how selectively a ligand binds to the intended target. This is normally done by performing competition assay screens with targets that are either similar to the intended target or most likely to cause confounding signal in vivo (e.g. non-target receptors co-localized with target receptors).

(d) Special care should be taken to verify that the readout of the binding assay is conducted at a time when equilibrium in the target ligand binding has been established. Alternatively, a time resolved assay may be used to determine association and dissociation rates. These values can then be used to calculate the affinity of the respective ligand. Note that binding assays conducted on material from transfected cell lines commonly result in affinity values that deviate from the values found in native cell lines, and from those in animals or human subjects.

(e) Binding studies are often carried out in fractionated extracts from target expressing cells (e.g. in membrane homogenates). However, they can also be carried out in intact cells or tissue slices.

3.1.2. Internalization and intracellular/subcellular distribution studies

Internalization assays provide insight into the ability of tracers to enter intracellular space by passive diffusion or active mechanisms. Internalization assays can aim to quantify the degree of tracer internalization, explain a concrete mechanism of internalization, or both. Intracellular distribution studies evaluate the specific localization of the tracer within the cellular system.

In many cases, but not all, the evaluation of tracer internalization can help to optimize the radionuclide choice (some nuclides will be retained inside the cell after internalization, while some will be excreted, for example) and to assess the similarity of the tracer to the parent/native ligand. Internalization and intracellular distribution assays can help to provide an understanding of the mode of action and potential efficacy, especially for therapeutic radiopharmaceuticals emitting α or β particles.

Internalization assays for radioactively labelled tracers are rather cumbersome to validate. It is recommended to confirm thoroughly that the assay is validated for the particular radiopharmaceutical prior to use. The most common internalization assay for radiopharmaceuticals is a cell uptake assay followed by an acid wash, where the acid wash presumably removes all surface bound tracer molecules. Quantification of the remaining radioactivity after acid wash indicates the amount of internalized radiopharmaceutical. The acid wash procedure may, however, disrupt the cell membrane and/or may not necessarily release the radiopharmaceutical from the surface; hence, there is a need for assay validation prior to use.

3.1.3. Cell uptake studies

Cell uptake studies evaluate the ability of the tracer to associate with a (target expressing) cell. The total tracer uptake is the sum of tracer uptake components due to surface binding and to internalization.

Uptake studies help to find out whether a proposed tracer is taken up sufficiently (or better than other tracers in development) to function as a useful in-organism tracer. Care has to be taken to evaluate cell uptake over time so as to estimate the optimal time interval from administration to accumulation in the region of interest.

3.1.4. Dissociation studies

Dissociation studies help to evaluate the retention time of the tracer in terms of its association to a cell. These are essentially extended cell uptake studies, where, after an initial period of cell uptake, the cells are washed with pure cell culture medium and the remaining bound activity is quantified over time, often many hours. Cell uptake and cell dissociation can often be combined in one assay. Cell dissociation assays test the ability of a radiopharmaceutical to stay associated with the target, after allowing the host organism to clear the unbound radiopharmaceutical from the blood stream, for example.

3.1.5. Blocking studies

Blocking studies assess the saturability and specificity of a radiopharmaceutical's binding to its intended target. These parameters are tested by introducing an unlabelled ligand that competes with the tracer for binding to a certain target. The unlabelled ligand is introduced in excess, so the binding of the tracer to the target is reduced to negligible values or blocked.

The selectivity of the tracer can be tested by making it compete with a ligand that binds to an intended target. If the presence of such a ligand decreases the total binding of the tracer, this signifies off-target specific binding.

In the simplest form of the blocking assay, a radiopharmaceutical is blocked with an excess of its unlabelled form (self-blocking). This assay will only prove the saturability of the binding; it is incapable of distinguishing between target and off-target binding. However, saturability is an important aspect because it proves that a radiopharmaceutical binds to one or more specific targets.

Ideally, the unlabelled blocking ligand, used to demonstrate specificity, should belong to a different structural class than the radiopharmaceutical that is being evaluated. This minimizes the chances that observed specific binding contains off-target components. It is also necessary to ensure that the blocking

ligand is provided at a sufficiently high molar concentration to occupy at least 90% of the target binding sites (i.e. minimum $10 \times K_D$) and that the incubation time is sufficient for the binding of both the radiopharmaceutical and the blocking ligand to reach equilibrium.

Blocking studies are typically carried out in fractionated extracts from target expressing cells (e.g. in membrane homogenates or in tissue slices). Using real tissue from the region of interest (whole or homogenized) allows the selectivity of binding to the intended target to be assessed in the presence of other targets that may cause an off-target signal.

3.1.6. Efficacy/functional assays

Functional assays test whether the tracer molecule launches or blocks a certain biochemical pathway after binding to its intended target. For therapeutic radiopharmaceuticals, in vitro efficacy assays test the ability of the radiopharmaceutical to exert therapeutic action on the target expressing cells at a relevant concentration. Usually, the ability of radiopharmaceuticals to destroy cancer cells or bacterial/viral pathogens is tested in such assays. Functional and efficacy assays are typically cell based.

3.1.7. Efflux pump assays and blood brain barrier permeability tests

The ability to penetrate the blood brain barrier (BBB) is essential for radiopharmaceuticals used for targets within the brain. Many radiopharmaceuticals may fail to penetrate the BBB because of insufficient solubility in the membrane bilayer or owing to being recognized by efflux pumps such as P-glycoprotein (or multidrug resistance mutation 1) or breast cancer resistance protein (or ABCG2). Lack of interaction with efflux pumps is also an important characteristic for therapeutic radiopharmaceuticals.

Susceptibility of the compound to be recognized and effluxed by P-glycoprotein or breast cancer resistance protein can be tested in cell based assays. More sophisticated in vitro BBB models can evaluate tracers for their ability to passively diffuse from the apical (blood) to the basolateral (brain) compartment through the layers of cells and avoid efflux pumps [17]. However, the ability of in vitro assays to predict a compound's behaviour in vivo is currently limited.

A number of physicochemical properties are believed to be important for optimal brain penetration. These include water/octanol distribution coefficients (log$P$ or log$D$ at pH7.4), polar surface area, hydrogen donor count and acidic/basic properties (pK_a). These properties can be calculated using proprietary or open source software packages, and some of them can be

measured experimentally. For example, log*D* is easy to measure by shake flask, HPLC based or nuclear magnetic resonance based methods [18–20]. The likelihood that a radiopharmaceutical will penetrate the BBB can be evaluated by in silico scoring systems on the basis of a composite of weighted calculated physicochemical parameters. Examples of such scoring systems are the multiparameter optimization score or the BBB score [21]. Such scores can help to preselect candidate tracers before in vitro evaluation.

3.1.8. In vitro autoradiography

In vitro autoradiography can be used to assess the affinity and binding selectivity of the tracer to its target in the tissue expressing it. It also provides information about the non-specific binding of a radiopharmaceutical. The basic autoradiography procedure involves incubating tissue sections mounted on a slide with radioligands under carefully defined conditions, washing and drying the sections with specifically bound ligands under conditions that preserve ligand binding, and visualizing and quantifying the distribution of the radioactive signal in the tissues [22].

Apart from binding and blocking assays, all in vitro assays that do not require functioning cellular machinery can be performed by means of autoradiography. One example is [^{35}S]GTPγS recruiting assay, which tests G-protein activation by ligand receptor binding.

Assessment of non-specific binding and its relation to tracer concentration and intrinsic lipophilicity (expressed, for example, as log*D*) is one of the special applications of in vitro autoradiography. The amount of non-specific binding remaining by the time of image readout can be manipulated by changing the intensity of washing the slides after incubation.

To perform autoradiography assays, the target should not dissociate from the tissue during the incubation with the radiopharmaceutical. Usually, targets such as membrane proteins or insoluble supramolecular aggregates (e.g. β amyloid plaques) fulfil this criterion. However, soluble enzymes usually cannot be imaged using in vitro autoradiography. Normally, in vitro autoradiography is performed in slices cut from snap-frozen tissue. In frozen tissue, the native structure of the proteins is conserved, which is crucial for the binding of most small molecule tracers. However, for some targets it is possible to perform autoradiography even in tissues that have undergone a certain preservation procedure. For instance, β amyloid tracers based on the thioflavin T structure recognize β amyloid plaques even in formalin fixed tissues [23].

The main advantage of autoradiography over assays in cell cultures or fractionated cell extracts (e.g. membrane homogenates) lies in the fact that the tracer is distributed in real tissue. Therefore, it is arguable that binding affinity,

binding site density, non-specific binding and other tracer characteristics measured in an autoradiography experiment most closely approximate the characteristics that the same tracer would show in living tissue.

3.1.9. Metabolite analysis

Metabolite analysis provides information about the possible metabolic transformations that the tracer will undergo in vivo. This information is important to validate the choice of the labelling position. For brain tracers, for example, the radiolabel should preferably be retained in metabolites that are much more hydrophilic than the parent compound. Metabolite analysis also helps identify weak points in the tracer structure, which may need to be revised if in vivo metabolic stability turns out to be too low.

Metabolic pathways can be studied for the organ that is mainly responsible for the metabolism of the circulating tracer (usually the liver) or for the target tissue or cell population. Cell cultures or tissue homogenates can be used for the assay.

Analysis of metabolism within the target cells gives useful information about the status of the tracer after uptake into the cell. It may be an indication of functional similarity of the tracer to the native biomolecule that it is designed to mimic. It also provides insight into the fate of the tracer, especially relating to postmetabolic release from the cell system, which is important from the point of view of usefulness and safety, for both diagnostic and therapeutic radiopharmaceutical formulations.

The time allowed for incubation with cells or homogenates has to be sufficient for any possible metabolic degradation of the tracer. The selected assay should be able to differentiate between the tracer and its various metabolites. Care needs to be taken to ensure that metabolites are not generated on account of microbial contamination or degradation of the tracer by factors such as air, light and generic components of the incubation medium.

3.1.10. Radiopharmaceutical stability during storage

Stability assays help to estimate the stability of a candidate radiopharmaceutical under typical storage conditions over a specified time (shelf life). It is essential that a radiopharmaceutical formulation retain its properties, such as purity and target affinity, during the course of a preclinical study or in clinical practice. Radiopharmaceuticals — in particular protein based ones — may degrade, aggregate, undergo radiolysis or otherwise change properties during storage. Such lack of stability will severely compromise scientific findings and/or clinical efficacy and safety. Tracer stability is most easily assessed by

repeatedly performing QC or conducting one or more preclinical assays over the proposed shelf life of the radiopharmaceutical. Should tracer performance cross the acceptance interval within the proposed shelf life, its stability should be further investigated and optimized.

3.1.11. Serum stability

Serum stability assays evaluate the stability of a radiopharmaceutical in the blood, the body fluid into which the radiopharmaceutical is most often administered. The objective of the assay is to predict the likelihood that the tracer retains its integrity when administered into the bloodstream of the test subject. It also allows assessment of plasma protein binding of the tracer. Serum stability assays are an important method by which to assess suitability of a radiopharmaceutical. The incubation time depends on the possible circulation time of the radiopharmaceutical. It is important to ensure that the tested fluid remains sterile to avoid microbial degradation of the tracer.

3.2. SELECTION OF ADEQUATE CELL LINES FOR IN VITRO ASSAYS

Radiopharmaceuticals generally need to be evaluated on a panel of suitable cell lines to minimize potential bias in the data obtained. This requirement exists because each cell line may not adequately represent cells within the patient population. Preferably, the cell lines derived from human tumours should be used, as these are better representatives for such studies than the transfected cell lines are. In addition, it is important to include negative control cell lines (at least one, and preferably more than one), that is, cell lines from the same or similar tissue that do not express the target receptor. This allows the evaluation of off-target binding and serves as a solid reference for positive cell lines.

3.3. SELECTION OF TISSUES FOR TISSUE BASED ASSAYS

Tissue based assays (autoradiography or metabolism studies) are typically performed using tissue from the same animal species that will be used for subsequent in vivo evaluation. This maximizes the translatability of in vitro results to the preclinical in vivo phase. However, the ultimate goal of radiopharmaceuticals is to be applicable to humans. Metabolic pathways in humans and test animals — for example, rodents, cats, pigs and non-human primates — often differ from each other. Animal models of certain diseases often

have limited validity or simply do not exist [24]. Therefore, it can be justified or even recommended to use human tissues for tissue based assays. For example, human liver microsomes are widely available and used for metabolic studies. Patient derived tumour models or human brain samples are also available.

3.4. ENVIRONMENTAL CONSIDERATIONS

Cells should be cultured and maintained according to instructions from the manufacturer or vendor, unless there are justified reasons against this. The typical cell culturing environment is 37°C with 85% relative humidity and an atmosphere containing 5% CO_2. Alternative buffering systems such as HEPES salt can be used if CO_2–bicarbonate buffering is unavailable or should not be used. It should be noted that HEPES salt is known to be toxic to a number of cell lines.

Composition of cell culture media and supplements is important for maintenance of cell characteristics and optimum growth cycle. Cell culture media usually contain mixtures of sugar/carbohydrate, different amino acids, vitamins, minerals and buffering systems. Foetal calf serum (FCS) is one of the most commonly used growth supplements. Certain studies may require the use of special starvation media (for example, folate deficient media for cell studies with ligands that target folate receptors). It is important to maintain uniformity in sourcing of all media and supplements (especially FCS, whose impact on cell growth can vary significantly depending on the source, making it mandatory to monitor and document the effects when changing suppliers or even lots) to maintain a baseline consistency in results obtained from cell studies. The passage number of cell lines should be documented and kept as low as practically possible, and special care should be taken to make comparative studies on cells that are of a similar passage number. Storage of frozen cells should be initiated at as early a passage number as possible, preferably not exceeding passage number ten.

3.5. GENERAL REMARKS ON IN VITRO METHODS

Most in vitro assessments of radiopharmaceuticals are based on tissue sections and homogenates or mammalian cell cultures, using either primary cell cultures or transformed cell lines. There are multiple relevant literature sources, including books, handbooks and monographs, which provide detailed insight into the basic principles and specific techniques of mammalian cell culture for biomedical research, including handling of cell lines, tissue sections and tissue homogenates [25, 26], and it would be redundant for this publication to delve into those details.

In general, a facility that aims to perform in vitro evaluation of radiopharmaceuticals should ensure the necessary conditions for the growth, maintenance and storage of cell lines. At the very minimum, this includes the following:

(a) Incubators to maintain mammalian cells under normal growth conditions (typically 37°C atmosphere containing 5% CO_2).
(b) Dedicated workplace for carrying out cell culture procedures with elevated cleanliness; for example, a laminar airflow cabinet.
(c) Optical microscope for examination of cells or tissue.
(d) Refrigerators and ultra-low temperature freezers (−20°C and −80°C) for storage of reagents and biological samples.
(e) Liquid nitrogen storage facility for long term storage of cell lines at −196°C.
(f) Apparatus for tissue homogenization and sectioning, if such work is anticipated.
(g) Personnel properly trained in the protocols related to general cell culture activities and the specific functions that they are expected to perform. Detailed standard operating procedures should be available for all processes. Any deviation from standard procedure or expected observation should be noted.
(h) General precautions for a cell culture/experiment facility. Entry needs to be restricted to authorized personnel. Rules pertaining to the use of sterilized outer garments, interlocking access doors, pass box for material transfer, and proper segregation and disposal of waste should be rigorously followed. Regular meticulous cleaning with surface disinfectant is required, and fumigation with a peroxide based microbicidal aerosol would be advantageous. Periodic validation of the laminar airflow filters should be performed by qualified agencies. Any issues of microbial contamination has to be dealt with immediately to protect the integrity of the facility.
(i) Separately designated areas for radiopharmaceutical evaluation studies, radioactive and non-radioactive work, in addition to general precautions to prevent cross-contamination and ensure worker safety. All necessary care has to be taken to prevent spillage or aerosol formation of stock radioactive solutions being evaluated in mammalian cells.

4. IN VIVO AND EX VIVO TESTING

4.1. GENERAL PRINCIPLES

In vivo and ex vivo testing comprise methods involving living laboratory animal organs, tissues or cells. In vivo refers to experiments using a whole, living organism (e.g. a mouse, a rat) and ex vivo to studies of a partial or dead organism after the injection of radiopharmaceutical.

4.1.1. Ethical considerations

All animal experiments need to follow applicable laws in the country where the experiment is conducted. It is recommended to have a well defined national structure to assure judicious use of animals in experimentation, as well as for licensing facilities for animal handling. Any animal experiment has to take into account the following ethical principles of laboratory animal science, known as the 3R principles: replacement, reduction and refinement.

(a) Replacement: animal experiments have to be replaced, where possible, with any other methods, such as computer simulation, mathematical models, in vitro studies and artificial tissues/organs.
(b) Reduction: only the minimum number of animals necessary to answer the stated research question may be used. This number should be based on statistical considerations, for example, on power analysis. It is important to note that using too few animals is as wasteful as using too many animals.
(c) Refinement: animal experiments have to be performed in a way that approaches minimum discomfort (i.e. the pain, suffering, distress or lasting harm that may be experienced by the animals).

Detailed guidelines on the implementation of the 3R principles are published by professional organizations in the field of laboratory animal science and governmental regulatory agencies (for example, see Ref. [27]).

4.2. STUDY DESIGN

Studies with newly developed diagnostic radiopharmaceuticals typically aim to test their PK (e.g. target identification, mechanism of action studies). Established diagnostic radiopharmaceuticals are used to image biomarkers or assess the pharmacodynamics of other drugs in a certain kind of intervention

(e.g. drug-drug interaction, therapy monitoring, disease monitoring, evaluation of a new animal model). For therapeutic radiopharmaceuticals, both PK and the therapeutic effect are of interest. Consequently, different experimental set-ups have to be used for each research question or treatment approach. The design should be aligned with results from in vitro studies and be supported by published literature. On the basis of this information, a comprehensive study can be designed. Some critical points to consider in the study design are the following:

(a) How PK/biodistribution data are obtained (i.e. ex vivo or imaging studies);
(b) Match between the decay half-life of the radionuclide and the kinetics of the process to be imaged;
(c) Administered dose (i.e. mass and radioactivity) of the radiopharmaceutical;
(d) Route of administration;
(e) Imaging time and type of scan (i.e. dynamic or static);
(f) Animal model, inclusion and exclusion criteria and type of anaesthesia;
(g) End points and samples to be collected upon termination;
(h) Selection of the animal model;
(i) Need for a preliminary pilot study.

It is advised to test a new radiopharmaceutical in more than one animal model, as results obtained can be heavily dependent on the model used [28–31].

4.2.1. Design and data analysis considerations

Because of limitations in the experimental design and statistical analysis of preclinical imaging studies, their reproducibility, robustness and translatability is an ongoing topic of discussion. Recent surveys suggest that >85% of published animal studies did not describe randomization or blinding and >95% lacked considerations of the necessary sample size needed for detecting true effects [32]. To bridge the gap between preclinical imaging studies and clinical trials, it is recommended to carefully optimize design and data analysis of preclinical imaging studies, including blinding considerations, power calculations and statistical analysis. It is recommended to follow the PREPARE guidelines for planning animal experiments [33].

4.3. ANIMAL RELATED FACTORS

Animal models are the basic component for preclinical studies and provide results that help to better understand the underlying biology of a human disease or the treatment effect. The model needs to reliably mimic the normal anatomy

and physiology of human organs and tissues of interest, as well as accurately reflect the morphology and biochemical aspects of disease pathogenesis. Rodents are mostly used in biomedical research owing to their small size, speed of reproduction, known genetic background and relative ease of procurement, handling and housing. Moreover, because of genetic manipulations, a wide range of different genetically modified models are now available to study specific factors of human diseases.

4.3.1. Animal models

Animal models can be divided into five groups: spontaneous, experimentally induced, genetically modified, negative and orphan models [34].

(a) Spontaneous models arise because of a naturally occurring genetic mutation. Some examples of spontaneous models include models for arthritis, diabetes or hypertension.
(b) Experimentally induced models are created in the laboratory, where laboratory animals are manipulated in some way to induce a condition or disease state equivalent to those occurring in humans. Common examples used for imaging are tumour models (e.g. syngeneic or xenogeneic, subcutaneous or orthotopic, chemically induced) and chemically induced models for neuroimaging (e.g. epilepsy, Parkinson's disease, multiple sclerosis).
(c) Genetically modified animal models are created by manipulation of the DNA in rodents, leading to transgenic models (rodents carrying inserted foreign DNA) or knock out models (rodents lacking one or more specific genes from their genetic code). Typically, human DNA parts are incorporated to create 'humanized' mouse models. Examples include the transgenic models of Alzheimer's disease.
(d) Negative models involve disease states that do not develop in the animal, so it can be used as a negative control or for the investigation of factors that prevent disease development.
(e) Orphan models concern disease states that occurs naturally in non-human species but have not been observed in humans.

Mouse and rat models can be obtained as inbred strains or outbred stocks. Inbred strains offer a defined genetic background, and the animals are thus nearly identical to each other, whereas outbred stocks show a higher genetic diversity, which is closer to the situation in most human studies. However, one has also to keep in mind that all commercially available rat and mouse models are inbred to

some extent, even if they are described as outbred, and that genetic drift occurs over time even within inbred strains [35, 36].

The final decision on which species or animal model will be used for a specific research study should be based on the following:

(a) The availability of the disease model;
(b) The physiology in comparison to human physiology;
(c) The structure and size of the organ or region of interest compared to the resolution and sensitivity of the imaging system;
(d) The desired information;
(e) The hypothesis (e.g. biochemical, behavioural, biodistribution) to be tested;
(f) The number of measurements required (related to the number of animals needed for the study);
(g) The requirements for staff and experimental facilities.

In addition, other factors — such as body weight, age and sex of the animals — that might have an influence on the study results have to be taken into account. The usefulness of an animal model should be evaluated on the basis of how well it is suited to answer the specific research question rather than how well it mimics the human disease.

Larger species such as pigs or non-human primates offer the advantage, because their physiology and behaviour is closer to that of humans, that dosimetry is more closely matched to that of humans and surgical interventions are simpler. Moreover, it is possible to obtain larger blood samples for analysis and their larger structure sizes can be resolved by most imaging systems. For brain imaging, the closer similarity of pig, primate and human neuroanatomy (compared with rodents) is an extra advantage. However, the disadvantages include higher costs and ethical concerns.

Smaller species such as mice and rats offer the possibility to perform dynamic whole body imaging with standard equipment. They are less expensive to purchase and house and easier to handle, and their genome is well studied and thus a big variety of rodent animal models are available. The disadvantages are the limited blood volume and thus limited sampling volume, the smaller size in comparison to the resolution of most imaging systems, more challenging surgical interventions and, finally, their physiology may not match human physiology.

Excellent and comprehensive reviews targeting cancer animal models [37–40] and animal models of neurological disorders [34, 41] are available.

4.3.2. Number of animals and randomization

4.3.2.1. Number of animals

The decision on how many animals should be included in the experiment is of critical importance. If the number of animals per group is too small, the study is underpowered, and the results can be misinterpreted. However, if the number of animals is too large, the reduction principle of laboratory animal science (minimizing the number of test subjects) can be violated (see Section 4.1.1). Therefore, it is important to carefully choose the right number of animals. The most common method to determine the necessary number of animals is to perform a power analysis. This analysis establishes a mathematical relation between the following:

(a) The magnitude of the effect of interest;
(b) The expected variability of the effect readout;
(c) The desired power of the test (the probability that the posited hypothesis will be confirmed if it is true);
(d) The desired significance level of the test (the probability that the alternative hypothesis will be rejected if it is true);
(e) An alternative hypothesis (typically the 'null hypothesis' in frequentist approaches);
(f) The sample size.

Software packages that provide free power analysis calculations for simple situations are available. An in-depth review regarding design and statistical analysis of animal experiments can be found in Refs [42–44].

4.3.2.2. Randomization

Randomization is an important method of experimental control that serves to decrease the chance of introducing bias in experimental studies. The following list gives some examples of how the risk of introducing bias can be minimized:

(a) Animals should not be separated into treatment groups based on the cages in which they are housed. It is better to have animals from different treatment groups housed together, as this will decrease the chance that environmental factors would interfere with the experiment.
(b) The same animal can be used as its own control where possible. For example, the effect of localized treatment can be studied on symmetrical body parts

(e.g. right and left paws). Different systemic treatments can be applied to the same animal at different points in time.

(c) Blinding to obscure from experimenters who evaluate the treatment which treatment group each animal belongs to. Blinding can also be implemented in processes such as animal allocation into treatment groups, administration of treatments, animal caretaking.

4.3.3. Administration and sampling

Administration of a radiopharmaceutical has to be carried out in a way that ensures reliable delivery of the radiopharmaceutical into systemic circulation with minimal discomfort to the animal. The most widely applied administration methods in a preclinical setting are intravenous and intraperitoneal injections. The use of a catheter is often recommended for intravenous injections of radiopharmaceuticals, since injection through a previously installed catheter minimizes the time spent handling a radioactive syringe. Moreover, the risk of a wrong (paravenous) injection is minimized. Injections and blood sampling should be performed using an aseptic technique. The equipment should be appropriate for the species. For example, smaller animals require thinner needles (Table 3). However, the viscosity of the administered substance has to also be considered in selecting needle size. Bigger needles are better suited for thick, viscous liquids. To minimize distress caused to animals during administration, test animals should be anaesthetized. Recommended administration volumes differ between species and administration routes. Table 3 gives some examples. The volume of any substance administered needs to be as small as possible and is naturally limited

TABLE 3. ADMINISTRATION ROUTES, VOLUMES AND RECOMMENDED NEEDLE SIZES FOR LABORATORY ANIMALS

Species	Intravenous		Intraperitoneal		Intramuscular		Subcutaneous		Oral
	Volume/ bolus (mL/kg)	Needle size (G)	Volume (mL/kg)	Needle size (G)	Volume (mL/kg)	Needle size (G)	Volume (mL/kg)	Needle size (G)	Volume (mL/kg)
Mouse	5	27–28	20	27	0.05	27	10	25	10
Rat	5	25–27	10	23–25	0.1	25	5	25	10
Rabbit	2	23–25	5	21–23	0.25	23–25	1	21–25	10
Rhesus monkey	2	21–25	20	21–23	5	23–25	2	21–25	4

by the size of the animal. Good practice guidelines for substance administration to laboratory animals are reviewed in Refs [45, 46].

In preclinical studies, blood sampling is regularly performed to obtain information about the radioactivity concentration in blood or plasma, to measure the free fraction of radiopharmaceutical in plasma and to test for radioactive metabolites. Blood sampling should be performed only by trained staff who are fully familiar with the chosen technique and equipment. It should also be performed while the animal is under anaesthesia. Depending on the volume of blood removed and the speed of withdrawal, blood sampling can elicit a physiological response in the animal. Therefore, the quantity of blood that is removed, as well as the speed of withdrawal, should be minimized. The amount of blood removed that is considered good practice is presented in Table 4 and is dependent on the circulating blood volume in each species.

Both arterial and venous blood can be sampled. Arterial blood samples are usually withdrawn from the femoral, carotid or the tail artery. However, arterial blood sampling requires invasive procedures (surgery), especially in rodents. Venous blood sampling is easier to carry out, since several veins are easily accessible; for example, the lateral tarsal (saphenous) vein, the marginal ear vein, the sublingual vein, the submandibular vein, the lateral tail vein, or the

TABLE 4. PRACTICAL BLOOD SAMPLE VOLUMES FOR LABORATORY ANIMALS

Species	Reference weight (g)	Blood volume (mL/kg)	Total blood volume, normal adult (mL)	Safe volume for single bleed (mL)	Bleed-out volume (mL)
Mouse	18–40	59	Male: 1.5–2.4 Female: 1.0–2.4	0.1–0.2	Male: 0.8–1.4 Female: 0.6–1.4
Rat	250–500	54–70	Male: 29–33 Female: 16–19	Male: 2.9–3.3 Female: 1.6–1.9	Male: 13–15 Female: 7.5–9
Rabbit	1000–6000	57–65	58.5–585	5–50	31–310
Rhesus monkey	5000	55–80	Male: 420–770 Female: 280–630	Male: 42–77 Female: 28–63	n.a.[a]

[a] n.a.: not applicable.

Note: Table adapted from Ref. [46] (see also https://www.nc3rs.org.uk/our-resources/blood-sampling).

retrobulbar plexus. For repeated or continuous blood sampling, catheterization of a major artery (e.g. carotid or femoral artery) is recommended. To record an arterial input function, which is required for kinetic modelling, protocols with arteriovenous shunts may be preferred, as they allow quantification of radioactivity concentration in blood without blood loss [47]. Temporary catheters such as butterfly needles can be used, whereas surgical implantation of a biocompatible catheter is required for long term use.

The choice of the sampling site will depend on a range of factors, including the following:

(a) The purpose of the blood collection;
(b) The need for an arterial versus venous sample;
(c) The duration and frequency of sampling;
(d) The impact on animal welfare;
(e) The health status of the animal from which blood is collected;
(f) Whether the sample is taken as part of a terminal procedure;
(g) The potential for stress induced effects on biochemical and haematological parameters;
(h) The training and experience of the staff involved.

4.3.4. Circadian rhythm

The circadian rhythm regulates many physiological parameters of the animal body. Changing the sleep–wake cycle is stressful for the animals and has to be considered in the data analysis and group comparison. Control and experimental groups need to have the same conditions. For example, rodents are usually active during the night (dark period) and sleep during the day (light period). Consequently, the food intake is also higher during the night and usually accounts for approximately two thirds of the total food and water intake during one full day [48]. Therefore, the blood glucose level varies in dark and light periods and can therefore influence the results obtained from [^{18}F]FDG PET scans. Because of the varied food intake during the day, the body weight of rodents is also different during the light and dark period. As such, it is important that rodents are weighed in the same period of the day if the body weight is used as an input parameter to calculate outcome measures such as the per cent injected dose per gram (%ID/g) [49]. Other parameters influenced by the circadian rhythm are hormone levels and body temperature [48].

4.3.5. Physiological monitoring

4.3.5.1. Body temperature

Body temperature can influence the outcome of an animal experiment. In an extreme case, the animal can even die because of hypothermia or hyperthermia. Therefore, it is strongly recommended to monitor the body temperature of a research animal, especially when it is anaesthetized. Animals usually have to be kept warm to maintain their normal body temperature when anaesthetized. This can be done by using, for example, a warming pad or warming lamps. Heating should be calibrated to avoid confounding of experimental results by variable warming of animals and to prevent animal death from hyperthermia. For imaging purposes, most vendors of small animal scanners provide a built-in solution, where the scanner is already equipped with devices to maintain and monitor the body temperature of the animals.

4.3.5.2. Respiratory and heart rate

The respiratory and heart rate of laboratory animals should be monitored in real time whenever possible. Anaesthetics can change these rates and influence the outcome of the experiment. Monitoring of these parameters in rodents is done relatively seldom owing to technical difficulties. Rodents are small and have very high respiratory and heart rates, which can be measured only by specific devices. However, in larger species such as pigs, dogs or non-human primates, respiratory and heart rates are almost universally monitored to allow the experimenter, for example, to adjust the concentration of anaesthetics or intervene in another way. Monitoring heart rate is essential for gated cardiac imaging, but also important for other imaging purposes, because the heart rate can influence the PK. Some small animal scanner suppliers deliver accessories to monitor the respiratory rate and heart rate.

4.3.5.3. Blood glucose level

The blood glucose level is a physiological parameter that can easily be measured using a small blood drop and a portable glucometer. The level of glucose in the blood is important, for example, for studies using [^{18}F]FDG. Glucose levels influence the accumulation profile of [^{18}F]FDG so it is important to have control over this parameter.

4.3.6. Fasting

Some experiments require fasting of the study animals. It can help to decrease blood glucose level, which may be necessary for imaging studies with [^{18}F]FDG. In general, fasting is not necessary for neurology studies; it may be necessary for oncology studies and it is necessary for cardiac studies. However, one should be aware that prolonged fasting leads to weight loss and decreased body temperature, heart rate and blood pressure. Furthermore, it changes hormonal and metabolic parameters. Fasting periods longer than 7 h can make animals enter a torpor state. As such, it is important to carefully consider whether fasting is needed, as it could influence the experiment's outcome. If fasting is necessary, it should be as short as possible and be performed in the period of low activity of the animals (i.e. during the light hours for rodents) to generate less discomfort [48].

4.3.7. Anaesthesia

Anaesthesia is normally necessary for preclinical in vivo PET/SPECT imaging, both to decrease animal discomfort and to keep the animal motionless throughout image acquisition to prevent artefacts caused by motion. The type of anaesthesia should be chosen on the basis of the experiment to be performed, especially regarding the organ of interest. Neuroimaging is particularly sensitive to changes induced by anaesthesia, and thus the method of anaesthesia has to be carefully chosen [50].

Anaesthesia decreases the heart rate, the respiratory frequency and the body temperature, therefore these parameters have to be monitored during anaesthesia. It can also be useful to control these parameters directly, for example by using heating mats or introducing artificial lung ventilation in animals being scanned.

Anaesthesia can be induced by injectable agents and, more commonly, by inhaled agents. Comparing injectable and inhalation anaesthesia, the inhalation is safer, especially in long procedures. Inhalation anaesthesia causes lower cardiovascular depression and also lower impact on liver and kidney function. Besides, it is much easier to control (i.e. a fast adjustment is possible in heart rate, breathing and/or body temperature change during the experiment) [51, 52].

Anaesthesia can change the PK and tissue accumulation patterns of tracers. For example, the uptake of [^{18}F]FDG in tissues is sensitive to the anaesthetic used, with brain and heart uptake being the most affected. Multiple studies have shown that uptake of [^{18}F]FDG is highly dependent on whether the animals are conscious or not; for example, awake animals show increased [^{18}F]FDG uptake in the brain, whereas the use of isoflurane increases the heart uptake [53–55]. Anaesthetics are also known to influence the binding of neurotransmitter receptor

tracers, which creates a confounding factor for the interpretation of results [50]. For example, both injectable and inhalation anaesthetics are reported to increase the binding of agonist tracers to dopamine $D_{2/3}$ receptors, while no such effect is observed for antagonist tracers [56–58].

The anaesthetic most used in the field of molecular imaging is the inhalation agent isoflurane mixed with either medical air or oxygen. The most popular type of injectable anaesthesia is the combination of the dissociative anaesthetic ketamine with a muscle relaxation compound such as xylazine or medetomidine. Table 5 gives an overview of commonly applied anaesthetics, applied doses and administration route in rodents.

TABLE 5. EXAMPLES OF ANAESTHETICS FOR IN VIVO ANIMAL IMAGING WITH RESPECTIVE DOSES AND ADMINISTRATION ROUTES

Anaesthetic	Dose and administration route	
	Mouse	Rat
Ketamine/xylazine	100 mg/kg/10 mg/kg IP[a]	90 mg/kg/10 mg/kg IP[a]
Ketamine/ medetomidine	75 mg/kg/1 mg/kg IP[a]	75 mg/kg/0.5 mg/kg IP[a]
Ketamine/diazepam	100 mg/kg or 5 mg/kg IP[a]	75 mg/kg or 8 mg/kg IP[a]
Pentobarbitone	40–60 mg/kg IP[a]	40–55 mg/kg IP[a]
Halothane	Induction concentration 4–5%	Maintenance concentration 1–2%
Sevoflurane	Induction concentration 8%	Maintenance concentration 3–4%
Isoflurane	Induction concentration 4%	Maintenance concentration 1.5–3%

[a] IP: intraperitoneal.

Note: Table adapted from Ref. [59].

4.3.8. Euthanasia

Euthanasia is the practice that ends the life of a study animal. Several procedures are permitted for this. The right procedure should be selected for each situation, considering the animal's well-being and the research question. The procedure has to be decided during the initial study design and to be approved by a local ethics committee. During the procedure, the animal should be anaesthetized to reduce animal discomfort. Those responsible for the procedure need to be well trained. Euthanasia is practised in the following ways:

(a) Anaesthetic overdose: this is the preferred protocol, since no invasive procedure is necessary, but it can influence the results of ex vivo analysis.
(b) Carbon dioxide saturation: this follows the same considerations as for anaesthetic overdose procedures.
(c) Cervical dislocation: this fast procedure is recommended for mice but not for bigger animals.
(d) Decapitation: this procedure is normally carried out with a guillotine while the animal is anaesthetised.
(e) Heart extirpation: this procedure requires surgery under deep anaesthesia and is recommended when organs will be used for ex vivo biodistribution and/or autoradiography.

4.3.9. Housing conditions and biosafety

Animal housing is an important parameter that guarantees the best conditions for research animals with respect to ethical considerations as well as non-biased results. Rodents, for example, should be housed in groups, since they are very social. Individual housing will cause distress and potentially induce bias. However, in some circumstances, the experimental design can justify individual housing. In such instances, this design has to be approved by the local animal care and use committee. Table 6 summarizes the prerequisites for rodent housing. Cages need to be secure and not allow animals to escape. They have to be made of a durable material, allow easy and frequent cleaning (including minimal corners and angles). Bedding material should allow the animals adequate time for rest and sleep, allow typical behaviour such as digging and burrowing, and facilitate thermoregulation. The bedding material needs to absorb urine and faeces.

Specialized housing systems are available and are used more frequently in animal facilities to maintain the animal in better sanitary conditions. The most common system, especially for rodents, is the individually ventilated cage (IVC) designed to minimize air exchange between cages, decreasing possible cross-contamination among cages in the same room.

TABLE 6. RECOMMENDED HOUSING SPACE FOR RODENTS

Animals	Weight (g)	Floor area/animal (cm^2)	Height (cm)
Mice	<10	38.7	12.7
	<15	51.6	12.7
	<25	77.4	12.7
	>25	≥96.7	12.7
Rats	<100	109.6	17.8
	<200	148.35	17.8
	<300	187.05	17.8
	<400	258	17.8
	<500	387	17.8
	>500	≥451.5	17.8

Note: Table adapted from Ref. [60].

It is extremely important to establish an identification system. This should include cage identification and individual animal identification using coloured stains, ear holes or piercing and subcutaneous transponders. Cages need to display the name and contact information of the responsible investigator and information about the animals: strain, gender, birth date, number of animals inside the cage, arrival date in the experimental vivarium (if different from the original animal facility), experimental group, initial date of treatment (if any) and description of the treatment (if any). It is also important that the identification tag of the cage shows alert/warning messages, if applicable, such as ‘infected animals’ or ‘radioactive material’. Further information — such as radionuclide or injected material used — should be clearly visible.

4.4. IN VIVO IMAGING STUDIES

In vivo imaging studies are defined as preclinical experiments involving (mostly unconscious) living animals during data acquisition. As described in Section 4.3, animals may need to be anaesthetized during the injection, distribution or imaging period.

4.4.1. Characteristics of preclinical PET and SPECT scanners

A variety of dedicated small animal imaging systems have been developed since 2000 and are now commercially available from different vendors. These include standalone PET and SPECT systems, combined PET/CT, PET/MRI (magnetic resonance imaging), PET/SPECT/CT, SPECT/CT and SPECT/MRI systems and, finally, integrated PET/MRI systems. The most important application of the CT and MRI scanner is to provide anatomical information. Moreover, CT and MRI information can also be used to perform partial volume, motion, scatter and attenuation corrections to improve tracer quantification. However, animal studies can also be performed using human scanners, with the disadvantage of reduced spatial resolution. Clinical γ cameras equipped with pinhole collimators complete the array of imaging modalities.

In most combined preclinical scanners, the separate modalities are mounted in-line, sharing the same bed or imaging chamber. When the bed moves in the axial direction, images of the different modalities can be acquired shortly after each other. Thus, the images are recorded at different times.

In integrated systems, the separate modalities are arranged one inside the other and share the same bed and gantry. Thus, a simultaneous acquisition of imaging parameters is possible (e.g. PET/MRI).

In contrast to clinical imaging of patients, multimodality imaging in small animals can be easily achieved using standalone scanners. In preclinical imaging, imaging chambers (or animal beds or animal cradles) are usually used, and the imaging chamber (including the animal) can be transported between imaging devices. This requires imaging chambers that can be easily, rigidly and reproducibly mounted on different scanners. Image registration can then be performed using multimodal fiducial markers attached to the animal or chamber or a predefined transformation matrix. Such side by side use of separate scanners offers higher flexibility in adding or replacing single modalities and might also increase the throughput by offering parallel imaging. However, maintaining anaesthesia may be challenging when transporting subjects between scanners that are far apart.

The requirements for nuclear imaging technologies for small animal imaging are the following:

(a) High detection efficiency;
(b) High spatial resolution;
(c) Low dead time;
(d) Good timing resolution;
(e) Good energy resolution.

These requirements are met in most preclinical PET scanners by using advanced scintillator materials such as lutetium oxyorthosilicate:cerium (LSO:Ce) or cerium doped lutetium yttrium orthosilicate:cerium (LYSO:Ce) crystals either in pixelated or monolithic arrangement attached to position sensitive photodetectors (photomultiplier tubes, silicon photomultipliers or photodiodes).

Preclinical SPECT scanners are based on pinhole collimation using one or multiple pinhole collimators and one or more detectors. For larger animals, other collimators (e.g. parallel hole collimators) are normally used. Pinhole collimators offer images with good spatial resolution — even superior to that of PET — however, at the cost of a reduced system sensitivity. Dedicated preclinical SPECT systems typically work with an increased number of pinholes to either improve the sensitivity and the angular sampling for a fixed field of view (FOV) or to enlarge it. It has to be mentioned that some systems work with multiplexing techniques (overlapping projections from different pinholes) whereas other scanners exhibit different arrangements of the pinholes to avoid multiplexing, which complicates the image reconstruction. The detection principle in preclinical SPECT systems is mostly based on a scintillator coupled to an array of photomultiplier tubes or silicon photomultipliers, or based on direct detection with a semiconductor detector material (e.g. CdZnTe). Current small animal SPECT scanners either rotate the detectors around the subject combined with axial bed translation (helical acquisition) or use stationary detectors and translate the bed in x, y and z directions to extend the FOV to include the whole animal, thereby improving sampling. Typical scanner parameters from preclinical PET and SPECT scanners are listed in Table 7.

In some scanners, the bore diameter and thus the radial FOV is limited to scan mice only. The axial FOV also varies between scanners and allows — depending on the scanner — whole body mouse or rat imaging. Scanners with smaller axial FOV typically operate with multiple bed positions or with continuous bed motion to cover the whole body of the animals. SPECT scanners are more versatile than PET scanners because, depending on the collimators applied, different animal species (e.g. mice, rats, rabbits) and different isotopes can be scanned. In contrast, most of the preclinical PET scanners are limited to mice and rats only. Comprehensive reviews on the instrumentation of preclinical PET and SPECT systems are available in Refs [61–64].

4.4.2. Image acquisition protocols

The ultimate goal in most imaging studies is to generate one or more images from which quantitative parameters can be extracted. The information that has to be extracted from the image will dictate how the data are collected. Thus, one of the first things that have to be determined is the image acquisition protocol. This includes the determination of the energy (PET and SPECT) and time window (PET),

TABLE 7. CHARACTERISTICS OF PRECLINICAL PET AND SPECT SCANNERS

	Preclinical PET	Preclinical SPECT
Detector material	LSO:Ce, LYSO:Ce, GSO:Ce	NaI:Tl, CZT
Axial FOV (mm)	50–150	7–300[a]
Radial FOV (mm)	80–120	12–250[a]
Energy resolution (%)	~20	5–10
Peak detection efficiency (%)	4–14	0.05–3[a]
Spatial resolution at CFOV (mm)	0.8–2	<0.5[a]

[a] Values are highly dependent on which collimator is used.
Note: FOV: field of view; CFOV: centre field of view.

the radionuclide, the collimator (SPECT) and the timing and duration of the scan. Usually, protocols are distinguished between static imaging and dynamic imaging. In static imaging, the radiotracer is applied, and at a certain time point (e.g. 60 min) after injection a short scan, typically 10–15 min, is recorded. Thus, the reconstructed image represents the average tissue activity concentrations at the recorded period. For dynamic imaging, the scan is started when the radiotracer is injected. Typical scan times are 60–90 min, depending on the radionuclide and tracer that are used. Data are typically collected as a list of recorded events over time. This list is then divided into a sequence of time frames, which are reconstructed separately, yielding a series of snapshots of tracer distribution in the tissues at different times. Determining the timing and duration of a scan strongly depends on the following parameters:

(a) Radiotracer:
  (i) Small molecules are usually labelled with short lived radioisotopes such as ^{11}C ($T_{1/2}$ = 20.4 min) and ^{18}F ($T_{1/2}$ = 109.8 min). As a general guide, the possible acquisition time is around two to three radionuclide half-lives, which translates into reasonable scan times of around 60–90 min for ^{11}C and 60–240 min for ^{18}F.
  (ii) Large molecules (e.g. peptides or antibodies) are usually labelled with longer lived radioisotopes such as ^{89}Zr ($T_{1/2}$ = 3.3 d) or ^{124}I ($T_{1/2}$ = 4.2 d). This is related to longer circulation time in the blood and lower PK and accumulation rates. With such radionuclides, scans can be performed up to one week after administration.

(b) Anaesthesia:
   (i) Using injectable anaesthesia limits the number of scans per day in one animal. As the recovery time is quite long, it is usually not possible to perform more than one scan per day.
   (ii) Inhalation anaesthesia is typically better tolerated, and thus animals can be scanned multiple (two to three) times per day (e.g. directly at 4 h and 8 h after injection).

(c) Animals:
   (i) If a surgical procedure performed on the animals is terminal (e.g. terminal catheterization for blood sampling), animals can be scanned only directly after the surgery.

The setting of the energy and the coincidence time window also influence image quality. Considering a typical energy resolution of 20% for a scintillator used in a preclinical PET scanner, a minimum energy window of 409–613 keV (511 keV ± 20%) is recommended. Usually, an energy window of 350–650 keV is applied in most preclinical PET scanners. When using a larger energy window (e.g. 250–750 keV), the sensitivity of a scanner is higher, as more counts are recorded. However, the scatter rate will also be increased and thus confound image quantitation. The coincidence time window has an influence on the singles rate of the scanner and thus on the random coincidences. For preclinical scanners, typical coincidence time windows are 2–6 ns. When using 'dirty positron emitters' — such as ^{124}I, which emits a high energy prompt γ ray at 603 keV — a reduction of the energy window might be advisable to reduce the background rate.

For SPECT acquisitions, it is common to set a 5–20% wide energy window around the photopeak of the respective radionuclide (e.g. ^{99m}Tc with 140 keV photopeak and energy window 126–154 keV).

In contrast to PET, SPECT offers the possibility to image multiple radiotracers simultaneously. By defining appropriate energy windows around the photopeak of the radionuclide that is used, it is possible to separate data according to the photon energy of the radionuclide of origin and perform separate reconstructions. On the other hand, quantification of dual or multiple radioisotope acquisitions is more complicated owing to the crosstalk between the two radionuclides. Thus, specific protocols have to be established before performing dual or multiple radionuclide imaging. An example of combining ^{111}In/^{177}Lu is provided in Ref. [65].

4.4.3. Dynamic versus static imaging

Dynamic and static imaging protocols have both advantages and disadvantages. Static imaging provides time averaged information about tracer distribution at a defined time interval after tracer administration. The advantage of static imaging protocols is their simplicity and ease of translation into the clinic, when mainly the relative tracer uptake in a specific organ (or tumour) is of interest. However, the choice of time interval for static imaging has to be validated, otherwise the results risk becoming biased. Conversely, in dynamic imaging protocols, the dynamic process of radiotracer uptake, retention or interaction with the target can be followed and even quantified using mathematical models (see Section 4.4.7.5). However, dynamic imaging protocols are more complex, produce more data that have to be stored and analysed, and are harder to translate into routine clinical practice.

Therefore, the choice between static and dynamic protocols depends on the radiotracer, animal model and the desired quantitative results.

In general, new radiopharmaceuticals should first be tested in the appropriate animal model using dynamic sequences in order to understand their PK. According to these results, simpler imaging protocols can be designed.

4.4.4. Image reconstruction

In image reconstruction, the recorded raw data are transformed to obtain temporally and spatially resolved images. Usually this transformation is separated into two steps. In the first step, the raw data are sorted to generate sinograms. Here, coincidence events from a PET image are grouped along a certain angle to compose projection images. In PET, each sinogram represents the data acquired for a specific axial plane across all projection angles, whereas in SPECT, each projection image represents the data acquired at a specific projection angle across all axial planes. As most o preclinical PET scanners operate in 3-D mode, which means that coincidences between the first and the last detection ring are allowed, rebinning of these oblique sinograms might be necessary. In this way, it is possible to reconstruct a 3-D dataset with conventional 2-D reconstruction schemes while maintaining the sensitivity of a 3-D sinogram dataset. Two approaches are mainly used in clinical routine: single slice rebinning and Fourier rebinning. Finally, images are reconstructed from the sinograms using either analytical or iterative image reconstruction algorithms. While analytical reconstruction methods calculate the tracer distribution directly from the measured projections, iterative reconstructions start with an estimate of the image and refine this estimate iteratively by comparison to the measured data. Analytical methods such as filtered backprojection are very fast and accurate; however, they usually provide

lower resolution images compared with iteratively reconstructed images. Iterative image reconstruction techniques such as maximum likelihood expectation maximization are routinely used in preclinical imaging. The disadvantage of iterative reconstruction is that it takes more time than filtered backprojection does and often leads to noise amplification. Advanced literature on PET [66] and SPECT image reconstruction [67] has been published.

Using commercially available preclinical scanners, the user has to define the histogram and reconstruction technique used. Some scanners offer predefined acquisition and reconstruction protocols for mice and rats. Before obtaining quantitative images, a series of correction factors have to be applied to the measured data. Some of these factors are absolutely mandatory (e.g. normalization, decay correction, dead time correction, random correction, calibration constant) and are usually applied in the process of data acquisition or image reconstruction. Others have to be applied only when performing kinetic modelling (e.g. blood/plasma input function, probe metabolism). In the following sections, the different correction factors are described.

4.4.5. Correction factors for PET

There are several corrections that have to be applied to the raw data to obtain a quantitative image. Depending on the scanner used, a series of corrections is normally carried out automatically during or directly after data acquisition, including decay correction, dead time correction and random correction.

4.4.5.1. Decay correction

During image acquisition, the tracer activity decreases owing to the radioactive decay of the radionuclide. Usually, the tracer activity is corrected to the scan start time (and/or time of injection) according to the isotope used. These corrections are typically implemented in the scanner acquisition programme and are performed automatically after selecting the right isotope.

4.4.5.2. Dead time correction

Another important aspect of a scanner is dead time (i.e. the time in which a coincidence cannot be registered because the system is busy handling a previous coincidence event). Several parts of the system contribute to the dead time; one of them is the detector. Depending on the detector system, the dead time behaviour as a function of count rate can be described by two different models. The non-paralyzable model assumes that each event is followed by a constant dead time of the detector, even if additional events reach the detector during

this time. If an event occurring during a dead time interval results in increased dead time, this can be described by the paralyzable model. For PET scanners, the dead time can be divided into dead time of the detector blocks (detector dead time) and of the electronics (system dead time). Dead time correction is usually performed automatically.

4.4.5.3. Random event correction

The random count rate is often estimated with the delayed coincidence count rate technique. For each line of response (LOR), the measured random count rate is subtracted before image reconstruction, either directly (on-line) or off-line, where the random event information is first stored in a separate sinogram dataset. Using the delayed coincidence method provides an identical electronic chain for both random and prompt events. In this case, both count rates have the same dead time characteristics. On the other hand, the random events are counted twice, thus increasing the data load on the coincidence controller. In addition, the low statistics of the random events may increase image noise. Other correction schemes for random events are the profile distribution and count rate methods. The profile distribution method uses the tails of the projection data outside the object to estimate the random event (and scatter) background. Various other corrections have to be manually defined by the user when setting up the reconstruction protocols in most preclinical systems. These corrections are then implemented in the reconstruction step, and they include normalization, attenuation and scatter correction.

4.4.5.4. Normalization

Normalization should be applied to the measured data to level out unavoidable variation in the LOR efficiencies due to variations in the crystal efficiencies (caused by different light output of the scintillator crystals, different light sharing within the detector blocks, fabrication tolerances, etc.), different positions of the LORs within the FOV, and variations in the photodetector gains. For most preclinical PET scanners, normalization is done using the direct normalization method.

4.4.5.5. Attenuation correction

To obtain quantitative images, the attenuation of photons through the subject has to be measured. Attenuation correction removes the effect of reduced activity found in the centre of a homogeneously filled cylinder. Photons emitted in the centre of the phantom have a higher absorption probability than photons

emitted at the edge of the phantom. A common method to correct for attenuation is to perform a transmission scan using an external source. When performing transmission scans, ‘hot’ and ‘cold’ transmission need to be distinguished: the cold scan is done before the radioactive tracer is applied to the subject, and hot transmission indicates the transmission measurement after the PET scan, when there is still some activity of the tracer remaining in the subject.

The following three methods are used to determine the attenuation:

(a) Coincidence transmission measurement

The conventional approach is to use a rotating ^{68}Ge rod or point source, considering the source position to accept coincidences only for LORs that pass through the source at a given moment. They are then sorted into a 2-D transmission sinogram dataset. This technique is called rod windowing or sinogram windowing, as it applies an electronic mask to the sinogram. The count rate of such transmission measurements is limited by the high count rate measured by the detectors in close proximity to the source.

(b) Single photon transmission measurement

An alternative method is to record single photons (e.g. using a ^{57}Co point source) instead of coincidence events. In this case, LORs are formed between the known source location at any time and the photons detected by opposing detectors. The advantage of this technique is the increased detected photon flux and hence increased signal to noise ratio, although this may come at the cost of increased sensitivity to scattered radiation.

(c) CT data

Here the so-called μ map from the CT transmission data is upscaled from the 80–120 keV used to 511 keV, and the attenuation map generated is used for correction.

4.4.5.6. Scatter correction

Compton scatter in the subject leads to reduction in the image contrast and depends on the material and structure of the object. Scattered events cannot be measured independently and are therefore included in the emission sinogram. Many schemes — such as simple background subtraction, convolution subtraction techniques, Monte Carlo modelling techniques, direct measurement techniques

and multiple energy window methods — have been proposed. Attenuation and scatter correction become more important the more material is in the FOV. This is related to the use of larger animals (e.g. rabbits), multiple animals ('mouse hotels', where two or more mice are scanned at the same time) or dense materials in imaging chambers (e.g. electronic board attached to MRI coils). For all these examples, attenuation and scatter correction is recommended.

4.4.6. Correction factors for SPECT

Besides establishing the proper scanner function, several correction factors can be applied to SPECT images to ensure that they are suitable and provide the highest quality data possible. These will increase in importance with the current drive towards more quantitative SPECT correction factors, which are highlighted in the following sections and discussed in detail in Ref. [68].

4.4.6.1. Uniformity correction

The uniformity of a SPECT system is its ability to produce a uniform image in response to a uniform source of γ radiation. Each projection image is corrected for uniformity before being used in image reconstruction. This correction is similar to normalization in PET data and is related to regional variations in sensitivity and uniformity due to intrinsic crystal response, non-linearities of the crystal and collimator, improper light guide coupling and variations of the photodetector yield over time. Non-uniformity is dependent on energy and perhaps an additional asymmetric energy window setting.

Uniformity correction uses the scanner computer running the acquisition to evaluate the high count flood and the mean counts per pixel. A pixel by pixel correction factor is created according to count variability within the matrix from the correction flood [69]. The resulting uniformity correction table is stored on the acquisition computer and can be applied to all future acquisitions in order to correct images for any non-uniformities present in the scanner.

4.4.6.2. Linearity and energy correction

The linearity correction and energy correction were the first approaches to correct the root causes of variations in count density associated with individual scintillation cameras. The linearity correction is based on the discovery that scanner variability is not due to issues of photon detection efficiency, but rather to the problem of mispositioning events [70]. The realization that such mispositioning is not random, but predictable for a given scanner, allows mapping of non-linearities. Linearity correction factors can be generated and applied

to future scans, repositioning detected events to their true position (within the confines of the resolution of the detector).

The energy correction method enables electronic adjustment of the position of the energy window in order to compensate for local variations in the position of the photopeak [70]. Energy correction tables can be generated and stored. These tables contain the energy correction map, including pulse height spectra for each pixel in a matrix within the detector area, and the photopeak for each individual spectrum. The map establishes an array of correction factors that can be used to adjust energy peaks from a given photon energy and can to correct future scans acquired on the same camera.

4.4.6.3. Centre of rotation

An accurate centre of rotation (COR) correction is critical for high quality SPECT imaging, as any errors in COR rapidly degrade SPECT scan quality [71]. By using a point source (usually ^{99m}Tc), COR is measured by acquiring a 360° scan. The scanner software will then determine whether COR is acceptable according to predefined specifications. The parameter is stable on most modern scanners and, if within specification, the scan can proceed as intended. For preclinical SPECT scanners, the COR correction is recommended for scanners with rotating detector heads. If COR is out of specification, service by a qualified person is recommended for the attenuation correction, the scatter correction and the collimator–detector response correction.

4.4.7. Image data analysis

This section discusses image analysis approaches that, from top to bottom, incorporate increasing degrees of quantitation. The greatest degree of quantitation involves kinetic modelling of the tracer distribution in the tissue. Before discussing tracer modelling, the simpler analysis approaches need to be described.

4.4.7.1. Image calibration

The first (and easiest) method of image analysis is visual examination of the radioactivity distribution in the body of the test subject. Immediately following reconstruction, image data are expressed in counts per second per voxel. However, images are typically calibrated in units of kilobecquerel per cubic centimetre (or per millilitre or per gram) of tissue. The calibration parameters, which are needed to convert counts per second per voxel into activity per unit of volume, can be obtained by scanning tracer filled vessels (phantoms)

of known activity and volume. The calibrated data are then converted into multicolour images, where each voxel is assigned a colour corresponding to a certain intensity according to a look-up table (also called colour map), which can be examined visually. Molecular imaging data are very often represented using so-called rainbow colour maps. It should be noted, however, that rainbow colour maps often create visual artefacts and obscure important features in the data, so the use of alternative colour maps is strongly recommended [72, 73].

4.4.7.2. Regions (volumes) of interest

A region of interest (ROI), or a volume of interest (VOI), is a group of voxels in the image that correspond to a specific organ (whole organ) or part of the organ (e.g. cortex of the brain). VOIs can be drawn by selecting the voxels manually or by various image analysis tools, such as region growing or segmentation algorithms. Activity concentration values of the voxels comprising a VOI are aggregated together for further analysis (e.g. a mean, median, modal or maximum/minimum value is calculated for the set). As VOIs are typically defined on the basis of anatomical landmarks, it is easier to define them if anatomical imaging data (CT or MRI) are available. In these cases, the VOI is defined on the anatomical image (CT or MRI) and afterwards transferred to the PET or SPECT image. The only prerequisite is a perfect alignment (fusion) of the two images. In dual coaxial scanners, where both modalities share one bed, which is translated from one modality to the other, this is mostly achieved by applying a transformation matrix to one dataset. Ideally, when fusing images, the PET or SPECT image should be the stationary one and the CT or MRI image the transformed one. Transformation usually includes translation, rotation and also re-slicing of the images. Thus, it could potentially change the values in the voxels.

4.4.7.3. Time–activity curves

If the reconstructed image is a dynamic image (i.e. consists of multiple time frames), then time–activity curves (TACs) for selected VOIs (organs or organ parts) can be extracted and analysed. The TAC is a plot of the mean radioactivity value in a VOI across a sequence of PET image frames (i.e. across time). Each data point corresponds to the mean voxel value in a common ROI at a time interval corresponding to a given image frame. The shape of the TAC provides information about the kinetics of influx and efflux of radioactivity into and out of the VOI.

4.4.7.4. *Semiquantitative image analysis*

To compare results from different subjects, there are two methods for dose normalization. The calculation of %ID/g takes into account only the total injected radioactivity and can be calculated as in Eq. (1):

$$\%\mathrm{ID/g} = C_\mathrm{t} \times \frac{V_\mathrm{t}}{W_\mathrm{t}} \times \frac{1}{D_\mathrm{inj}} \tag{1}$$

where

C_t is the tissue concentration (given in activity/volume);
V_t is the tissue volume;
W_t is the tissue weight;

and D_inj is the ID (activity).

Another method often used is the standardized uptake value (SUV), as seen in Eq. (2). Here the subject weight (W_P) is also taken into account.

$$\mathrm{SUV} = (\%\mathrm{ID/g}) \times W_\mathrm{p} \tag{2}$$

When choosing the right unit for image quantification, there are several issues that should be considered. In %ID/g, the weight of the animal is unaccounted for, which might be a problem when comparing subjects with different weights. If the injection was not perfect, extravasation at the injection site can be substantial and can thus influence the PK of the tracer (slow infusion of the paravenously injected tracer). The injection method itself (intravenous, subcutaneous or intraperitoneal) will influence tracer uptake (intravenous, subcutaneous, intraperitoneal, bolus or infusion). For intraperitoneal injection, the intestine might be penetrated. Finally, it is important always to measure the residual activity in the syringe, as especially lipophilic tracers tend to stick to the walls of the syringe and remain in the needle.

4.4.7.5. *Tracer kinetic modelling*

Dynamic reconstructed images can be further analysed using kinetic modelling to determine the parameters of interest. These types of studies require TACs for one or more VOIs representing relevant organs or regions. Some methods also require additional data, such as the time course of unmetabolized tracer concentration in arterial plasma (metabolite corrected input function).

One of the most generic parameters of interest that are determined by kinetic modelling is the (total) volume of distribution (V_D or V_T) which represents the ratio between the tracer concentration in the VOI and in the plasma at equilibrium. Reversible binding of tracers to their targets is typically described by the binding potential, which is equal to the ratio between target bound and free tracer concentrations in the VOI at equilibrium [74]. If the tracer condition is satisfied, this ratio is equal to the product of the density of binding sites (B_{max}) and the affinity of the tracer ($1/K_D$) towards them. For tracers that bind to their targets irreversibly, the binding potential cannot be estimated. Instead, transfer (or 'clearance') rate constants are estimated as primary parameters of interest. These constants represent the rate of tracer trapping in the tissue and, similar to the binding potential, are proportional to the target site density or activity.

Tracer kinetic modelling is widely used in brain imaging but is also applicable to cardiac and tumour imaging. Several different kinetic modelling approaches have been proposed for preclinical applications [75, 76]. Model driven approaches describe the distribution of tracer in terms of its transfer between virtual compartments representing free tracer in the plasma, tracer specifically bound to its target inside the tissue, etc. In data driven approaches, for example in graphical analysis, parameters of interest are estimated through data transformations, which do not entail any assumptions regarding a concrete distribution model.

4.5. EX VIVO TESTING

Ex vivo testing includes all experiments that are performed on radioactive samples derived from living animals. These samples are obtained after injection of a radiotracer into an animal that is then euthanized. They typically represent the radioactivity content in the sample at the time point of collection.

4.5.1. Organ biodistribution

4.5.1.1. Description

Ex vivo organ distribution studies aim to quantitatively map the distribution and retention of the radiopharmaceutical inside the animal at a defined time interval after injection. In such studies, the radiopharmaceutical is administered into an animal, the animal is euthanized after a specified distribution period, and the relevant organs and tissues are excised for the measurement of accumulated radioactivity.

4.5.1.2. Procedure

After radiopharmaceutical administration, animals can be returned to their individual cages if needed. In this case, it is recommended to house one animal per cage in order to reduce contamination issues. Metabolic cages, which allow for segregation of urine and faeces, can give a better differentiated excretion pattern, but they are expensive and space consuming. At the end of the specified incubation period, the animals should be euthanized. Blood should be collected immediately after euthanasia or under terminal deep anaesthesia through cardiac puncture (delay can lead to clot formation); up to 1 mL of blood in mice can be drawn through this technique. It is recommended to use a 23–26 G bore size needle for blood collection to avoid haemolysis of red blood cells. After blood collection, the animal can be dissected, and relevant organs and tissues excised for radioactivity measurement. These can be measured whole on a dose calibrator or a flat bed geometry detector, or a representative tissue sample can be taken inside a counting tube and measured in a well type γ counter.

4.5.1.3. Considerations

The time points for biodistribution studies are selected according to the radioactive half-life of the tracer and the intended application. The time points should aim to cover the pattern of uptake and retention of the tracer in the ROI, as well as non-target uptake, followed by potential washout. Taken together, the ex vivo biodistribution study should provide a clear image of the in vivo distribution and PK of the tracer. For a short to medium range half-life, it is advised to study the in vivo distribution over at least one half-life of the radionuclide, depending on the original activity. Myocardial perfusion tracers accumulate in the heart within minutes of injection, while radiolabelled antibodies for tumour targeting may take 24 h or more for optimal uptake in the ROI.

The selection of organs or tissues excised for measurement of accumulated radioactivity also depends on the nature of the radiopharmaceutical (and its possible metabolites or degradation products) and the proposed clinical application. Typical considerations for the selection of organs and tissues for excretion are the following:

(a) Presence of the target (e.g. in specific brain regions for brain receptor tracers, implanted tumours for tumour tracers) or complete absence of the target to estimate target/non-target contrast (e.g. receptor free regions of the brain, skeletal or cardiac muscle, whole blood or plasma).

(b) Involvement in the excretion of the radiopharmaceutical (e.g. kidneys and bladder for urinary excretion, liver, gall bladder and intestine for hepatobiliary excretion).

(c) Involvement in the recognition of the radiopharmaceutical by the immune system (e.g. liver, spleen, lungs, and lymph nodes, especially relevant for large molecules or nanoparticles).

(d) Tissues acting as radionuclide sinks, after the radionuclide is cleaved from the radiopharmaceutical by metabolic enzymes (e.g. bone for ^{18}F labelled compounds, liver for ^{64}Cu labelled compounds, stomach and thyroid gland for radioiodinated compounds).

(e) If there is a reason to suspect that a non-negligible fraction of the radiopharmaceutical became trapped at the injection site (e.g. owing to imperfect intravenous injection), then the excision of the injection site (e.g. piece of the tail) may also be warranted.

A typical list of organs excised to assess ex vivo biodistribution includes the liver, intestine, gall bladder, stomach, kidney, heart, lungs, spleen, brain and thyroid. During the course of dissection, care should be taken to minimize rupture of blood vessels. It is recommended to give the organs a mild rinse in saline or phosphate-buffered saline (PBS) to wash off any surface blood and lightly pat them on absorbent material to remove excess surface moisture. Individual organs are weighed, and the associated radioactivity is measured on a γ counter or γ ray spectrometer. All radioactivity measurements should be corrected for background. It is also important to subtract from the total ID, the fraction of radioactivity not cleared from the site of injection (typically the tail vein, for intravenously administered radiopharmaceuticals), after ensuring that this value does not account for more than 5–7% of the total injected radioactivity. For the measurement of total radioactivity associated with blood, muscle and bone, representative samples of each tissue are taken, weighed and counted carefully. The measured radioactivity of the samples is extrapolated to estimate the total dose to these tissues, using the assumption that in rodent models 7–8% of the total body weight is accounted for by blood, about 10% by skeletal tissue and around 40% by muscle tissue [77, 78].

There are two ways to measure the total ID during ex vivo biodistribution studies: (a) measurement of activity in the syringe before and after injection to estimate total injected activity, which is then decay corrected to the time at which organs are assessed and multiplied by the sensitivity of the γ counter; and (b) preparation of a standard solution using a known weight of radiopharmaceutical solution, dispensed in 1–2 mL saline/PBS in a 5–10 mL vial. In this protocol, each syringe has to be carefully weighed before and after injection to obtain the exact weight of radiopharmaceutical injected. The standard

solution is counted along with each set of organs and used to calculate the actual ID for each animal. While this method needs additional work, it removes the need to correct the total injected activity for radionuclide decay and γ counter sensitivity. The measured organ weights and associated radioactivity are then processed to represent the distribution of administered radiopharmaceutical in terms of the percentage of total ID (%ID) per whole organ and/or per gram of tissue (%ID/g). Automated organ counters may have built-in software to process the data and provide the results.

A tabulated list of organs and associated activity should be prepared for each time point. The tables can then be converted into graphs showing %ID/g or total %ID for each organ. Tracer distribution patterns at different time points can thus be compared to each other to obtain an overall picture of the PK of the radiotracer. Mean values of %ID/g or %ID per organ are normally plotted with the standard deviation drawn as error bars. For tumour targeting radiopharmaceuticals, ratios of %ID/g values of tumour to blood and tumour to muscle provide a measure of specific accumulation in tumour tissue. The %ID/g ratios of heart to lung and heart to liver give information about the usefulness of the tracers for myocardial perfusion imaging.

In the simplest type of biodistribution experiment, only the radiopharmaceutical is administered. In addition, the specificity and/or saturability of in vivo uptake of a radiopharmaceutical can be assessed through the use of blocking agents. A blocking agent can be the unlabelled form of the tracer itself or a different molecule with known affinity for the target recognized by the tracer. To ensure full target blockade, blocking agents are administered in much higher doses (>100-fold) than the tracer. They may be given either with the radiopharmaceutical or injected beforehand, so that they have time to reach the target sites and block them.

4.5.2. Autoradiography

4.5.2.1. Description

Autoradiography is a technique to image the radioactivity distribution in tissue slices obtained from in vitro or ex vivo experiments. The advantage of ex vivo autoradiography over in vivo imaging is that it can provide much higher resolution (<0.2 mm) than typical small animal PET/SPECT scanners (~2 mm or more). The difference between in vitro autoradiography and ex vivo autoradiography is that in the case of ex vivo the tracer is injected into a live animal, so the tracer–target interaction has already happened, and the tissue is already radioactive by the time of sectioning.

Radionuclides suitable for ex vivo autoradiography include both standard PET/SPECT isotopes (i.e. γ and β^+ emitters), as well as β^- emitters, α emitters or low energy β emitters that are not suitable for in vivo imaging. For example, ^{3}H or ^{14}C labelled analogues can be used instead of ^{11}C labelled compounds, and ^{125}I can be used instead of ^{124}I. ^{14}C and tritium (^{3}H), in particular, are ideal radioisotopes for autoradiography experiments, because the low energy of the emitted electrons provides very high image resolution [79].

4.5.2.2. Procedure

Autoradiography is frequently used for ex vivo evaluation of radiopharmaceuticals with respect to their tissue distribution. The same tissue can be used for autoradiography and histochemistry. The fresh tissue (after the animal is euthanized) should be frozen and prepared for sectioning in a cryostat. In experiments with short lived radionuclides, slices for ex vivo autoradiography should be thicker than slices for histochemistry, in order to contain enough radioactivity to produce an image by the time that all radioactivity has decayed. In general, slices of 30–40 μm are recommended for autoradiography and 5–20 μm for histochemistry.

Once the slices are ready, they should be dried before being placed in contact with the imaging plate (a helium, nitrogen or air flow can be used to accelerate that). For long lived radionuclides ($T_{1/2} > 1$ d), it is recommended to fix the slices with paraformaldehyde vapours (incubation of the slices in a closed container with dry powdered paraformaldehyde) overnight in order to increase their mechanical strength. Finally, the slices are put in contact with the imaging plate or X ray sensitive film. It is essential to ensure that the tissue is tightly and uniformly pressed against the plate/film, especially for low energy β emitters, such as ^{3}H or ^{14}C, which have a short β particle penetration depth.

Exposure time (the time that tissue remains in contact with the plate) depends on the radioisotope and the activity contained in the sample. As the exposure time increases by one half-life of the radioisotope used, the image contrast obtained becomes 50% closer to the maximum possible value. After the end of exposure, the film or the imaging plate is read in a scanner. It is important to keep the plates or films in a dark place until after reading, because visible light can interfere with the image generation.

4.5.2.3. Considerations

Patterns of tracer distribution obtained from autoradiography can be matched with staining patterns from histochemistry. Imaging data can also

be quantified using generic open source image processing software and/or proprietary software shipped together with the imaging equipment.

The relationship between the radioactivity concentration in the tissue and the obtained exposure value is often non-linear. If quantitative analysis of radioactivity distribution in the tissue is aimed for, it is strongly recommended to expose calibration standards (i.e. samples with known radioactivity concentration per unit of area) together with the tissue slices. If the software used to analyse the data is dedicated to the hardware used for imaging and is capable of automatic linearization of imaging data, calibration standards may be omitted.

All in all, it is important to consider each case individually. As different kinds of equipment are available for autoradiography, with different resolution and sensitivity, these parameters should be known before planning the experiment. The radionuclides and activity contained in the sample are also important factors, and they need to be considered when selecting the incubation time or exposure time of the sample to the imaging plate.

4.5.3. Radiometabolite analysis

4.5.3.1. Description

Radiopharmaceuticals are metabolized in vivo after injection, mostly in the liver. Metabolites that still carry the radioisotope are called radiometabolites. Non-radioactive metabolites do not interfere with nuclear imaging, but radiometabolites still generate radioactive signal, while their PK and target affinity can be significantly different from the parent tracer (i.e. the intact injected molecule). Radiometabolites can confound the interpretation of imaging data; therefore, they need to be measured and considered in the image quantification. In the context of targeted radiotherapy, radiometabolites can increase the radiation burden on healthy tissue and their creation is usually unwanted.

4.5.3.2. Procedure

In most cases, radioactive metabolite analysis entails withdrawing blood samples from the test subject, separating the plasma and measuring the percentage of total radioactivity corresponding to the metabolites in the plasma samples. In nuclear imaging, metabolite analysis of arterial blood samples is usually of primary interest, because arterial blood can be assumed to have the same metabolite content in all systemic arteries. Subtraction of radiometabolite activity from the total radioactivity concentration in plasma allows one to produce the so-called arterial input curve: the dataset describing the time course of parent tracer radioactivity concentration in arterial plasma. Arterial input curves are

essential for many kinetic modelling approaches (see Section 4.4.7.5). If the goal of radiometabolite analysis is not kinetic modelling, but simply the assessment of the kinetics of tracer metabolism, venous plasma can also be used.

Radioactive metabolites can also be measured in the target tissue. For example, all kinetic modelling approaches used in neuroimaging assume that radiometabolites do not cross the BBB. Therefore, when a new PET tracer for brain imaging is being evaluated, it is often necessary to check the validity of this assumption. This can be done by homogenizing and extracting the tissue and assessing the presence of radiometabolites in the extract obtained. Apart from measuring the amount of radiometabolites relative to the parent tracer, it is important to assess whether they are more or less lipophilic than the parent. Lipophilic metabolites are more capable of penetrating the BBB, which is relevant for brain tracers (see above).

The gold standard technique for measuring radioactive metabolites and characterizing their lipophilicity is reversed phase HPLC with radioactive detection. In the case of routinely used radiopharmaceuticals, the analysis protocol can be replicated from the literature, even if it is published only with human data. The biggest difference between radiometabolite analysis in small animals and humans is the amount of sample that can be obtained. The amount of plasma taken from a rodent is relatively small, meaning that a higher sensitivity is needed for radioactive detection. If the radioactive detectors coupled to the HPLC system are not sensitive enough, the eluate can be collected into fractions and measured in the γ counter. New radiopharmaceuticals require the development of new analysis protocols, including HPLC methods. It is essential that the HPLC method be able to differentiate the parent tracer from the metabolites. For the optimization of the protocol, non-radioactive reference compounds can be used to establish the retention time of each compound and select the best column and mobile phase.

For metabolite analysis, it is important to adequately prepare the sample for injection. Blood samples have to be centrifuged for plasma separation. After that, the radiopharmaceutical and its metabolites need to be separated from plasma proteins. This is often done by precipitating the proteins with acetonitrile. A more advanced approach is column switching, typically combined with radio-HPLC: plasma is passed through a trapping cartridge (also called a capture column), and subsequently the radiopharmaceutical and metabolites are eluted from the cartridge onto an HPLC column for separation and analysis. If HPLC is used for radiometabolite analysis, it is recommended that the sample be filtered before being injected into the loop to prevent system clogging due to residual plasma proteins. Sample preparation and analysis methods for radiometabolites have recently been reviewed [80].

Another option for the determination of radiometabolites is thin layer chromatography (TLC) in combination with autoradiography. This method is an economical alternative to HPLC, with the drawback of poorer analyte resolution and low sensitivity, thus requiring long exposure times. The supernatant of the plasma or homogenized organ sample is spotted on TLC plates and the plates are developed in solvent (e.g. dichloromethane, methanol, ethyl acetate). It is advisable to optimize the mobile phase for each radiotracer. Thereafter, the TLC plates are air dried and imaged with the autoradiographic detection system. With this method, the percentage of unchanged radiotracer can be calculated.

4.5.3.3. Considerations

HPLC is the gold standard method for radiometabolite analysis, as it provides the highest resolution, making possible the quantification and identification of various radioactive metabolites. On the other hand, HPLC analysis has high costs (expensive equipment and large amounts of solvents used) and relatively long analysis time per sample and requires highly trained personnel. Alternative techniques for radiometabolite analysis, such as TLC, solid phase extraction and liquid–liquid extraction are faster, cheaper and simpler to execute, resulting in lower sample dilution and thus providing higher sensitivity in terms of radioactivity detection compared to HPLC. Therefore, such methods may be more suitable in the preclinical set-up if high throughput is necessary. However, the resolution of these methods, especially non-chromatographic ones, is much lower than the resolution of HPLC, so proper separation of parent tracer from radiometabolites has to be validated by comparison with the gold standard.

Intrinsic biases of analytical methods have to be taken into account: some radiometabolites can become trapped on HPLC or solid phase extraction columns, leading to an overestimation of the unmetabolized radiopharmaceutical fraction. Volatile radiometabolites can evaporate from the TLC plate, again confounding the results.

All in all, the choice of the technique depends on the desired throughput and resolution, the existing knowledge about the nature of radiometabolites and the radionuclide used. With long lived radionuclides, lower sample amounts can be used and more time-consuming sample preparation and analysis methods can be applied. The opposite is true of radionuclides with shorter half-lives. The optimal strategy is to start with HPLC, continue with TLC and then, if more simplicity and throughput are needed, develop and validate methods based on solid phase extraction or liquid–liquid extraction.

4.5.4. Plasma protein binding

Interaction of tracers with plasma proteins (e.g. serum albumin) prolongs their circulation time and protects them from metabolic enzymes, but also hinders their penetration into tissues. Moreover, the degree of plasma protein binding may be different across animal species. Therefore, it is often important to measure the 'free fraction' of a tracer (i.e. the percentage of unmetabolized tracer in the plasma that is not bound to proteins). Standard methods for free fraction measurement are equilibrium dialysis, ultrafiltration and high performance frontal analysis [80].

4.6. EFFICACY STUDIES

For therapeutic radiopharmaceuticals, in vivo efficacy studies are essential to demonstrate that they possess the desired pharmacological activity and that the benefits of this activity outweigh potential toxic effects. Radiopharmaceuticals that have high efficacy and low toxicity bear enough 'clinical promise' to be translated to humans.

Two major applications for therapeutic radiopharmaceuticals are the treatment of cancer and of infectious diseases. Therefore, in vivo experimental set-ups most relevant for therapeutic radiopharmaceuticals are tumour growth inhibition studies and various animal models of severe infection.

Considerations that need to be taken into account in the design of preclinical efficacy studies for therapeutic radiopharmaceuticals include all considerations valid for analogous non-radiolabelled drugs plus radiotoxicity/dosimetry considerations. The latter are discussed in Section 6.

There are no overarching guidelines for the design and execution of preclinical therapeutic efficacy studies, but systematic reviews of area specific guidelines exist and are recommended for consultation [81]. One is also urged to consult the guidelines for the reporting of in vivo preclinical data [82] and a systematic review of threats to the validity of preclinical efficacy studies [83].

5. TOXICOLOGY

5.1. RATIONALE AND GENERAL PRINCIPLES

Before clinical phase I studies, the safety profile of any new radiopharmaceutical has to be evaluated independently of whether the radiopharmaceutical has been developed for research or for commercial purposes. Non-clinical risk assessment should be performed to estimate the risk–benefit profile of the new radiopharmaceutical. This risk assessment should include mitigation procedures. This section discusses only toxicology concerns stemming from the biological activity that the radiopharmaceutical possesses as a drug. Radioprotection and radiotoxicity are discussed in Section 6.

Usually, diagnostic radiopharmaceuticals are administered at tracer levels (micrograms per kilogram of body weight); namely the compound is administered in such a low dose that no biological effect is induced. Consequently, this dose typically results in no toxic effects. However, some toxins are known to be lethal even at these dose levels, or even lower. For example, the LD_{50} of botulinum toxin in mice ranges from 1 to 5 ng per kilogram of body weight [84]. Consequently, it is essential to study the toxicological effects of any new radiopharmaceuticals.

Non-clinical toxicological studies should be performed in agreement with the specific regulatory requirements in each country where the experiments are to be carried out, and the experimental plan should be carefully discussed with national regulatory authorities. The study design should comply with the 3R principles. In some cases, literature data that document the necessary toxicology parameters might be available. If these data suggest no toxicological concerns, they are usually sufficient to receive permission from authorities for human application. Finally, non-clinical toxicology studies should be performed in agreement with good laboratory practice (GLP) procedures and conducted in a GLP certified laboratory.

Toxicity studies should be presented in the clinical trial application file and included in the ‘investigational medicinal product dossier’ together with information such as quality specifications, non-clinical pharmacology, efficacy (diagnostic or therapeutic) and dosimetry. In the European Union (EU), the investigational medicinal product dossier should contain a statement on the application of GLP or equivalent standards, as referred to in Article 25(3) of Regulation (EU) No. 536/2014: “Non-clinical information submitted in an application dossier shall be based on data derived from studies complying with Union law on the principles of good laboratory practice, as applicable at the time of performance of those studies” [85]. Non-clinical safety studies should also contain an estimation of the no observed adverse effect level (NOAEL),

which is necessary to identify the starting dose for clinical phase I studies. For radiopharmaceuticals, the dose of radioactivity that should be administered to patients is usually estimated from biodistribution and dosimetry studies.

Finally, it should be noted that the test material (i.e. radiopharmaceutical formulation) used in toxicity studies should be representative of what will be used in the clinical trial in terms of qualitative and quantitative content of the radiolabelled and unlabelled pharmaceutical substance and impurities. For this reason, the quality of any test material should be fully described and the final formulation should be specified to the GLP laboratory responsible for the conduction of toxicological studies and the preparation of non-clinical safety reports.

5.2. EXISTING GUIDELINES AND RECOMMENDATIONS

Guidelines and recommendations for non-clinical toxicity studies are in general not specifically focused on radiopharmaceuticals. Recently, the European Medicinal Agency has published a guideline on the non-clinical requirements for radiopharmaceuticals [86]. This is an important development for scientists and industries working with radiopharmaceuticals. Thanks to a large international debate between regulatory authorities and the scientific community, the concept of tracer and tracer theory and the term 'microdose' have been associated with imaging radiopharmaceuticals. The concept of the microdose was originally proposed to simplify the non-clinical safety dossier requested for a new medicine administered at very low doses and was included in the Guideline of the International Conference for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [87]. The ICH aims to standardize regulatory requirements for marketing authorization of drugs under development. These guidelines have regulatory value when adopted by local regulatory authorities. In Ref. [87], diagnostic radiopharmaceuticals are specifically mentioned in section 7, which is dedicated to exploratory studies based on the administration of microdose agents [87].

Microdose exploratory studies, and in particular Approach 1 and Approach 2 described in section 7 of Ref. [87], described below, can be used as the basis for the design of safety studies of radiopharmaceuticals or as a starting point for local authorities. Approach 1 can be applied when the total dose administered is ≤100 μg and the total dose is ≤1/100 of the NOAEL and ≤1/100 of the pharmacologically active dose (scaled in mg/kg for intravenous and in mg/m^2 for oral administration). Approach 2 is applied when more than one administration is required. The total cumulative dose has to be ≤500 μg, a maximum of five administrations are permitted with a washout between doses

(representing six or more actual or predicted half-lives), each dose has to be lower than ≤100 µg and each dose should result in <1/100th of the NOAEL and in <1/100th of the pharmacologically active dose calculated for Approach 1.

In Approach 1, toxicity studies are based on an ‘extended single dose toxicity study’. The study has to be conducted in a minimum of one species (usually rodents). The extended single dose toxicity study should evaluate haematology, clinical chemistry, necropsy data and histopathology in a minimum of ten test animals per sex per group for all groups on day 2. Additional data should be provided for five test animals per sex per group on day 14 in the group that is planned to support the clinical dose [87]. A maximum dose of 1000 times the clinical dose on a mg/kg basis for intravenous administration can be used. For Approach 2, a seven day repeated dose toxicity study in one species, usually in rodents, should be carried out. Haematology, clinical chemistry, necropsy and histopathology data should be included in this dataset.

For both approaches, genotoxicity studies are not requested, but appropriate PK and dosimetry should be performed. ID can be determined from biodistribution studies and from the specific activity of the radiopharmaceutical at the time of injection. It has to be proven that the dose used in the prospective microdose studies is ≤1/100 of the NOAEL. This means that a dose 100 times higher than what is intended for use in humans should not cause any adverse effects.

The dose limit for microdose studies is defined in micrograms, not in moles. This regulation may represent a bias against biological drugs with large molecular weight. This issue is clearly stated in the new European Medicines Agency guideline, which, at the time of writing, is still under public consultation [86]. In August 2018, the United States Food and Drug Administration (FDA) also published a document on non-clinical safety evaluation of diagnostic radiopharmaceuticals [88]. This publication is also based on Ref. [87]. The guideline clearly states that radiopharmaceuticals are administered in low doses, and adverse events related to the unlabelled compounds are not likely to occur. Furthermore, the guideline indicates that toxicity should be evaluated via an extended single dose toxicity protocol: 14 days observation, interim necropsy and evaluation of body weights, clinical signs, clinical chemistries, haematology and histopathology (high dose and control only if no pathology is seen at the high dose). The protocol specifies the use of a single species (both sexes) with the same route of administration as intended in humans and with a formulation as similar as possible to the formulation intended for use in clinical trials [88]. The study should demonstrate that the unlabelled compound does not induce adverse effects at a dose larger than that intended for clinical trial (i.e. at 100 times the human dose).

5.3. GUIDANCE ON THERAPEUTIC RADIOPHARMACEUTICALS

When the microdose concept cannot be applied, there are a set of other guidelines to consider; for example: (a) the guidance of the FDA for industry [89], (b) the ICH guideline in Ref. [87], and (c) other ICH guidelines depending on the product and application [90–92]. The guidance in Ref. [89] outlines a process for deriving the maximum recommended starting dose (MRSD) for FIH clinical trials of new molecular entities in adult healthy volunteers and recommends a standardized process by which the MRSD can be selected.

The goals of this guidance are as follows:

(a) Establish a consistent terminology for discussing the starting dose;
(b) Provide common conversion factors for deriving a human equivalent dose (HED);
(c) Delineate a strategy for selecting the MRSD for adult healthy volunteers, regardless of the projected clinical use.

The major elements in the assessment of MRSD are the determination of the NOAELs in the tested animal species, conversion of NOAELs to HED, selection of the most appropriate animal species and application of a safety factor. The process of calculating the MRSD should begin after the toxicity data have been analysed. Although only the NOAEL should be used directly in the algorithm for calculating the MRSD, other data (exposure–toxicity relationships, pharmacologic data or prior clinical experience with related drugs) can affect the choice of the most appropriate species, scaling and safety factors. The NOAEL for each species tested should be identified and then converted to the HED using appropriate scaling factors.

5.4. CALCULATION OF HUMAN EQUIVALENT DOSE

The body surface area normalization and the extrapolation of the animal dose to human dose should be done in one step by dividing the NOAEL in each of the animal species studied by the appropriate body surface area conversion factor. This conversion factor is a unitless number that converts mg/kg dose for each animal species to the mg/kg dose in humans, which is equivalent to the animal's NOAEL on a mg/m^2 basis (Table 8). The result is the HED value.

A safety factor should then be applied to the HED to ensure that the first dose in humans will not cause adverse effects. In general, one should consider using a safety factor of at least 10. The MRSD should be obtained by dividing the HED by the safety factor. Importantly, NOAEL is not the same as 'no observed

TABLE 8. CONVERSION OF ANIMAL DOSE TO HUMAN EQUIVALENT DOSE USING BODY SURFACE AREA [93]

Species	Conversion factor, K_m (kg/m^2)[a]	Conversion factor, X[b]
Human (60 kg)	37	n.a.[c]
Mouse	3	12.3
Rat	6	6.2
Rabbit	12	3.1
Dog	20	1.8

[a] To convert animal dose in mg/kg to human equivalent dose in mg/m², multiply the animal dose by K_m.

[b] To convert animal dose in mg/kg to human equivalent dose in mg/kg, divide the animal dose by X.

[c] n.a.: not applicable.

effect level' (NOEL). The definition of NOAEL, in contrast to that of NOEL, reflects the view that some effects observed in the animal may be acceptable pharmacodynamic actions of the therapeutic and may not raise a safety concern. Species conversion of doses for medicinal studies is also reviewed in Ref. [93].

6. DOSIMETRY

6.1. STUDY DESIGN

Preclinical studies with radiopharmaceuticals offer a helpful guidance before setting up in human trials. Dosimetry assessments in animals lead to estimates for the recommended amount of activity that can be safely injected for initial clinical testing of the radiopharmaceutical drug in humans. Biodistribution studies should be performed to determine the TACs in physiological organs, either by dissection of several animals or by quantitative imaging at several time points after injection. Organs at risk can be identified both by observation of toxicity in mice and by comparing the absorbed dose in mice with the corresponding expected value in humans. Absorbed dose limits for normal organs are well known for external beam exposures and increasingly also known for radionuclide therapy internal

exposures. Often, once translation of a radiopharmaceutical occurs, true human dosimetry can be determined in the first healthy volunteers who are scanned.

6.1.1. MIRD principle for dosimetry calculations

The absorbed dose of an organ or tissue expresses a measure of the damage caused by ionizing radiation in that organ or tissue. The absorbed dose is defined as the energy absorbed per unit mass and is expressed in grays, corresponding to joules per kilogram. For external exposures, the absorbed dose can be measured directly through tissue equivalent dose meters, such as ionization chambers. For internal dosimetry the absorbed doses are derived from dosimetry models according to the MIRD, as shown in Eq. (3) [94]:

$$D(\text{target}) = \sum_{\text{source}_i} \tilde{A}(\text{source}_i) \times S(\text{target} \leftarrow \text{source}_i) \quad (3)$$

where

D is the absorbed dose;
$\tilde{A}$ the cumulative activity in source organ i (source_i);

and S stands for the absorbed dose rate to the target organ per unit activity in the source organ.

To determine the cumulative activity, the TAC in a source should be known. The cumulated activity is the integral over time of the TAC, or equivalently the area under the TAC curve, as seen in Eq. (4):

$$\tilde{A}(\text{source}) = \int_0^{\infty} A(\text{source}, t)\,\mathrm{d}t \quad (4)$$

The time integrated activity $\tilde{A}$ is the sum of the total number of nuclear disintegrations in the source, or the area under the TAC curve. Many times, the time integrated activity coefficient $\tilde{a}$ is used, formerly also known as residence time. It indicates the number of disintegrations per administered activity (A_0), as shown in Eq. (5):

$$\tilde{a} = \tilde{A}/A_0 \quad (5)$$

By using $\tilde{a}$ instead of $\tilde{A}$ in Eq. (3) the absorbed dose per administered activity is obtained. Biodistribution data and SPECT or PET imaging quantification data are usually given as activity concentrations (in percentage of injected activity (%IA/mL) or amount of activity (kBq/mL) per volume). The TAC curve for an organ can then be based on the activity concentration over time, either multiplied with the (mean) actual organ mass or with mass according to the standard phantom used for the *S* factors.

For the preclinical setting, various *S* factors have been determined for standard geometries using the scalable mouse phantom MOBY and the rat phantom ROBY as input [95]. These models have been used to generate *S* values for several combinations of source and target organs with various types of radionuclides [96, 97]. In the context of radiotherapeutics, the range of β particles in humans can be considered to be minimal in relation to the source organ dimensions and, usually in human applications, β radiation is considered to be non-penetrating (i.e. all β energy is locally absorbed within the source organ) [94]. In small animals, however, the range of β particles can become comparable to the organ dimensions. For instance, the β particles from ^{90}Y (with a mean energy of 933.4 keV and an end point energy of 2280.1 keV) have a range in tissue of 4 mm (mean) to 11 mm (maximum) according to the continuous slowing down approach[1]. As electron tracks do not follow a straight line, but a random scattered path, a better parameter to estimate their range is X_{90}, which is the radius of the sphere in which 90% of the β energy is absorbed [98]. The X_{90} value in water for ^{90}Y is 5.4 mm, while the low energy β emitter ^{177}Lu (mean energy of 133.5 keV and end point energy of 498.3 keV) has X_{90} = 0.62 mm in water. A typical 25 g mouse bears a 0.302 g kidney with approximate dimensions of 3.5 × 3.5 × 6 mm^3 [96]. Here ^{90}Y will cause radiation exposure also to the surrounding tissue when it is taken up in the mouse's kidneys, whereas β radiation from renal uptake of ^{177}Lu will be mainly absorbed within the kidneys with a much smaller exposure to surrounding tissues from its γ ray component.

The range of α particles in tissue is of the order of micrometres. For example, the 5.83 MeV α particle emitted by ^{225}Ac has a continuous slowing down approach range of 48 μm, and the 8.38 MeV α particle emitted by ^{213}Po has a continuous slowing down approach range of 85 μm in tissue (α particle ranges determined with the Astar code[2]). The high mass of α particles, or He^{2+} nuclei, leads to straight α particle tracks by conservation of momentum in (electron) scatter events. These ranges reach only neighbouring cells adjacent to the cells

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[1] Calculated with the Estar code, available at https://physics.nist.gov/PhysRefData/Star/Text/ESTAR.html.

[2] Available at https://physics.nist.gov/PhysRefData/Star/Text/ASTAR.html

with the α emitter uptake. The consequence is a possible non-uniformity of the dose distribution by α particle emitters.

6.1.2. Pharmacokinetic modelling of in vivo data to derive time–activity curves

The TAC ($A(t)$) in each organ with physiological uptake and in animal models (e.g. transgenic models of neurodegenerative disease, tumour-bearing mice) should be derived either from biodistribution data or from quantitative imaging studies and can be used to perform pharmacokinetic modelling (Fig. 2). For biodistribution studies, the timing of animal euthanasia and the scan moments should be carefully chosen. For the PK determination of the compound under investigation, essentially four basic kinetic models can be anticipated [99]:

(a) Instantaneous uptake without biologic clearance — physical decay (λ_{phys}) only;
(b) Instantaneous uptake with biological clearance and physical decay;
(c) Gradual uptake without biologic clearance — physical decay only;
(d) Gradual uptake with biological clearance and physical decay.

Reference [99] gives excellent advice on the sampling points needed. Selection of PK temporal sampling points can be based on the physical half-life (T_p) of the radionuclide or the effective half-life (T_{eff}) of the

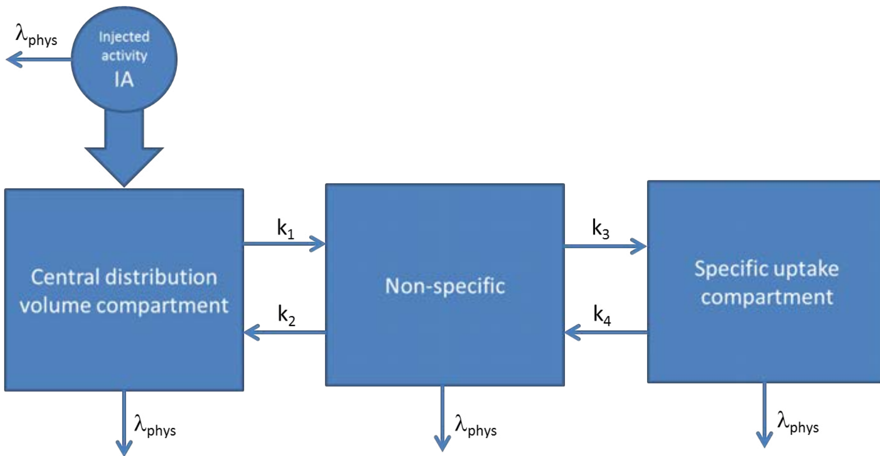

FIG. 2. Pharmacokinetic model (courtesy of M.W. Konijnenberg, Erasmus Medical Centre, Netherlands).

radiopharmaceutical in the blood, organ or total body. The effective half-life is calculated by combining both the physical decay and the biological clearance half-life (T_b), through Eq. (6):

$$T_{eff} = \frac{T_p \times T_b}{T_p + T_b} \quad (6)$$

A minimum of three sampling times should be chosen to determine each clearance or uptake compartment (Fig. 2). Each PK distribution compartment can be described by an exponential function, hence the need for three time points. Reference [99] recommends one or two data points to be taken at fractions of T_{eff}, one to be taken near T_{eff}, and one or two other data points to be taken at 3–5 × T_{eff}. Radiopharmaceuticals following kinetic model (d) may therefore need six to nine time points for an accurate TAC and PK model. When nothing is known on the kinetic model to be expected, a dozen time points are needed, distributed between the time of injection and 3–5 × T_p.

6.1.3. Biodistribution data organ concentrations

The TAC result from a multiple time point biodistribution study is usually expressed as activity concentration. The concentration TAC can also be integrated to calculate the TAC concentration in the source volume, which is given by Eq. (7):

$$\frac{\tilde{A}}{m}(\text{source}) \quad (7)$$

Total volumes of the excised organs are needed to choose the right dosimetry models or phantoms for *S* value calculation. For oncology radiopharmaceuticals, it is common to determine dosimetry in tumour-bearing animals. In neuroscience and other applications, it is more typical to conduct dosimetry studies in control animals rather than transgenic animals, probably owing to the high cost and long ageing timelines (≥18 months in some instances) required to access the latter.

6.1.4. Quantitative data of small animal SPECT/PET imaging

The use of a preclinical SPECT or PET camera to determine the TAC in source organs can lead to a reduction in the number of animals needed for the experiment. The animal scanner needs to be calibrated (see Section 4.4.7) for the radionuclide under investigation. The delineation of organs in the SPECT or PET image can be quite cumbersome and, if available, additional anatomic imaging

by CT or MRI could be useful in assisting with defining VOIs. VOIs should be drawn over the source organs to collect the mean activity per gram of tissue at each time point.

6.1.5. Phantoms and models for small animal dosimetry

The use of 'standardized' reference dosimetry calculation phantoms has considerably improved the absorbed dose results in preclinical experiments. The use of these phantoms has to be chosen with care, however, as the radiopharmaceutical under research may show a highly non-uniform uptake in some organs and, in particular, in tumours. For instance, uptake of activity in the cortex of the kidney instead of homogeneous uptake in the whole kidney may increase the absorbed dose by a factor of 1.5–2. In addition, the anatomy of the mice in the experiment might be different from the standardized MOBY anatomy. Relative differences of 10–40% have been found between dosimetry codes claiming to use the same MOBY phantom, mostly owing to differences in spatial sampling matrices [100].

An example of small animal dosimetry is shown in Fig. 3 [101] from a study investigating the dose response effect of Gelofusine (manufactured plasma volume substitute) on renal uptake and retention of radiolabelled octreotate in rats with CA20948 tumours [101]. Single-exponential curves were fitted through the data using the least squares method and demonstrated pronounced effects by the kidney protection agents in the renal uptake, but not in the tumour. When conducting preclinical mouse dosimetry, there can be advantages to having more than three time points. Inclusion of additional time points (beyond 48 h) can enable a two compartment model that fits a constant compartment, indicating the specific uptake within the tumour, and a second slower washout exponential in the kidneys and pancreas. Statistical decision criteria, as the F test or the Akaike information criterion, can be used to decide whether the additional compartment is necessary.

6.1.6. Voxel based dosimetry models

Voxelized uptake patterns from SPECT or PET imaging can be used directly to determine the absorbed dose at the voxel level. Depending on the voxel size and the range of the β emission spectrum, the absorbed dose can be calculated by assuming local energy deposition in the voxels; else the radiation transport has to be taken into account, which can be done with a point kernel method for homogeneous media or by Monte Carlo calculations for heterogeneous media. However, all voxel dosimetry methods do need a voxelized TAC, which would require reliable image registration of all SPECT or PET images taken over time.

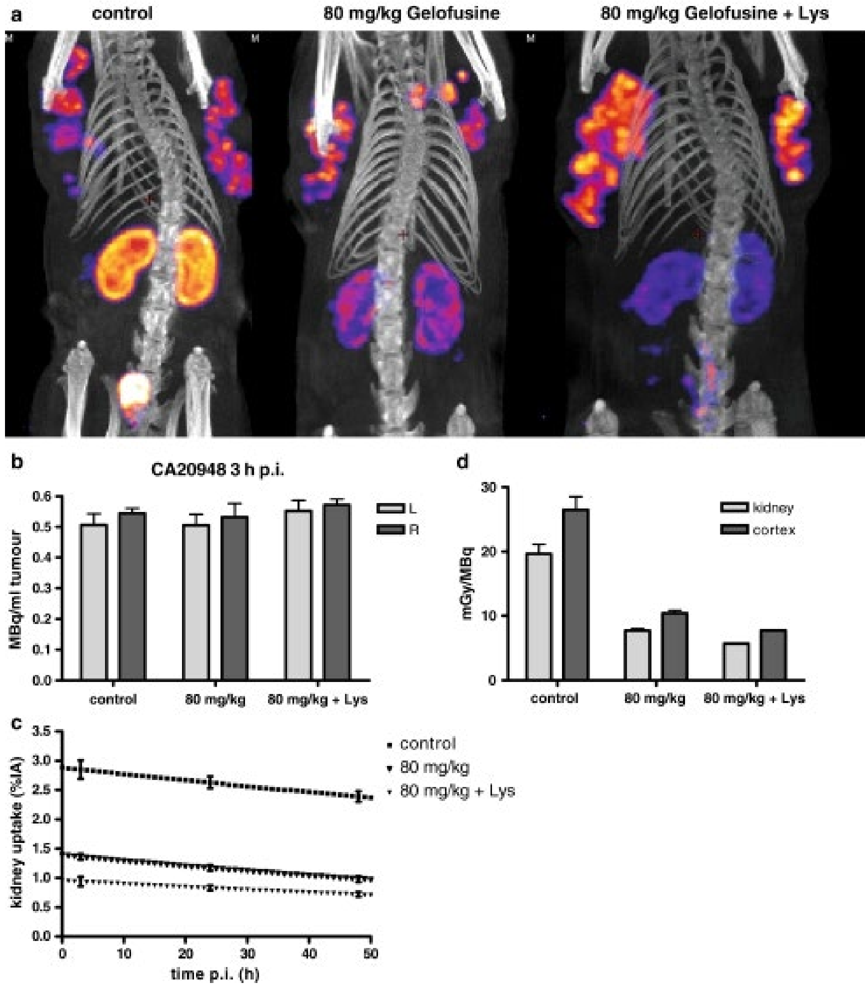

FIG. 3. NanoSPECT/CT imaging, quantification and dosimetry (adapted with permission from Ref. [101]). (a) NanoSPECT/CT images of CA20948 tumour-bearing rats, 3 h after injection of 15 µg ^{111}In-DOTA,Tyr^{3}-octreotate labelled with 60 MBq of ^{111}In, without (left) and with (centre and right) co-injection of 80 mg/kg of Gelofusine (and 400 mg/kg Lys). (b) Quantification of retained radioactivity in CA20948 tumours of imaged rats using InVivoScope software. Each tumour nodule inside a CA20948 tumour was analysed separately. No significant differences were found. (c) Residence time of ^{111}In in kidneys, as determined in NanoSPECT/CT images taken 3, 24 and 48 h post injection (p.i.) of 15 µg ^{111}In-DOTA,Tyr^{3}-octreotate. The washout of ^{111}In is plotted for three rats: control, with 80 mg/kg of Gelofusine alone and combined with 400 mg/kg Lys. (d) Dose calculation of ^{111}In-DOTA,Tyr^{3}-octreotate for the whole kidney or renal cortex only, expressed in mGy/MBq ^{111}In. Renal radiation dose in a control rat is compared with rats receiving 80 mg/kg of Gelofusine alone or co-administered with 400 mg/kg Lys.

6.1.7. Small scale dosimetry models

Especially for short ranged particle emitters, such as α particles or low energy electrons, more detailed models may be needed to calculate the absorbed dose *S* values in organs (or tumours), with either functional subunits or stem cell volumes defined as target regions. The sub-organ/tumour distribution can be derived from autoradiography and, in the case of α emitters, also with an α camera. Especially for the kidneys, more detailed standardized dosimetry models now exist. The most simple example is for the renal cortex, using autoradiography at one time point as the source distribution [102]. More detailed dosimetry models for the kidneys now include glomeruli and proximal and distal tubules as source and target organs [103].

6.1.8. Extrapolation of animal dosimetry to human dosimetry

Preclinical experiments should identify potential organs at risk and provide indications for absorbed dose threshold values for risk induction. Translation of absorbed dose limits found in the preclinical setting to the human patient should be performed as guidance, taking into account not only the difference in morphology but also the equivalence of toxicity endpoints in humans and small animals. Also, translation of PK from small animals to humans should be considered cautiously.

The FDA has issued guidance for studies needed for drug approval of oncology therapeutic radiopharmaceuticals [104]. It is advised to use organ TACs derived from preclinical experiments in relevant animal models to estimate the %IA, residence time and time integrated activity in human organs. The estimated human values should be used to guide the decision on an ideal prescription dose for FIH use of the therapeutic. Increasingly, however, therapeutic radiopharmaceuticals are developed as a complement to a diagnostic analogue, which has been tested FIH. The human PK of this diagnostic can be used to determine the safe initial activity for its therapeutic pair drug.

6.1.8.1. Uptake scaling

Several models exist to extrapolate animal biodistribution data to human PK expectation values. The most commonly used scaling depends on the relative organ (with mass *m*) to total body weight (mass *M*) ratio in mouse and human, leading to the mass scaling option in Eq. (8):

$$\left(\frac{\%\text{IA}}{\text{organ}}\right)_{\text{human}} = \left[\left(\frac{\%\text{IA}}{\text{g}}\right)_{\text{animal}} \times M_{\text{animal}}\right] \times \left(\frac{m}{M}\right)_{\text{human}} \tag{8}$$

Another option is to assume physiological uptake in animal organs equal to what is to be expected in humans, leading to the invariant organ option in Eq. (9):

$$\left(\frac{\%\text{IA}}{\text{organ}}\right)_{\text{human}} = \left(\frac{\%\text{IA}}{\text{organ}}\right)_{\text{animal}} \tag{9}$$

When the time kinetic scale in animals is the same as in humans, the time integrated activity coefficients would translate from animals to humans according to the mass scaling option in Eq. (10):

$$\tilde{A}_{\text{human}}(\text{source}) = \left[\left(\frac{\tilde{A}}{m}\right)_{\text{animal}}^{\text{source}} \times M_{\text{animal}}\right] \times \left(\frac{m}{M}\right)_{\text{human}} \tag{10}$$

and in the invariant organ uptake option shown in Eq. (11):

$$\tilde{A}_{\text{human}}(\text{source}) = \left(\frac{\tilde{A}}{m}\right)_{\text{animal}}^{\text{source}} \times m_{\text{animal}} \tag{11}$$

6.1.8.2. Time scaling

Allometric scaling of physiology from animals to humans has not yielded exact relations. Empiric rules have been set out assuming that the heart rate is proportional to body weight *M*. Under this assumption, the allometric time rule has been empirically determined by Gerlowski et al. to be as in Eq. (12) [105]:

$$t_{\text{human}} = \left(\frac{M_{\text{human}}}{M_{\text{animal}}}\right)^{0.25} \times t_{\text{animal}} \tag{12}$$

where t_{human} and t_{animal} are the time scales corresponding to humans and animals, respectively.

When time scaling is applied together with mass scaling of the uptake, extrapolation of the time activity coefficients from animals to humans would be as in Eq. (13):

$$\tilde{A}_{\text{human}}(\text{source}) = \left(\frac{M_{\text{human}}}{M_{\text{animal}}}\right)^{0.25} \times \left[\left(\frac{\tilde{A}}{m}\right)_{\text{animal}}^{\text{source}} \times M_{\text{animal}}\right] \times \left(\frac{m}{M}\right)_{\text{human}} \tag{13}$$

The extrapolated time integrated activity coefficient value needs to be multiplied by a human S value for the required source and target combination to reach an estimate of the human dosimetry from the preclinical animal data. Human S values and the whole dosimetry calculation can be obtained using several dosimetry packages, including Olinda/EXM [106] and IDAC-Dose [107].

7. DATA REPORTING AND MANAGEMENT

7.1. DATA REPORTING

Reporting of preclinical imaging data in scientific publications, study reports or academic publications should be performed in such a way that it is possible for a reader to understand the aim of the study, the materials and methods used and, ideally, to replicate the experiment. It is also encouraged to make raw data available (e.g. in an article's supporting information) to facilitate the replication of a preclinical imaging study. Accurate reporting is fundamental for reproducibility and thus the reliability of scientific results.

An excellent guideline on reporting animal experiments (the ARRIVE guideline) was published by Kilkenny et al. [108], and guidance for describing methods used in preclinical imaging papers has been presented by Stout et al. [109]. It is recommended to strictly follow these two guidelines, as they help to convey what was done and why. These guidelines also provide insight on how to assess the biological relevance of the study and finally the reliability and validity of the results obtained.

7.2. DATA MANAGEMENT

7.2.1. Study and animal identification

For each study and animal identification, it is recommended to establish an individual code in each imaging laboratory. This code can either be based on consecutive numbering of a study or numbering of the animals. It might also include abbreviations of the species used (e.g. M for mice, R for rats or NHP

for non-human primates), tracer used and/or imaging modality, animal type (e.g. wild type, transgenic, tumour-bearing) or even the date of the measurement. It is recommended to have individual numbers for each animal used to avoid mistakes in naming. Sometimes, animals already have a unique identification when included in a study; in that case, it would be advisable to rename them but keep the records of the old identifications and a translation sheet. Considerations of the blinded studies described in Section 4.2.1 should also be taken into account when identifying animals (e.g. those with the key to unblind a study should not be performing data analysis).

7.2.2. Data tracking

The establishment of a basic quality management system is advisable for easier data tracking. In such a system, templates for protocols can be created and printed out shortly before use. This will ensure that all users are following the same protocols and, ideally, documenting the same parameters.

A database for generating study identifications, animal identifications and web access, and for entering session specific information (such as injected probe, injection time and reconstruction parameters) might be a helpful tool. However, if the documentation is stored in file folders (either hardware or software) for each individual or study, data tracking is usually also simplified.

7.2.3. Archiving

At the outset, archiving criteria should be defined for all available and created data. This includes animal specific data (e.g. documentation on breeder, birth certificate, species, strain, transgenic information) and data on the radiopharmaceutical, acquisition and reconstruction protocol for all the acquired image data. It is recommended that the archiving system define which data, in what form, where, when and how long should be archived, as well as how archives will be backed up. Ideally, the archiving system will support the study and/or animal identification system so that studies can be easily located years after completion.

If the study is performed as a contract research study or as per GLP, the predefined archiving system and duration criteria have to be fulfilled. Such studies often define additional specific archiving requirements that need to be adhered to (e.g. documents should be stored in a fireproof safe and/or on a secure server for 10 years after completion of the study).

It is now also possible to convert current imaging files from preclinical studies into DICOM formats for archival and retrieval from PACS systems [110]. The benefits of using existing PACS solutions for preclinical imaging datasets

are the standardized data exchange interface, structured data content search and retrieval functionality, as well as mature vendor supported hardware and software solutions.

7.2.3.1. Archiving documents

Ideally, all the protocols, reports and documents are archived in a file folder named with the study identification. For easier retrieval, subdividing the folder into animal identifications or scan dates is advisable and further reiterates the importance of clear systems for study, animal and experiment identification from the outset.

7.2.3.2. Archiving files

Created imaging data can either be archived on a computer, a large disk array storage system (e.g. NAS (network attached storage) or SAN (storage area network) arrays), a file server, an external flash drive or hard drive, burned onto DVDs or, increasingly, institutional or third party cloud storage. For contracted studies, storage and/or security requirements for primary data and any backups should be accounted for.

Nuclear imaging data are typically present in three different file formats: raw data files (e.g. in list mode format), sinogram data files and reconstructed image data files. List mode and sinogram files easily exceed 20 GB, whereas image data files are in the order of 6–150 MB, depending on whether they are from dynamic or static studies. According to the available archiving system, it might be possible to store only the image files or to store all the files created. Storing list mode files offers the possibility to reconstruct images with changed parameters at a later time (e.g. using a different algorithm or different time frames). It is recommended to archive the analysed image files together with the analysis method (e.g. the defined VOIs) and the quantitative parameters obtained from the analysis.

Paperwork can be scanned and included in the electronic archiving system. It is recommended to use a consistent folder structure and identification in the archiving system.

7.2.4. Data retrieval

For image data analysis, it should be possible to retrieve the data hours, days or even years after image acquisition. Depending on the policy of each laboratory, data retrieval might be restricted to specific people or groups. A properly designed and supported computer network is required to ensure quick and easy flow of data for viewing and analysing.

8. FACILITIES REQUIREMENT

8.1. FACILITY DESIGN

Facility design and requirements are dependent on the intended scope of preclinical testing. The Organisation for Economic Co-operation (OECD) guidelines state (in section 3.1 of Ref. [111]):

> "1. The test facility should be of suitable size, construction and location to meet the requirements of the study and to minimise disturbance that would interfere with the validity of the study.
> 2. The design of the test facility should provide an adequate degree of separation of the different activities to assure the proper conduct of each study."

Moreover, the test facility should have a sufficient number of rooms or areas to assure the isolation of test systems and the isolation of individual projects, involving substances and/or organisms known to be or suspected of being biohazardous. Suitable rooms or areas should be available for the diagnosis, treatment and control of diseases, in order to ensure that there is no unacceptable degree of deterioration of test systems.

In the context of in vivo studies, suitable animal housing facilities should be available. OECD states in section 5.2 of Ref. [111]:

> "Newly received animal and plant test systems should be isolated until their health status has been evaluated. If any unusual mortality or morbidity occurs, this lot should not be used in studies and, when appropriate, should be humanely destroyed. At the experimental starting date of a study, test systems should be free of any disease or condition that might interfere with the purpose or conduct of the study."

> "During use, housing or containers for test systems should be cleaned and sanitised at appropriate intervals. Any material that comes into contact with the test system should be free of contaminants at levels that would interfere with the study. Bedding for animals should be changed as required by sound husbandry practice. Use of pest control agents should be documented."

It is particularly important to note that additional specialized animal facilities could be required when handling immunodeficient animals such as tumour-bearing animals or transgenic animals with neurodegenerative disorders.

Finally, waste disposal protocols have to be considered that are in line with local biohazard regulations.

Provisions should also be made for radioactivity when designing a facility. This includes everything from safe and secure receipt and handling of radiopharmaceutical doses, to facilities (and trained personnel) for managing animals that have been dosed with radioactive material, to waste disposal. Special animal facilities are traditionally not required for animals receiving short lived radionuclides (^{18}F, ^{11}C), as they can usually decay in the study room before being returned to their regular housing. Depending on local legislation, such short term storage of animals may be authorized in 'standard' laboratories, provided that suitable cabinets are in place. Local regulations should be consulted. Special housing provisions are often required by radiation safety policy for animals that have received longer lived diagnostic (^{89}Zr, $^{125/131}$I) or therapeutic (^{225}Ac, ^{177}Lu) radionuclides. Specific waste disposal strategies for radioactive waste should also be in place. These can include appropriate authorized radioactivity space for discarded radioactive laboratory supplies (such as syringes and paper towels) and animal waste (such as carcasses and bedding) to decay to background levels, before entering the regular laboratory hazardous/biohazardous waste disposal chain, or specific provisions for disposal of waste that is still radioactive. All waste entering the regular waste disposal chain should be surveyed prior to disposal to ensure that radioactive waste has indeed decayed to background levels. Anything that remains radioactive should be stored for longer or disposed of as radioactive waste.

There should be suitable storage rooms or areas available for supplies and equipment. Storage rooms or areas should be separated from rooms or areas housing the test systems and should provide adequate protection against infestation, contamination and deterioration.

Lastly, OECD guidelines also prompt archive facilities to ensure that data, plans, reports, etc. are safely stored and may be retrieved at any time for traceability.

8.2. EQUIPMENT

Major equipment used in imaging centres includes PET and SPECT scanners, γ counters, isotope calibrators, centrifuges, HPLC, TLC and autoradiography systems, cryostats/microtomes, cell culture equipment, sterile benches, cell counters, incubators, individually ventilated cages, microscopes, staining facilities, anaesthesia systems, physiological monitoring systems, automated fraction collectors, micro balances, laminar flow cabinets, personal computers and data archiving for images and data.

If the aims and scopes of experimental preclinical studies are focused on basic research, and not intended to contribute to a (radio)pharmaceutical registration or

licensing procedure, the requirements are less strict. However, some of the principles set by OECD should still apply, although informally, as they are related to an ordered organization of the working space aimed at reducing risks for personnel and animals, ensuring that operations are conducted in a proper way and reducing errors. Therefore, the size of the facility has to be consistent with the instrumentation to be installed. Ideally, scanners should be placed in separate rooms and have appropriate areas for handling of radioactive doses (e.g. appropriate shielding, dose calibrator) and injection (e.g. access to anaesthesia). If radioactive doses are to be prepared on site, suitably shielded hot cells should be installed (see Refs [112, 113]; a detailed discussion of radiopharmaceutical synthesis is outside the scope of this publication).

8.3. STAFFING REQUIREMENTS

The required number of professionals and their expertise depend on the activities performed in a laboratory. Some recommendations for trained personnel and their areas of expertise and responsibility are as follows:

(a) Radiopharmacist and/or radiochemist: radiopharmaceutical production and QC;
(b) Biologist and/or veterinarian: animal care, in vitro and in vivo assays;
(c) Physicist: imaging equipment calibration, QC and radioprotection;
(d) Biotechnologist: image acquisition;
(e) Image processing and quantification expert: image processing and quantification;
(f) Responsible scientist: long experience and in-depth knowledge in radiopharmaceutical evaluation;
(g) Technicians: assistance with basic activities such as animal care (food and housing care), laboratory material preparation and cleaning.

8.4. STAFF TRAINING

Staff training depends on national requirements. Basic recommendations include animal handling, biosafety, radiation safety, chemical safety, GLP (if applicable) and instrument specific training.

People involved in preclinical studies with radiopharmaceuticals should be well trained in laboratory animal manipulation and care, radioactive material manipulation and biosafety. Such training should be official and documented and should take place at least once a year.

8.5. SAFETY CONSIDERATIONS

8.5.1. Biosafety

Diseases transmitted from animals to humans are called zoonoses or zoonotic diseases and the infectious agents responsible can be viruses, bacteria or parasites. Laboratory animals should be supplied by a reputable commercial breeder to avoid colonies with zoonosis that would present risks to the personnel and investigators involved in the use of these animals.

Animals can also be infected as part of the research protocol, as infection models. When this is the case, only trained people should be involved in the experimentation, and attenuation of the infectious agent has to be considered to reduce the virulence of the agent.

Genetically modified organisms should also be considered in biosafety, since transgenic laboratory animals (especially mice) are in greater use in biomedical research [114].

Regarding biosafety, it is also important to take into account allergies, which are common among people working with laboratory animals (11–30%) [115].

When working with microorganisms, personnel safety aspects need to be considered. The basic requirements are knowledge of the infection agent, training and adequate infrastructure.

A useful guide for biosafety is the World Health Organization's Laboratory Biosafety Manual [116] which provides basic biosafety concepts and includes a section dedicated to laboratory animal facilities. Tables 9 and 10 are adapted from Ref. [116] and give important information about the requirements that need to be satisfied prior to executing a potentially hazardous experiment.

Infective microorganisms are classified into four risk groups:

(a) Risk group 1 (no or low individual and community risk): a microorganism that is unlikely to cause human or animal disease.
(b) Risk group 2 (moderate individual risk, low community risk): a pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available and the risk of infection spread is limited.
(c) Risk group 3 (high individual risk, low community risk): a pathogen that usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available.

(d) Risk group 4 (high individual and community risk): a pathogen that usually causes serious human or animal disease and can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available.

TABLE 9. RELATION OF INFECTIVE ANIMAL RISK GROUPS, LABORATORY TYPE, BIOSAFETY LEVELS, PRACTICES AND EQUIPMENT

Risk group	Biosafety level	Laboratory type	Laboratory practices	Safety equipment
1	Basic, Biosafety Level 1	Basic teaching, research	Good microbiological techniques	None; open bench work
2	Basic, Biosafety Level 2	Primary health services, diagnostic services, research	Good microbiological techniques plus protective clothing, biohazard sign	Open bench plus biological safety cabinet for potential aerosols
3	Containment, Biosafety Level 3	Special diagnosis services, research	As Level 2 plus special clothing, controlled access, directional airflow	Biological safety cabinet and/or other primary devices for all activities
4	Maximum containment, Biosafety Level 4	Dangerous pathogen units	As Level 3 plus airlock entry, shower exit, special waste disposal	Class III biological safety cabinet, or positive pressure suits in conjunction with Class II biological safety cabinet, double-ended autoclave (through the wall), filtered air

Note: Table adapted from Ref. [116].

TABLE 10. ANIMAL FACILITY CONTAINMENT LEVELS — SUMMARY OF PRACTICES AND SAFETY EQUIPMENT

Risk group	Containment level	Laboratory practices and safety equipment
1	ABSL-1	Limited access, protective clothing and gloves.
2	ABSL-2	ABSL-1 practices plus hazard warning signs. Class I or II biological safety cabinets for activities that produce aerosols. Decontamination of waste and cages before washing.
3	ABSL-3	ABSL-2 practices plus controlled access. Biological safety cabinets and special protective clothing for all activities.
4	ABSL-4	ABSL-3 plus strictly limited access. Clothing change before entering. Class III biological safety cabinets or positive pressure suits. Shower on exit. Decontamination of all wastes before removal from facility.

Note: Table adapted from Ref. [116]. ABSL: animal biological safety level.

When designing the facility, the following biosafety concepts should be considered [114]:

(a) Research line and scientific questions;
(b) Types of animal to be used;
(c) Hazardous materials (biological, chemical and radioactive) to be manipulated;
(d) Equipment necessary;
(e) Physical infrastructure (e.g. heating, ventilation, air-conditioning, electrical outlet, bench material);
(f) Standard operating procedures;
(g) Staff training (in radioprotection and biosafety).

8.5.2. Radiation safety

8.5.2.1. General concepts

Radiation safety is based on the ‘as low as reasonably achievable’ (ALARA) principle. ALARA is designed to minimize the radiation dose to workers (Table 11) (and members of the public) and the environmental release

TABLE 11. TYPICAL ALARA LIMITS FOR OCCUPATIONAL EXPOSURE

	Regulatory limit	ALARA I	ALARA II
Whole body	50 mSv in a year	5 mSv	15 mSv
Extremity	500 mSv in a year	50 mSv	150 mSv

TABLE 12. ENVIRONMENTAL RELEASE OF RADIOACTIVITY

	Regulatory limit	ALARA I	ALARA II
All radionuclides	Local limits (e.g. Refs [117–120])	20% of local limits	50% of local limits

of radioactive material (Table 12) [117]. ALARA is predicated on legal dose limits for regulatory compliance and is a requirement for all radiation safety programmes [117–120].

8.5.2.2. ALARA and time, distance and shielding

The ALARA principle acknowledges that even in small doses, exposure to radioactivity that has no direct benefit should be minimized as much as possible. To accomplish this, the following three basic radiation safety protective measures need to be taken:

(a) Time: the amount of time that a person spends near a radioactive source. Proximity to a radioactive source should be limited to the time required to perform the work and not longer. If a member of staff is in an area where radiation levels are elevated, they should complete work as quickly as possible and then leave the area to minimize exposure. There is no reason for a worker to spend more time around sources of radioactivity than absolutely necessary.
(b) Distance: how close a person is to a source of radioactive material. Staff should maximize their distance from a radioactive source as much as possible. This is an easy way to protect themselves, according to the inverse relationship between distance and dose. When conducting a preclinical experiment, increasing the distance could involve moving away from the experiment, for example the PET scanner, once the animal has been injected.

(c) Shielding: putting something between the worker and the radiation source. The most effective shielding depends on the kind of radiation emitted by the source (e.g. α, β or γ), keeping in mind that many radionuclides emit more than one kind of radiation. For preclinical imaging, doses tend to be quite low (e.g. compared with radiopharmaceutical production, clinical imaging) but there is still potential for significant hand and whole body doses, even when handling small amounts of activity. Common shielding might involve syringe shields for animal doses, but they can often be impractical for the small syringes used to inject rodents, for example. Often, the gain from using such shielding is negated by slowing down injections and increasing the time that the staff member holds the dose in their hand. Thus, any solution will be a balance between time, distance and shielding. Other shielding could be between workers and animal scanners and, in the case of hybrid scanners, should account for both PET/SPECT and CT components.

8.5.2.3. Monitoring radiation exposure with personal dosimeters and dosimetry badges

Workers' exposure to radiation is typically monitored with personal dosimeters and dosimetry badges. Personal dosimeters are electronic devices that enable real time detection of the radiation field in a radioactive area. They have visual, audible and vibrating alarm indicators that are programmed to activate when defined radiation limits are exceeded. Dosimeter badges (e.g. Landauer whole body dosimeter badge, finger ring dosimeters) measure radiation exposure to workers over a given time (e.g. week, month or quarter). They employ optically stimulated luminescence and detect radioactivity exposure due to X rays, γ rays and β particles. Optically stimulated luminescence radiation detectors consist of a small strip of aluminium oxide. After the dosimeter period, the badge and rings are returned to the manufacturer for analysis. During this analysis, the aluminium oxide strip is stimulated with light. The light causes the dosimeters to emit an amount of luminescence that is proportional to both the frequency of the stimulating light and the amount of radiation to which the dosimeter has been exposed. The exposure data are then sent in a report to the radiation worker — and the facility's radiation safety officer — allowing continual monitoring of radiation exposure to staff during their day to day activities and indicating employees for which action might be required to reduce radiation exposure if possible.

Facilities establish ALARA I and II action limits so that the facility's radiation safety officer can intervene before legal limits are exceeded and before a staff member would be required to stop working with radioactive material for the rest of the calendar year. ALARA interventions include review of work being conducted in order to improve work practices and reduce radiation exposure

(i.e. apply time and distance principles), as well as identify any additional shielding (e.g. syringe shields, lead shielding) that might be required (shielding principle). Moreover, new procedures (e.g. new injection routes) should be practised without radioactivity to ensure safe handling is learned.

8.5.3. Animal safety

Preclinical imaging programmes should be committed to the safety and security of the personnel handling animals, as well as to all animals in care. Although minimal, there are some hazards associated with working in a laboratory and on experiments that include close contact with animals.

8.5.3.1. Safety for personnel handling study animals

(a) Animals and allergies

Although allergies affect only a small percentage of staff, allergic reactions (e.g. respiratory and skin disorders, rashes and eye, nose or throat irritation) are among the most common occupational hazards to be aware of while working with laboratory animals.

Symptoms typically develop within 12 months of beginning research with animals but can also appear after years of working with them. In order to prevent the development of an animal allergy, the following precautions are recommended:

- — Extremely well ventilated areas should be used for animal housing and experiments.
- — Appropriate personal protective equipment should be worn (e.g. gloves, protective clothing, face masks) to minimize direct exposure to animals, waste (e.g. urine) and/or animal dander.
- — Animal cages should be changed frequently while wearing personal protective equipment.

(b) Zoonotic diseases

As mentioned in Section 8.5.1, zoonotic diseases are diseases that can be transmitted between animals and humans. Although the risk of acquiring infections from laboratory animals is low, many zoonotic diseases can be serious. For example, rhesus macaques are often infected with the herpes B virus, which can be transmitted to humans by exposure to the animal’s saliva or other body fluids or tissues. Most infections are spread through skin injuries such as bites,

scratches, needle stick lacerations or through splashes of body substances. To minimize the risk of contracting a zoonotic disease such as herpes B, it is critical that animal handlers follow safety procedures and wear appropriate personal protective equipment. In the event of an exposure incident, details should be documented and reported to responsible persons, and medical care provided as necessary.

(c) Physical hazards

Physical hazards from animal work can include grabs, bites, scratches, contaminated needlestick injuries or, in the case of larger animals, traumatic injury (e.g. being hit or stepped on by a larger animal). Care should be taken when working with animals, including proper planning of activities in advance, wearing appropriate personal protective equipment and following all appropriate procedures and safety rules. It is important to note that even though scratches or bites may appear inconsequential, workers who have sustained an animal related injury should seek medical attention to avoid any potential complications such as allergic reactions and zoonotic diseases, as discussed above.

(d) Use of hazardous materials in animal research

Animal experiments involving hazardous materials should be carefully planned to ensure that the appropriate control measures are in place before beginning any experiment. Depending on the type of hazardous material used (e.g. radiation, biohazard), additional regulatory approval may be required before work can start. Any activities that involve the use of hazardous materials should meet the following requirements:

(a) Be conducted in accordance with established animal use protocols;
(b) Require use of appropriate personal protective equipment;
(c) Follow applicable safety policies and standard operating procedures.

Additional safety training may also be required for personnel working with hazardous materials. Training requirements are determined on a case by case basis depending on the individual’s basic training, the type of animal and specifics of animal handling, and the type of chemical and/or hazard that may be encountered during the research. In the context of preclinical imaging, radiation safety training will also be required for any animal worker handling or administering radioactive doses.

8.5.3.2. Animal safety

Animal safety and well-being should be a high priority in facilities conducting animal research activities and is essential for achieving certification from organizations such as Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), as explained in Section 8.6.3. The following aspects of animal safety should be considered:

(a) Veterinary care

To ensure the highest levels of care in animal research, dedicated veterinary staff (including licensed veterinarians and veterinary technicians with experience in laboratory animal medicine) should be available either as employees of the facility or, at the very least, as third party consultants. The veterinary staff should oversee daily observations of all animals in the research facilities to ensure their health and well-being. Animals in pain or distress should be treated or, if needed, euthanized using approved protocols.

(b) Institutional animal care and use committee

Facilities should consider establishing an institutional animal care and use committee (IACUC). The IACUC is responsible for overseeing the animal care and use programme and its components. It can be considered the preclinical equivalent of the institutional review board responsible for overseeing clinical research.

(c) Composition

The IACUC should have a minimum of three members, usually appointed by the head of a research facility. If the IACUC consists of four or more members, no more than three members can work in the same group at the institution. The IACUC has to be composed of a chairperson and at least two other members. Membership requirements should include the following:

— The members need to be qualified to regulate animal care;
— One of the members has to be a doctor of veterinary medicine with experience in laboratory animal research;
— One member should have no other relationship with the institution.

(d) Oversight

The IACUC's oversight of an institution's animal care and use is accomplished as follows:

- By reviewing animal research protocols;
- By inspecting facilities to ensure compliance with legal requirements.

The IACUC has to be able to ensure compliance and correction of problems in animal care when these are discovered. As such, fair treatment of whistleblowers who report animal welfare violations to the IACUC is mandatory.

8.6. ACCREDITATION

8.6.1. External review

From a practical perspective, preclinical studies involving radiopharmaceuticals are complex research studies that necessitate expertise in many different rules and regulations across multiple disciplines. As discussed in this section, knowledge of safe handling of radioactivity, biosafety considerations, health physics and animal regulations are all essential to conduct preclinical research with radiopharmaceuticals. Oversight for compliance at the institutional level is ensured by radiation safety officers, the IACUC, etc. However, since the rules and regulations are usually mandated by law, it is not uncommon for facilities working in the field to receive external review of areas such as radiation safety and animal handling from government agencies and/or third party organizations to ensure institutional compliance with the pertinent legal requirements in each of these disciplines.

For example, in the United States of America (USA), the Nuclear Regulation Commission is responsible for ensuring compliance with radiation safety requirements such as ALARA and dosimetry, while AAALAC and the United States Department of Agriculture oversees laboratory animal research activities.

8.6.2. Good laboratory practice

GLP is a quality management system composed of principles intended to ensure the quality, integrity, robustness and reproducibility of preclinical studies that are conducted in support of research or drug development. It was first

introduced in New Zealand and Denmark in the 1970s. GLP studies are usually required in drug submissions to agencies such as the FDA.

The OECD has established principles of good manufacturing practice [111] that include stipulations about the following:

(a) Test facility organization and personnel:
- Management responsibilities;
- Study director responsibilities;
- Principal investigator responsibilities;
- Study personnel responsibilities.

(b) QA programmes:
- General GPL principles;
- QA personnel responsibilities.

(c) Facilities:
- Test systems;
- Handling of test and reference items;
- Archives;
- Waste disposal.

(d) Apparatus, materials and reagents.

(e) Test systems:
- Physical/chemical;
- Biological.

(f) Test and reference items:
- Receipt, handling, sampling and storage;
- Characterization.

(g) Standard operating procedures.

(h) Performance of the study:
- Study plan objectives;
- Content of study plan;
- Running of the study.

(i) Reporting of study results:
- General GPL principles;
- Content of final report.

(j) Storage and retention of records and materials.

The FDA has rules for GLP specified in Ref. [121]. Preclinical trials on animals in the USA use these rules prior to clinical research in humans. Research in the USA that is not conducted under these regulations, or research done outside USA not conducted according to the OECD guidelines (or Ref. [121]), might be inadmissible in support of regulatory filings such as the FDA new drug applications.

Similarly, the European Council has adopted two basic directives concerning GLP principles: Directive 2004/10/EC and Directive 2004/9/EC [122, 123]. These directives state that Member States should designate the authorities responsible for GLP inspections in their territory and should follow the relevant OECD guidance [111] during laboratory and/or study audits. Other states are strongly encouraged to adhere to regulations such as the FDA and OECD rules for conducting GLP studies when running preclinical work.

In order to be considered a GLP laboratory, in some jurisdictions it is necessary to obtain GLP accreditation for demonstrated adherence to OECD GLP principles. In the USA, no accreditation programme for GLP is sponsored by the FDA (instead, facilities are inspected on a case by case basis for adherence to Ref. [121]), whereas GLP accreditation programmes exist in the EU and other countries (e.g. Australia, Canada, Japan and South Africa). The EU also has mutual recognition agreements with Israel, Japan and Switzerland [124]. However, under this GLP programme, accreditation for preclinical imaging is not currently available.

8.6.3. Association for Assessment and Accreditation of Laboratory Animal Care International

Animal care and handling may be accredited through associations such as AAALAC (a private, non-profit organization that promotes the humane treatment of animals in science through a voluntary accreditation programme), a Program Status Evaluation service and educational programmes. Although not legally binding, the above accreditation is nonetheless a suitable measure of the quality level of the intended preclinical facility.

Animal experiments in the EU are regulated by Directive 2010/63/EU, which follows the principles of replacement, reduction and refinement, which aim to "ensure that, wherever possible, a scientifically satisfactory method or testing strategy, not entailing the use of live animals, shall be used instead of a procedure" [125]. In the USA, "The transportation, care and use of animals should be in accordance with the Animal Welfare Act (7 U.S.C. 2131 et seq.)" [126]. Other specific legislation acts are currently enforced in countries that are members of the OECD. Among other general provisions, this legislation sets general standards for the use of animals for scientific research, emphasizing that special care has to be taken in case of endangered species of non-human primates, and setting requirements for facilities, animal handling, breeding, care, anaesthesia and euthanasia. Although radiopharmaceutical preparation and preclinical testing departments are usually not committed to handling animals, except for short term housing and storage (typically, no more than a few weeks), they need to comply with the above rules. During planning of a preclinical testing laboratory in which radioactive compounds are expected to be used and administered to small animals, legislation on radiation protection has to

be considered. The main legislation in the EU is Directive 2013/59/Euratom, which states the principles of radiation protection for professional operators and for the population. Although the main operations involving radioactivity carried out in such laboratories may vary, depending on the available instrumentation and on the aims and scope of the research, they generally include the following:

(a) Radioactive dose preparation;
(b) Radioactive dose administration to small animals;
(c) Signal acquisition (scan);
(d) Animal storage after tracer injection;
(e) Animal euthanasia and organ/tissue withdrawal for subsequent treatments (e.g. radioactive determination).

The aims and scope of animal experiments with radiopharmaceuticals may be roughly divided into two categories: (a) experiments aimed at gathering basic information on biological, biochemical and physiological functions (basic research), and (b) experiments designed to test chemical and biological substances applied to non-clinical health and environmental safety studies required by regulations for the purpose of registering or licensing pharmaceuticals. In the latter case, GLP principles apply. GLP was established by the FDA in 1976 to strengthen controls on the quality of non-clinical studies aimed to provide data for drug registration purposes. FDA regulation was then used as the basis for more general OECD guidelines that are currently enforced in the EU and in other OECD members. GLP principles may be seen as a general quality system for the organization of non-clinical studies; they define, for instance, the various degrees of responsibility assigned to the study director, the principal investigator and the operating personnel. An integral QA system is also aimed at guaranteeing traceability. GLP should be certified following inspections. GLP status is typically valid for two years, after which the facility has to be re-certified. In the EU countries, application of GLP principles is regulated by Directive 2004/10/EC [122], which includes the OECD guidelines in annex 1. From the above description, it is clear that in most preclinical applications of radiopharmaceuticals GLP is not required, but there are several situations in which GLP principles apply. For instance, in the case of studies aimed to provide data and information on a radiopharmaceutical proposed for marketing authorization or used in the development of other medicines that undergo marketing authorization.

Other regulations/legislation that may apply to preclinical testing of radiopharmaceuticals are biosafety rules, to be considered for biological material handling, storage and disposal. The main legislation act in this field in the EU is presented in Directive 2000/54/EC, which addresses the protection of workers from risks related to exposure to biological agents at work.

9. QUALITY ASSURANCE AND QUALITY CONTROL

9.1. QUALITY ASSURANCE AND QUALITY CONTROL FOR LABORATORY AND EQUIPMENT

An essential aspect for generating robust and repeatable scientific data is ensuring that laboratory equipment is in good working order, appropriate for the intended purpose and regularly calibrated and qualified. Adequate QA and QC oversight for laboratory equipment is therefore critical in preclinical laboratories, and especially those adhering to GLP. It is recommended that all laboratory equipment undergoes installation qualification (IQ), operational qualification (OQ) and performance qualification (PQ). The equipment manufacturer typically performs IQ/, while PQ is usually performed by the end user through, for example, method validation or process qualification. After IQ/OQ/PQ, equipment can be used in preclinical experiments. Additional checks may be required, such as daily system suitability or constancy checks, annual preventive maintenance or annual recalibration. Records of any IQ/OQ/PQ, preventive maintenance or other testing should be kept, as they may be requested during, for example, a GLP inspection.

9.1.1. Balances

Balances should undergo IQ/OQ/PQ upon installation. It is then recommended to perform checks using calibrated weights, such as ASTM (American Society for Testing and Materials) class 5 weights, on each day of use. Balances should be recalibrated annually, or if they fail a daily system suitability check.

9.1.2. Pipettes

Volumetric pipettes are used in most facilities that perform preclinical testing of radiopharmaceuticals. They can be used for a range of activities, including pipetting TLC samples (1 μL) and endotoxin testing samples (25 μL). Since samples can be used for quantitative analysis, calibration is of critical importance. If work according to GLP principles is being conducted in a laboratory, then the calibration should be traceable to internationally acceptable standards such as those of the National Institute of Standards and Technology, and ISO 17025 accredited pipette calibration laboratories should be used to recalibrate pipettes annually (or as needed).

9.1.3. Dose calibrators

Dose calibrators (pressurized gas filled ionization chambers) are used to assay radioactivity amounts in, for example, dose vials and syringes. Since their use is critical in preclinical testing of radiopharmaceuticals, routine QC tests are essential to ensure their proper function. After installation or service of a dose calibrator, its geometry (position and volume) dependent response have to be measured and its volume dependent correction factors relative to the standard volume (e.g. 10 mL) need to be derived. Additionally, constancy and accuracy checks are recommended daily, as well as at least quarterly checks of linearity.

For the constancy test, a National Institute of Standards and Technology traceable reference source (e.g. ^{137}Cs) is positioned in the dose calibrator, and then the radioactivity is measured on each scale and recorded. Daily readings should agree within ±10%.

For the accuracy test, at least two National Institute of Standards and Technology traceable reference sources (e.g. ^{137}Cs, ^{68}Ge, ^{57}Co) should be measured separately in the dose calibrator on each scale, and the radioactivity measurements recorded. For each source, the measured activity on each scale should agree with the source's true activity within ±10%.

The quarterly check of linearity involves measuring a highly radioactive source (e.g. 37 GBq of ^{99m}Tc or ^{18}F), independently calibrated, at various intervals over 12 half-lives of the radionuclide (approx. 24 h for ^{18}F or 72 h for ^{99m}Tc). The measured radioactivity is then plotted against time (using a semilogarithmic scale), and the line of best fit for the data is determined. For each data point, the difference between the measured radioactivity and the radioactivity on the line of best fit should be ≤10%.

9.1.4. Well counters

Well counters are used instead of dose calibrators when high sensitivity counting of samples containing very low levels of radioactivity is required, such as blood samples taken during preclinical experiments (e.g. to determine arterial input function or analyse for the presence of radioactive metabolites), or wipes for surveys of removable contamination that are required by regulatory agencies.

Routine QC tests for well counters include checking the background, constancy and efficiency (i.e. sensitivity). If the counter is also equipped with a multichannel analyser, the photopeak energy window should also be monitored.

9.1.5. Gamma counters

Gamma counters are used for a variety of applications in an imaging centre, including counting wipes and analysing samples from biodistribution studies. Generally speaking, γ counters require relatively low maintenance, but some general guidelines for routine maintenance include the following:

- Cleaning the housing of the γ counter and wiping the γ counter;
- Cleaning the conveyor belt with a clean cloth;
- Checking the background periodically to monitor for contamination, and decontaminating if needed;
- Performing a calibration curve for every radioisotope used by measuring a series of decreasing radioactivity concentrations.

As for all instruments discussed in this publication, it is recommended that annual preventive maintenance is performed.

9.1.6. Phosphor imagers

Similar to γ counters, phosphor imagers tend to require relatively low maintenance beyond manufacturer recommended IQ/OQ/PQ and annual preventive maintenance. Other than that, it is recommended that the instrument and the laboratory, as well as the phosphor imaging plates, be kept clean. Phosphor imaging plates are subject to wear on the black side during normal handling and use. They can appear scratched, while the sensitive white side remains relatively smooth and clean. Scratches on the black side have no effect on the quality of the image and are indicative of common wear and tear. If the white phosphor side becomes scratched, the instrument operators need to ensure that plates are being handled correctly. Plates can be washed with soap and water and dried, taking care not to scratch them.

9.1.7. Chromatography systems

Chromatography systems, such as gas chromatography (GC), HPLC and TLC systems, are used to conduct QC and stability testing of radiopharmaceutical doses, for example to analyse blood samples for presence of radiometabolites. They are sophisticated analytical instruments that should be properly installed and maintained. These instruments undergo IQ/OQ/PQ when they are installed and each time they are moved. It is also highly recommended that they undergo annual preventive maintenance, ideally performed by the manufacturer. Besides these scheduled activities, it is recommended that system suitability testing be

completed at each use. This is usually accomplished by running two standard injections; the measured peak area of the two peaks should agree within 5% for automated injections or within 10% for manual injections. The results are averaged and used with the standard concentration to obtain a calibration factor, which is used in subsequent sample injections on that day. If this test fails, an intervention is required (e.g. replacing septa or liners, cleaning, conditioning or replacing the HPLC or GC column). The tailing factor and resolution should be determined from one of the two chromatograms and noted in the experiment record (e.g. lab book, batch record).

9.1.8. Laminar airflow hoods

Laminar airflow hoods (LAFs) are the typical class 5 [127] environments available in imaging centres. Air is taken in through a HEPA filter and blown in a very smooth laminar flow towards the user. LAFs are used for conducting aseptic manipulations, such as aseptic assembly of radiopharmaceutical dose vials prior to synthesis, sterility testing and cell culture.

IQ/OQ/PQ should be performed upon installation to verify the laminar airflow (e.g. 28 m/min ± 20%). After installation, the HEPA filter of the LAF should be inspected and certified every six months. The HEPA filter removes bacteria from the air, so it is critical to verify that it is functioning correctly. It is typical to also conduct air sampling every six months to ensure a class 5 environment [127] (≤3520 particles per cubic metre or ≤100 particles per cubic foot).

LAFs should be cleaned with sterile 70% isopropyl alcohol before each use and given sufficient time to dry (~10 min). Additional cleaning with a sporicide (e.g. Spor-Klenz) is recommended once per week.

Critical aseptic manipulations should be accompanied by appropriate environmental monitoring using touch and settle plates. Action levels are usually set at >3 colony forming units per plate and could mandate cleaning and/or recertification of the LAF and/or retraining of the operator [128].

9.1.9. Incubators

Incubators used in imaging centres can be used to incubate environmental monitoring plates, media for sterility testing or cell cultures, which are temperature sensitive experiments that require incubators in good working order. Following installation per the manufacturer guidelines, it is recommended that the following are performed routinely:

— Cleaning and disinfecting;
— Refilling CO_2 levels and calibrating as necessary,;
— Refilling water levels as required;
— Cleaning per manufacturer guidelines;
— Replacing the HEPA filter every 6–12 months;
— Replacing gas inlet filters every 6–12 months;
— Heat sterilization (e.g. once per month or once every six months).

9.1.10. Cryostats and microtomes

Cryostats and microtomes are used to section post-mortem brain tissue sections or tumours for autoradiography studies. Correct maintenance ensures safe and consistent operation. It is recommended that preventive maintenance be conducted by the manufacturer.

(a) Cryostats

For routine cleaning after every use, remove debris from the cryostat chamber and discard with biohazardous waste. Clean the blade and all exposed surfaces and instruments with 70% ethanol.

On a monthly basis, power down the cryostat. Rinse the cabinet and the microtome with 70% ethanol and then defrost overnight. Place a waste bucket (containing a disinfectant such as 5% sodium hypochlorite) under the drain outlet of the cryostat. After defrosting, remove the microtome from the cabinet, wash in hot water containing detergent, then rinse in absolute ethanol and dry. Lubricate all sliding parts and oil holes with low temperature oil. Wash the inside of the cabinet with warm water containing detergent. Rinse the cabinet with absolute ethanol and then allow to dry. Replace the microtome when dry and reconnect the power.

(b) Microtomes

Clean all components daily, including the knife holder and specimen holder. Brush away any loose debris, and use a soft cloth or gauze to remove debris that is stuck to surfaces. Baby oil (or other light mineral oil) can help to remove residual paraffin. Oil residues should be removed after cleaning using a clean cloth. Debris should be disposed of as biohazardous waste, as it includes remnants of brain or tumour samples.

9.2. QUALITY ASSURANCE AND QUALITY CONTROL FOR PRECLINICAL IMAGING SCANNERS

Routine QA and QC are important to maintain good performance of the imaging equipment. Reference [129] provides guidelines for preclinical imaging specialists in setting up an appropriate QA/QC programme for their facility, including guidelines for PET, SPECT, optical imaging, CT and MRI. The QA and QC recommendations of Ref. [129] for PET and SPECT scanners are reproduced in the following sections.

For PET and SPECT scanners, QC falls into two categories: system set-up and routine quality assessment of scanner performance. System set-up is recommended at the acceptance testing procedure or after replacement of hardware or repairs and involves determining detector look-up tables, energy settings, time alignment and other specifications. The performance of PET scanners should be assessed using the National Electronics Manufacturing Association (NEMA NU 4-2008) standardized methods to obtain image quality and quantitation metrics. For SPECT scanners, such a method does not exist.

9.2.1. Quality assurance and quality control for PET scanners

For QA/QC measurements, a series of radioactive sources or phantoms are needed. For routine QC, long lived radioisotopes such as ^{68}Ge or ^{22}Na, encased in epoxy or plastic, are typically used. These long lived radioactive sources are needed for quick system checks (quick scans), for system set-up and for normalization measurements. Shorter lived radioisotopes such as ^{18}F, ^{11}C, ^{68}Ga and ^{124}I are typically imaged in refillable containers (e.g. small bottles or vials) and used for cross-validation of the PET camera with a dose calibrator and a γ counter. Most vendors recommend the set of sources and phantoms needed. According to the NEMA standards for image quality assessment, the NEMA NU 4-2008 image quality phantom should be used.

9.2.1.1. Daily quality control tests for PET scanners

Visual assessment and/or efficiency scans (quick scans) describe a basic daily check of the function and imaging performance of PET scanner systems. A long lived source with diameter and activity similar to those of typical animal scans is placed in the centre FOV. A short acquisition (5–10 min) is recorded and an image is reconstructed. The image should be examined visually for artefacts, and quantitative data from an ROI should be analysed (mean and standard deviation. Data should not vary from the baseline by more than 20%.

Another method for a daily QC test is to check the response of individual detector blocks by performing an efficiency scan (quick scan) to verify that all the electronics are working properly and that each crystal is functioning correctly.

The daily QC test also includes a measurement of the consistency of the dose calibrator used. These are usually constancy and linearity measurements, which are specified by the vendor.

9.2.1.2. Monthly quality control tests for PET scanners

Testing of the PET calibration constant is performed to relate the scanner measurement to the actual amount of radioactivity, taking into account the sensitivity and performance of the PET scanner. Using these values, the amount of activity drawn in the dose calibrator can be related to the activity in the PET images and to data from blood and tissue samples from the γ counter. The ideal calibration measurement replicates the conditions of most in vivo imaging experiments. This means using the specified activity normally used in routine imaging, a refillable container close to the size and shape of the animal, and the same scan and reconstruction parameters (e.g. energy window, timing window, scan time, reconstruction algorithm). The calibration constant is then calculated by using the ratio of the measured activity from the PET image, obtained from a cylindrical ROI in the image, and the known activity per unit volume from the dose calibrator.

Gamma counter calibration is performed by measuring samples with very small activities, typically less than 37 kBq (1 μCi). Using small aliquots of the radioactive solution used in the PET calibration constant phantom, multiple samples can be drawn and measured to create the γ counter data. As pipetting small samples is inherently inaccurate, samples need to be weighed using an analytical balance to determine the sample volume. From the data obtained (in counts per second (cps)) and the known activity concentration, the γ counter efficiency value can be calculated.

9.2.1.3. Semiannual and annual quality control tests for PET scanners

The PET system set-up is performed using manufacturer specific procedures and protocols (as seen in Table 13). This usually includes recalibration of detector high voltage settings, calculation or calibration of pixel location maps, adjustment of electronic calibration factors and determination of energy look-up tables. After the full system set-up, a normalization (and blank) scan should be performed and the calibration constant should be measured.

Normalization should also be performed annually. For most of the preclinical PET scanners, normalization is done using the direct normalization

TABLE 13. TYPE AND FREQUENCY OF QA/QC TESTS FOR PET SCANNERS

Daily	Monthly	Semiannually/annually
Visual assessment	PET calibration constant	Blank measurement (semiannually)
Efficiency (quick) scan	Gamma counter calibration	Full PET system set-up
Dose calibrator constancy		Normalization
		Dose calibration linearity

Note: Table adapted from Ref. [129].

method. That involves statistical calculations on scans acquired with a homogeneous source that provides uniform irradiation of the detectors. Each LOR is illuminated with the same amount of activity. For a given sinogram bin, the inverse of the measured number of counts is proportional to the normalization coefficient. A usual normalization scan is done for 4–6 h using a ^{68}Ge cylinder that covers the whole axial FOV of the scanner.

When attenuation data are acquired using a transmission scan in a PET scanner, then a blank measurement should also be performed semiannually. For the blank scans, the phantom and the animal bed have to be removed from the FOV. Then, a scan using only a rotating point or rod source is acquired with the same acquisition and histogram parameters (e.g. energy window, rebinning method) to obtain a blank sinogram. The ratio of the blank counts to the transmission counts during an animal scan yields a correction factor for each emission LOR.

Finally, the dose calibrator linearity should be tested. This can be performed using a high starting activity (3.7 GBq or 100 mCi) of a short lived isotope filled in a phantom. Measurements should be performed repeatedly until the activity has decayed well below any amounts measured, typically below 0.37 MBq (10 μCi). The data covering the normal range of use are then plotted on a semilogarithmic graph, and the results are expected to show a linear loss of activity over time.

9.2.2. Quality assurance and quality control for SPECT scanners

The preclinical SPECT scanner, frequently in combination with either CT or MRI, is an important tool in oncology, cardiology and neurology research. To capitalize on its capabilities, it is imperative that scanners be in good working order. QC procedures are used to evaluate the proper function of SPECT γ cameras [130, 131]. In a clinical setting, these procedures will be performed on a documented schedule (e.g. daily, weekly, monthly). They should also be performed routinely on preclinical scanners, on a schedule that is appropriate for the frequency of scanner use. These procedures normally include the following:

(a) Uniformity floods;
(b) Spatial resolution evaluation;
(c) COR assessments;
(d) SPECT phantom evaluation.

9.2.2.1. Phantoms for SPECT scanners

As for PET, QA/QC measurements of a series of radioactive sources or phantoms are needed. A point source phantom with an activity of around 10–250 μCi (depending on the SPECT scanner) should be available. This can be either a commercially available fillable sphere or a partially filled syringe or capillary tube. A line source (or a series of line sources) is needed for resolution measurements consisting of filled capillary tubes. Finally, a uniform cylinder with a diameter similar to the animals being scanned should be available.

9.2.2.2. Daily quality control tests for SPECT scanners

The first test is used to monitor the drift of the photopeak for each isotope used for imaging. It should be performed without collimators, using a point source. The amount of activity should be enough to acquire the desired counts in a reasonable time frame without exceeding a 20% dead time threshold and should ideally maintain a flux of 4500 counts/cm² (but not more than 10 000 cps). The source should be placed in the centre of the FOV with maximum extracted detector heads. The distance from the centre of the FOV to the detector face needs to be five times the detector's FOV to ensure uniform exposure of the detectors. This can be a challenging requirement to meet on closed preclinical γ and SPECT camera scanners.

After this, a planar flood image should be acquired using the same source and detector configuration. For each detector head, the count density should be 10 000 counts per pixel. Image quality should then be assessed by

visual inspections of the projection data from each detector. It is recommended to calculate the integral and/or differential uniformity in the useful FOV and centre FOV.

9.2.2.3. Weekly quality control tests for SPECT scanners

Weekly QC tests include a physical assessment of the collimator and detector stability to determine the effects of normal wear and tear.

9.2.2.4. Monthly quality control tests for SPECT scanners

On a monthly basis, a more detailed examination of the interchangeable collimators is recommended for signs of wear and tear. This includes dents, fractures and checks of the attaching mechanism.

The integral and/or differential uniformity should be checked on a monthly basis at least, if it is not possible on a daily basis.

In SPECT cameras that include attenuation and scatter correction, the calibration factor should be determined on a monthly basis for all isotopes used. The workflow is similar to that for PET systems. This means using the specified activity that is normally used in routine imaging, a refillable container close to the size and shape of the animal, and the same scan and reconstruction parameters (e.g. energy window, scan time, scatter and attenuation correction, reconstruction algorithm). It is recommended to measure the filled phantom for 1–2 h to acquire a large number of counts. The calibration constant is then calculated by using the ratio of the measured activity from the SPECT image, obtained from a cylindrical ROI in the image, to the known activity per unit volume from the dose calibrator.

9.2.2.5. Semiannual and annual quality control tests for SPECT scanners

The quality of the image reconstruction over time should be assessed annually using a spatial resolution measurement. It is recommended to measure the resolution using a ^{99m}Tc line source placed in the centre FOV with a selected collimator and acquisition protocol. After reconstruction with a typical reconstruction protocol, a line profile should be drawn through the hottest central voxel in all directions and then fitted with a Gaussian plus to calculate the full width at half-maximum. The resolution should not vary from the baseline by more than 10%.

The recommended semiannual QC tests also include a check of the rotational uniformity, which should be done for scanners with rotating detectors (see Table 14). Using the same source arrangements as for the daily uniformity tests, a long (e.g. overnight) scan covering 360° should be acquired.

TABLE 14. RECOMMENDED QUALITY ASSURANCE AND QUALITY CONTROL TESTS AND FREQUENCY FOR SPECT SCANNERS

Daily	Weekly	Monthly	Semiannually/annually
Photopeak drift	Collimator stability	Collimator durability	Resolution
Uniformity	Detector stability	Uniformity (if not done daily)	Full SPECT system set-up
		Calibration constant	Rotational uniformity (semiannually)

Note: Table adapted from Ref. [129].

Finally, once a year, a full SPECT system set-up is recommended. This includes full calibration of electronics and detector settings. After the full system set-up, normalization scans and measurement of the calibration constants should be performed.

10. PROTOCOLS

10.1. IN VITRO EVALUATION PROTOCOLS

10.1.1. Protocol for cell uptake assay

The preliminary cell uptake study is a good starting point to assess the uptake/binding characteristic of a tracer under evaluation. A typical protocol for conducting a cell uptake assay is as follows:

(a) The cells are harvested from culture and dispensed into reaction tubes or well plates in cell suspension media containing 2% foetal bovine serum (or recommended supplement). Typically, between 10^4 and 10^6 cells per reaction may be used. When working with adherent cells in well plates, it is important to adjust the number of cells so to obtain a uniform monolayer on the well surface without overcrowding or dissociation of cells. The reaction should be performed at least in triplicate, so ensuring adequate cell mass

for the whole assay is important. In the case of adherent cells harvested by trypsinization and/or mechanical scraping, it would be useful to allow the cells to rest in the suspension medium overnight, or at least a few hours prior to starting the reaction, so that they can recover from the trauma of the harvest procedure.

(b) The radiolabelled tracer is prepared under aseptic conditions and diluted with buffered saline or cell suspension media. The concentration of the tracer used can be equal to the K_D affinity. If K_D is not known, generally the experiments can be conducted with 5–10 nM concentration.

(c) The tracer is added to the reaction wells/tubes. It is advisable to keep an additional set of reaction tubes to which, along with the tracer, a 100-fold concentration of unlabelled ligand is added, to assess non-specific adsorption of the tracer to the surface of the cells or the reaction vessel. It is important to keep the tracer volume as low as possible and not to introduce any changes into the cell medium. The reaction volume has to be kept constant by adding buffered saline or appropriate medium. While allowing for adequate coverage of the cell mass, it is recommended to minimize the reaction volume in order to maximize the interface between the tracer and the cells; for example, 500–1000 μL is adequate suspension volume for a reaction carried out in a 24 well plate for up to 24 h.

(d) In each individual reaction set the cells are incubated in triplicate for different lengths of time (suggested intervals of 0.25 h, 0.5 h, 0.75 h, 1 h, 2 h, 4 h, 8 h). The experiment can be repeated with shorter time intervals to obtain optimal results.

(e) Binding reactions are typically carried out at physiological temperature (37°C). To inhibit kinetics of cellular internalization/turnover of the tracer and specifically assess binding to membrane receptors, the reaction set may be incubated at 4–8°C. In such a case, at the end of the incubation the cells should be carefully examined under a microscope to assess possible damage from exposure to the lower temperature.

(f) At the end of the incubation period, 1 mL of ice cold plain culture media or buffered saline is added to the wells. For adherent cultures, it may be sufficient to simply decant the supernatant, while for suspension cultures the cell mass will require centrifugation at 800*g*–1000*g* for 5 min. It is recommended to perform this step twice for satisfactory ‘washing’ of the cells. This washing step stops the binding reaction and removes non-bounded tracer activity. It is recommended to perform the washing steps rapidly and gently to minimize dissociation of bound tracer from the cell mass.

(g) The cells are then harvested (by trypsinization or by lysis with dimethyl sulfoxide/1 M sodium hydroxide solution) and radioactivity associated with the cell mass is measured on a suitable radioactivity counter.

For measurement of pure β emitting radioisotopes by liquid scintillation, it is important to ensure that the scintillation cocktail used is compatible with the solution/solvent used for cell harvesting.

(h) A curve may be plotted of incubation time against bound radioactivity. It is expected that the curve plateaus when the incubation time reaches binding equilibrium. The earliest convenient time point at which equilibrium is achieved can be used as the incubation time for future studies including saturation binding and competitive binding.

10.1.2. Protocol for saturation binding assay

The protocol for a typical saturation binding assay is as follows:

(a) The cells are harvested from the culture and dispensed into reaction tubes or well plates as described in Section 10.1.1.

(b) The radiolabelled tracer is prepared under aseptic conditions and diluted with buffered saline or cell suspension media into a series of different level concentration covering at least a 100-fold difference. The lowest tracer concentration should be at least below one tenth the affinity K_D (often in the nanomolar range or lower) and the highest concentration should be at least ten times higher than K_D.

(c) The different tracer concentrations are added to the respective reaction wells/tubes in the same manner as described in Section 10.1.1, keeping in mind the instructions regarding tracer volume and total reaction volume. It is advisable to keep an additional set of reaction tubes to which, along with the tracer, a 100-fold concentration of unlabelled ligand is added to assess non-specific adsorption of the tracer to the surface of the cells or to the reaction vessel.

(d) The cells are incubated with the tracer for the time required to approach equilibrium. To assess binding to membrane receptors specifically, the reaction set may be incubated at 4–8°C to block cellular internalization or turnover of the tracer.

(e) At the end of the incubation period, the cells are washed with cold culture media or buffered saline. It is recommended to perform the washing steps rapidly and gently to minimize dissociation of bound tracer from the cell mass.

(f) The cells are then harvested (by trypsinization or by lysis with dimethyl sulfoxide/1 M sodium hydroxide solution) and radioactivity associated with the cell mass is measured on a suitable radioactivity counter. For measurement of pure β emitting radioisotopes by liquid scintillation, it is

important to ensure that the scintillation cocktail used is compatible with the solution or solvent used for cell harvesting.

(g) The readings obtained in triplicate for each tested concentration are corrected for background, and the average and standard deviation are calculated. Using the average values, a standard curve of tracer concentration against bound radioactivity is plotted, which is expected to show a region of saturation. The curve can be used to obtain a value for K_D using regular software packages. Alternatively, a Scatchard plot can be created to manually determine K_D.

10.1.3. Protocol for competitive binding assay

The competitive binding assay is performed subsequent to the saturation binding assay. Here, the binding of the radiotracer is challenged with a range of concentrations of the native biomolecule or ligand that is known to bind with the target receptor, so as to assess the percentage of radiotracer that binds to the target receptor in the presence of competitive ligand. The competitive binding assay is conducted under reaction conditions analogous to those used in the saturation binding assay (Section 10.1.2) and many of the steps are similar. A stepwise description of a typical competitive binding assay is as follows:

(a) Cells are harvested and dispensed into reaction wells as described in Section 10.1.2.

(b) A single tracer concentration, for example corresponding to 50% of saturation binding value, is used for the binding reaction. A range of concentrations of unlabelled native biomolecule/receptor targeting ligand is prepared, which can extend from 0.1 to 100 times the tracer concentration. It is recommended to have six data points or more.

(c) Different protocols give different advice about the sequence of adding tracer and unlabelled ligand. They may be added immediately after each other or the cells may be preincubated with the unlabelled ligand for 0.5–2 h before adding the tracer. All other conditions have to be maintained as in Section 10.1.2.

(d) The cells are incubated according to optimized reaction conditions. After the incubation period, they are washed, harvested and counted as described in Section 10.1.2.

(e) If a curve is plotted of unlabelled ligand concentration against bound radioactivity, the concentration at which the binding of the tracer is inhibited by 50% is known as the IC50 (inhibitory concentration 50%) value of the ligand. IC50 provides a measure of the inhibition of the tracer by the unlabelled ligand, and it can be used to calculate K_i.

The competitive binding assay can also be used to screen potential new ligand analogues in exploratory testing. In this case, a tracer with known binding properties can be used as a standard against which the competition for receptor binding by the new molecules can be measured using the above protocol.

10.1.4. Protocol for retention/dissociation assay

The retention/dissociation assay is a measure of the binding strength of the tracer to the receptor, as observed by the pattern of washing out over time. A typical protocol for performing a dissociation assay is as follows:

(a) Cells are harvested and seeded in reaction wells/tubes (10^4 to 10^6 per well) as described in Section 10.1.1.
(b) The tracer is added at a concentration approximately correlating to $10 \times K_D$ as calculated in a saturation binding assay. If K_D is not known, 10–20 nM may be used as a starting point.
(c) The cells are incubated with the tracer until the reaction nears equilibrium, which may take 0.5–2.0 h. In general, higher tracer concentrations require lower incubation times.
(d) The incubation is then interrupted by performing two or more washing steps as detailed in step (f) of Section 10.1.1. Care should be taken to avoid excessive washing that may lead to loss of bound tracer.
(e) After washing, complete culture medium is added to the wells (as much as the binding reaction volume), with the time of addition noted as 'time zero', and dissociation takes place in individual reaction sets (in triplicate) for different intervals (suggested intervals of 0, 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, 16 to 20 h).
(f) At the end of the respective dissociation intervals, the cells are washed twice or more and harvested by trypsinization or lysis (using 1 N NaOH/ dimethyl sulfoxide), as described in Section 10.1.1. The radioactivity associated with the cell mass is measured.
(g) A curve of dissociation time against radioactivity bound to cells is plotted. The time taken for the activity to fall to 50% of the activity at time zero is a measure of the dissociation of the tracer. The dissociation time can be a useful parameter for refining assay protocols, especially to avoid excessive or time consuming washing steps.

10.1.5. Protocol for internalization assay

The internalization assay, as evident from the name, gives a measure of internalization of the tracer into the cells, by stripping away the tracer molecules

bound on the cell surface prior to measuring radioactivity associated with cells. The following steps describe a typical internalization assay:

(a) Cells are harvested and seeded in reaction wells/tubes as described in Section 10.1.1. All the precautions described there should be adhered to in the internalization assay as well.
(b) The tracer is prepared under aseptic conditions and diluted in culture medium. It is recommended to use a tracer concentration approximately 10 times the calculated K_D. If K_D is not known, then 10–20 nM concentration of the tracer can be employed.
(c) The tracer is added to the cells and incubation is performed in triplicate for a set of time intervals up to the estimated time for binding equilibrium (suggested intervals of 0 h, 0.25 h, 0.5 h, 0.75 h, 1 h, 2 h).
(d) At the end of the respective incubation periods, the reaction wells may be washed once or twice with ice cold plain culture medium or buffered saline to remove unbound tracer, as described in Section 10.1.1.
(e) After washing off the unbound tracer, the cells are quickly exposed (30–60 s) to a stripping buffer (using the same amount as the total binding reaction volume). Several recipes are available for the stripping buffer; a typical one uses 0.2 M glycine with 0.15 M NaCl (pH3.0). This step may be performed twice.
(f) After stripping, the cells are washed again with plain culture medium or buffered saline, harvested by trypsinization or cell lysis and counted to measure tracer radioactivity associated with the cell mass.

The above protocol assumes that the stripping procedure is sufficient to release surface bound tracer without significant damage to cell membrane integrity. However, it would be useful to verify these aspects to avoid errors in the results. Prior to doing the assay, unlabelled cells exposed to the stripping regimen and then washed can be examined with a microscope after cell staining to check for membrane integrity. It is more tedious but equally important to confirm that the stripping protocol is effective in removing surface bound tracer to avoid falsely high internalization values even with short binding incubation periods. In such a case, it is recommended to vary the stripping conditions (buffer composition and incubation time) to ensure correctness of the measurements.

10.1.6. Protocol for tracer stability assay

It is important to establish the innate stability of the tracer over its shelf life. To achieve this, the basic cell uptake assay (Section 10.1.1) can be repeated at different intervals after preparation. This will provide insight into how long a

tracer can be used for evaluation studies before the results are affected by tracer degradation. The selection of intervals in which to repeat the assay for a specific tracer will depend on the radionuclide half-life and the proposed clinical application. This can vary from a few hours to some days.

10.1.7. Protocol for plasma/serum stability assay

Plasma/serum stability is a special case of tracer stability that concerns the stability and protein binding of the tracer in vitro, which is an indication of the tracer's behaviour when administered via the bloodstream. A typical protocol for a plasma/serum stability assay is described below:

(a) Plasma/serum is collected aseptically, dispensed into single use (0.5–1.0 mL) aliquots and stored at –20°C or lower temperatures. It is important that the aliquots do not undergo repeated freeze–thaw cycles before use. The source of the fluid can be an adult animal/human; it is recommended to avoid using any type of foetal or immunodeficient serum. Some ligands could have differential stability in human and animal serum; it would be ideal to have a comparative study taking into account the proposed ex vivo/in vivo testing models.
(b) For the assay, as many aliquots are taken as necessary to perform the assay for the required number of intervals.
(c) The same amount of tracer is added to each of the aliquots, and they are incubated at 37°C. Care should be taken to ensure that the tracer activity is sufficient for measurement after the incubation intervals and dilutions performed in the protocol.
(d) At the end of each incubation period, the vials are removed and an aliquot of reaction stopping or protein precipitating agent equal to the total reaction volume is added. Methanol and ethanol are common precipitating agents.
(e) After centrifugation to separate out the precipitated protein (at 20 000*g* for 10 min), the supernatant may be analysed by TLC/HPLC (using the protocol used to characterize the tracer) to assess the stability/metabolism of the tracer in the serum/plasma. The radioactivity associated with the precipitated protein fraction provides insight into the plasma/serum protein binding tendency of the tracer for the tested incubation periods.

10.1.8. Protocol for in vivo stability assay

The in vivo stability assay is necessary to evaluate whether a radiopharmaceutical is metabolized in vivo and whether radiometabolites

distribute in the target organ. An example protocol for metabolite analysis in a rat is described below:

(a) The analysis is generally performed using a dedicated HPLC system. The column and the elution (mobile phase and flow rate) are selected on the basis of methods set up for a QC and purification procedure of the radiopharmaceutical. Prior to the analysis, HPLC, column, mobile phase and flow rate are evaluated with the unlabelled standard to check that the retention time of the parent compound is suited to the radionuclide's half-life.
(b) On the day of the experiment, animals are injected with the radiopharmaceutical using doses higher than the ones used for biodistribution studies (37 MBq for rats and 7–8 MBq for mice) and euthanized at the time of maximum radioactivity uptake in the tissue of interest. Blood and target tissues are collected. Blood is centrifuged in heparinized tubes to obtain plasma samples.
(c) Aliquots of plasma (of volume depending on the radioactivity concentration — typically 500 μL for rats and 200 μL for mice) are extracted using CH_3CN (1:1 v/v) and filtered. Tissue samples are homogenized in saline solution (1:1 v/v) using a Potter–Elvehjem tissue homogenizer (for brain or soft tissue) or a rotating homogenizer (for fibrous tissue such as heart or tumour) and processed as described for plasma in Section 10.1.7.
(d) Pellet and CH_3CN extracts are counted in a γ counter and the extraction efficiency is measured.
(e) Plasma and brain extracts are injected in the HPLC scanner. Eluted fractions are collected in tubes with an automatic collector every 30 s for long enough to collect the radiolabelled parent compound. The tubes are counted with a γ counter to obtain the activity in the fractions (in counts per minute (cpm)), which is corrected for decay with a retention time corresponding to the parent compound and is divided by the total amount of radioactivity injected for HPLC analysis. If an HPLC radioactivity detector with high sensitivity is present, collection in tubes is not necessary.

For ^{11}C labelled compounds, the maximum number of samples per analysis is five. If radiometabolites are used to correct the input function for kinetic modelling, the analysis is performed on plasma samples collected at different times after injection. This may be done in the same animal only if the concentration of radioactivity is sufficient to collect a small volume of blood. Alternatively, plasma should be pooled from different animals.

10.1.9. Protocol for ex vivo autoradiography

Autoradiography studies are used when the regional distribution in the target organ should be compared with that of a marker tested using immunohistochemistry or to compare two different radioligands in the same animal (the latter only if the radioligands are labelled with radionuclides with different half-lives). Below is an example of the protocol:

(a) For single radiopharmaceutical autoradiography, animals are injected in the tail vein with the radiopharmaceutical and euthanized at the optimal time in terms of target uptake.
(b) Tissues are rapidly removed, immediately placed in liquid nitrogen and cut with a cryostat. In the case of soft tissue such as brain tissue, samples can be cut with a tissue slicer without freezing. Slices are exposed to a phosphor screen in a dark place for approximately 3 h for ^{18}F or overnight for ^{11}C and developed with phosphor imager. ROIs are drawn on the required areas to obtain values of density light units per square millimetre, which are then converted into percentage of injected dose per square millimetre of tissue (%ID/mm^2) using a calibration factor previously estimated using the protocol described in step (c).
(c) Increasing radioactive concentrations of the radionuclide used in the studies are placed on different discs of paper with the same diameter (1 cm). Each concentration is prepared in duplicate. One disc is counted with a γ counter and the other is exposed to the phosphor screen to build a straight line of density light units per square millimetre versus radioactivity concentration, which is used to convert density light unit values into units of radioactivity.

In the case of dual label autoradiography, the radiopharmaceuticals are injected at different times so as to sacrifice the animal at the time of optimal kinetic behaviour of each radiopharmaceutical. Slices are exposed as described above and developed with phosphor imager to obtain the distribution of both radionuclides. After the radionuclide with the shorter half-life is completely decayed (six half-lives), the screen is exposed again to obtain the distribution of the second radionuclide. The second set of images is then subtracted pixel by pixel from the first set to obtain the image of the distribution of the first radionuclide. In the process of conversion from density light units to radioactivity units, the pixel values should also be corrected for radionuclide decay.

10.1.10. Protocol for γ counter calibration

Gamma counter calibration is necessary in a QA system to obtain reliable data. A γ counter system is used to measure small amounts of radioactivity such as those obtained during plasma counting, metabolite analysis or biodistribution studies. In the case of positron emitting radionuclides, a γ counter able to measure activity at 511–1022 MeV is required. The absolute γ counting unit is the becquerel but γ counts are generally expressed in disintegrations per minute (dpm). Steps for γ counter calibration are as follows:

(a) Calibration test: this procedure allows estimation of the γ counter efficiency and conversion of the activity detected by the instrument from counts per second or per minute into becquerels or disintegrations per minute. It is performed with a calibrated radioactive source, and the efficiency (E) is calculated as follows: E = [(activity measured (cpm))/(activity present in the calibrated source (dpm))] × 100.
(b) Stability: the calibrated source should be measured before each experiment to verify that the system is stable.
(c) Saturation test and linearity: this test indicates the range linearity between the activity counted and the response of the γ counter. The test is performed by counting different concentrations of radioactivity to detect the range of linearity of the instrument.

10.2. IN VIVO EVALUATION PROTOCOLS

Figure 4 gives the typical format for documenting the required information before and during a preclinical imaging experiment. It is recommended to have a standard basic protocol approved as per institutional procedures. The individual experimental variations should be noted for each study.

10.2.1. In vivo preclinical imaging study with [^{18}F]FDG considerations for different applications

10.2.1.1. General considerations

All procedures should be approved by the IACUC prior to conducting animal imaging studies.

PROTOCOL WORKSHEET

Project: ______________________________

Principal investigator: ______________________________

Study name: ____________________ Date: ____________

Researcher: ____________ Researcher: ____________

Animal details

Species: ________ Strain: ________ Sex: ________

Date of birth: ________ Age: ______ Weight: ________ g

Fasting: ________ h

Biochemical parameters: ______________________________

Remarks: ______________________________

Pretreatment: ______________________________

Anaesthetic: ________ Time: ________

Route: ________ Dose: ______ Volume: ______ Flow: ______

Radiotracer details

Radiotracer name: ________ Isotope: ________ Chemical form: ______

Date and time of expiry: ______________________________

Injection details

Drawn by: ________ Injected by: ________

Pre-injection activity: ______ MBq Time: ________ Drawn volume: ______ μL

Pre-injection activity: ______ MBq Time: ________ Drawn volume: ______ μL

Post-injection activity: ______ MBq Time: ________ Injected volume: ______ μL

Post-injection activity: ______ MBq Time: ________ Flush volume: ______ μL

Injection activity: ______ MBq Inj. time: ________

Scanning details

Scanner name: ________________ Operator: ____________

Bed position: Horizontal: ______ mm Vertical: ______ mm

Scan region: ____________ Animal position: ____________

Attenuation Attenuation type: ________ (Co-57 source, Ge-68 source, CT)

	Scan duration		Acquisition start time:	Energy window:	Timing:
Acquisition 1	________	s			
Acquisition 2	________	s			

Emission

	Scan duration		Acquisition start time:	Energy window:	Timing:
Acquisition 1	________	s			
Acquisition 2	________	s			

Remarks: ______________________________

FIG. 4. Example PET/SPECT protocol worksheet.

10.2.1.2. Animal preparation

New animals are left in the vivarium for at least seven days for acclimation. The conditions of humidity and temperature in the room are controlled and animals are given as much food and water as they want.

On the day of the experiment, animals are taken from the vivarium and put in the PET scanner room. They are anaesthetized with 5% isoflurane in 100% oxygen (or air) and then maintained with 2–3% isoflurane over a warmed table. Blood glucose level is measured after taking a droplet aliquot from the tail.

The animal is weighed in a calibrated balance and all characteristics (e.g. number, weight, experimental group, date, treatment, blood glucose level, fasting) are recorded.

10.2.1.3. Brain [^{18}F]FDG study procedure

The [^{18}F]FDG dose is prepared in a 0.5–1 mL syringe, for example 20 MBq in 0.5 mL. The animal (a male Wistar rat weighing 300 g) is intravenously injected with [^{18}F]FDG in the tail vein. The injection time is noted. The activity of [^{18}F]FDG is measured in a calibrated dose calibrator before and after injection and the numbers are registered in the form with their respective measurement times.

The rat is allowed to wake up after injection, is put back into the cage and kept warm (keeping the animal awake during the distribution phase of [^{18}F]FDG increases brain uptake and keeping it warm decreases uptake by the muscle and brown adipose tissue).

Forty-five minutes after injection, the animal is anaesthetized again and positioned in a warmed bed with its brain in the centre of the FOV, and a static image is acquired for 30 min. The animal is taken from the equipment and allowed to wake up in a warmed cage. An image is reconstructed using an OSEM-3-D algorithm with 20 iterations and four subsets. Images are fused with an MRI template for drawing the VOI in different brain regions; the result is expressed in kBq/mL. The radioactivity concentration is then normalized for ID and animal body weight to express the uptake in SUV units.

10.2.1.4. Heart [^{18}F]FDG study procedure

The animal should be prepared in the way described in Section 10.2.1.3. However, fasting needs to be considered in this case. All procedures are the same as in Section 10.2.1.3, except for the positioning; in this case, the heart should be in the centre of the FOV.

Using isoflurane is a good option for cardiac studies because this anaesthetic agent increases heart uptake.

Image quantification should use VOIs in the different myocardium axes and be presented as the percentage of uptake related to the maximum region and/or SUV. In cardiac studies it is customary to use a polar map as a guide for the myocardium regions.

10.2.1.5. Tumour [^{18}F]FDG study procedure

Animal preparation, dose preparation and image acquisition parameters are as in Section 10.2.1.3. An important aspect to consider in tumour models is the location of the tumour. The positioning has to consider the correct location of the tumour and adjacent organs. For example, the tumour should not be too close to the bladder. However, if the model presents a spontaneous tumour that is close to the bladder, a hand massage can be given to the animal's bladder before putting the animal in the equipment to minimize urine content in the bladder.

The choice of anaesthetic should also be taken into account. Isoflurane increases heart uptake. Therefore, if the tumour is close to the heart (breast cancer model, for example), the use of ketamine with xylazine can be considered.

During image quantification, manually drawing VOIs in the tumour may be considered if the tumour has irregular borders and size. The results are commonly expressed in SUV_{max} (maximum SUV value inside the VOI) and in tumour to muscle ratio.

10.2.2. In vivo preclinical imaging

This section presents a dynamic dual primate (rhesus monkey) PET neuroimaging study with [^{11}C]dihydrotetrabenazine ([^{11}C]DTBZ) to determine the per cent occupancy of vesicular monoamine transporter 2 (VMAT 2) by different doses of a pharmaceutical test article (DTBZ).

10.2.2.1. General considerations

In this procedure, monkeys receive a baseline scan with [^{11}C]DTBZ and a challenge scan with [^{11}C]DTBZ in the presence of a pharmaceutical test article (e.g. 1 mg/kg DTBZ) [132]. All procedures should be approved by the IACUC prior to conducting imaging studies. The minimum time between study sessions in the same animal should be one week.

10.2.2.2. Animal husbandry and housing

Animal facilities should comply with local regulations, such as those defined by the United States Department of Agriculture. For example, monkeys

should be housed in steel cages (at least 83.3 cm high × 152.4 cm wide × 78.8 cm deep per individual) equipped with foraging boxes. As monkeys are sociable, if possible, they should be housed together in a cage with minimum space for each animal. However, this can be problematic if aggressive incompatibility is an issue. Cages should be metal, with gridded floors so that radioactive waste is safely drained through the gridded floor and is easier to clean. Temperature and humidity should be carefully controlled, and monkeys should be kept on a 12 h light–12 h dark schedule. Monkeys should be fed an appropriate diet approved by concerned food authorities, which may be supplemented with fresh fruit and vegetables daily as necessary. Water and enrichment toys (items that can be handled and food based treats) should be available continuously in the home cage.

10.2.2.3. Animal preparation

The monkey should fast for at least 12 h prior to the study. The animal is initially anaesthetized with ketamine (e.g. 15 mg/kg intramuscular) in the home cage, weighed and transported to the PET imaging suite. The monkey is then intubated and placed on continuous inhalation of 2% isoflurane in oxygen or air, and a percutaneous catheter is inserted into a hindlimb for administration of the radiopharmaceutical. The monkey should be placed supine with the head positioned and secured in the PET scanner, ensuring that the brain is in the FOV. The body temperature (37°C) should be maintained by an appropriate heating pad (e.g. electronic, water), and the respiratory rate, heart rate and blood oxygenation should be continuously monitored during imaging. Fluid balance can be maintained using saline injections.

10.2.2.4. PET imaging

Following a measured transmission scan, a dynamic emission PET scan is performed for 60 min. At the start of the dynamic sequence [^{11}C]DTBZ (~1.0 mL) is injected intravenously followed by a 1 mL flush with saline. For each dual study, 50 min after completion of the first emission scan, the pharmacological test article is injected intravenously (in ~0.5 mL of saline), followed 10 min later by injection of [^{11}C]DTBZ and initiation of a second 60 min emission PET scan. Emission data should be collected using a standard framing sequence (5 × 2 min, 4 × 5 min and 3 × 10 min).

10.2.2.5. Image reconstruction

PET scans should be corrected (for radioactive decay, dead time, random and scattered coincidences, and attenuation) and reconstructed (e.g. using

ordered subset expectation maximization for rapid initial convergence and 3-D maximum a posteriori algorithms that take into account the point spread function of the detectors) to generate a reconstructed image. Images for the baseline and challenge scans should be co-registered using, for example, SPM12 software[3] and can also be registered to MRI scans for the animal if available.

10.2.2.6. Image analysis

For quantitative analyses, 3-D VOIs should be established for brain ROIs (e.g. striatum), along with the reference region to be used (e.g. occipital cortex), on the average image of both scans of the monkey. VOIs should be defined using either PET scanner software or in-house developed software by first defining a boundary surrounding each brain region and then setting a threshold to exclude all voxels below the threshold value. If multiple monkey scans are to be analysed, defining VOIs on the average of all co-registered scan images ensures that the exact same VOIs are used for each scan. Striatal and occipital cortex tissue TACs can then be generated.

The image derived tissue time–radioactivity data can be further analysed using the Logan graphical method with the occipital cortex as the reference region [133] to calculate striatal distribution volume ratios, from which binding potentials can calculated, as seen in Eq. (14):

$$\mathrm{BP}_{\mathrm{ND-Logan}} = \mathrm{DVR} - 1 \tag{14}$$

where $\mathrm{BP}_{\mathrm{ND}}$ is the non-displaceable binding potential and DVR is the distribution volume ratio.

Finally, the per cent occupancy values of VMAT 2 can be calculated using Eq. (15):

$$\%\mathrm{occupancy} = 100 \times \frac{\mathrm{BP}_{\mathrm{control}} - \mathrm{BP}_{\mathrm{test}}}{\mathrm{BP}_{\mathrm{control}}} \tag{15}$$

[3] Found at http://www.fil.ion.ucl.ac.uk/spm/software/spm12/

10.2.3. In vivo testing — efficacy of therapeutic radiopharmaceutical

10.2.3.1. General considerations

All procedures should be approved by the IACUC prior to conducting animal imaging studies. For radionuclides that do not emit γ rays, a β counter should be available for ex vivo biodistribution studies. Injected animals should be maintained in an animal cabinet or facility adequate to protect people from β emissions.

10.2.3.2. Animal model preparation

Animals are subcutaneously injected with the sufficient number of tumour cells, either on the basis of existing literature or as deduced from animal model standardization experiments. They are monitored three times a week with a caliper until the tumour reaches an approximate volume of 1 cm^3.

10.2.3.3. Animal treatment

The administered dose for assessing therapeutic efficacy is calculated on the basis of biodistribution studies. It should be recorded in a manner that specifies radioactivity content, mass of cold ligand, injection volume and route of administration (e.g. 18.5 kBq, 12.5 μg, 100 μL given via tail vein). Post-administration, body weight and tumour size measurements are recorded three times a week until the end of the experiment. Euthanasia criteria/humane endpoints are: tumours that ulcerate or become necrotic/infected, tumour diameter >20 mm, weight loss >20% from maximum recorded weight and/or any sign of distress or pain (e.g. rough coat, unkept appearance, malaise). Animals are monitored until euthanasia or for a maximum of 45 days. For control, the same protocol is followed but animals are treated with the cold precursor.

10.2.3.4. Efficacy measurement

Efficacy is measured as reduction in tumour volume or overall survival. Tumour volume is derived from caliper measurements using the formula $V = (W^2 \times L)/2$ (V: volume; W: width; L: length). The therapeutic effect is expressed in terms of tumour growth inhibition. The overall survival effect is evaluated using Kaplan–Mayer curves [134].

10.2.3.5. First in human dose selection

When proposing a dose of a therapeutic radiopharmaceutical for an FIH study, the radioactive administered dose and the mass dose of the pharmaceutical should be considered. With respect to the administered radiation dose, factors such as scientifically available evidence, clinical experience and the results of animal dosimetry studies should be used to decide on the initial radiation dose. The available literature on dosimetry from external radiation beam therapy may also be considered; however, the reported dosimetry values should be adjusted for the type of radionuclide used and the associated relative biological effectiveness. The planned mass dose of the cold pharmaceutical should be based on the results of the general toxicology studies (see Section 5) and prior experience with the ligand in both animal and human studies.

11. CONCLUSION

Radiopharmaceuticals have advanced from simple radiochemicals and the early labelled molecules, whose biological profile is a passive reflection of their physicochemical characteristics, to intricate molecular and supramolecular constructs with active homing abilities to selectively deliver the radioactive payload in the region of disease or dysfunction. This is a natural consequence of the increased sophistication of nuclear medicine requirements, demanding targeted approaches that engage at the level of cells, organelles and even individual biomolecules. The need to rigorously assess these capabilities while adhering to increasingly stringent research safety and ethical norms means that laboratory evaluation of potential nuclear medicine candidates for diagnosis or therapy has become a correspondingly complex endeavour. This publication provides a useful primer to both existing and upcoming radiopharmaceutical research facilities regarding the elaborate sequence of activities that come under the collective appellation of 'preclinical development and evaluation'.

With its multitude of cell based assay recommendations, Section 3 details the measurement of a new compound's affinity, avidity and specificity to its target, as well as functional assays that can assess pharmacologic effect where relevant. A robust cell based testing platform based on the principles discussed herein can provide scientists with high throughput screening of candidates, reducing the burden of testing in animal models. This is highly desirable not only for adhering to the 3R principles of laboratory animal research, but also because it is more convenient and time and cost effective when dealing with a

large array of molecules. Specialized in vitro efflux pump and BBB permeability assays provide a model quantifiable assessment of penetration and distribution behaviour, avoiding extraneous factors that sometimes create ambiguities during in vivo studies. In vitro autoradiography can be a low cost precursor step to animal studies to assess the distribution of a tracer within a tissue of interest, while metabolic assays may provide hints about potential redistribution of the radiolabel. It should be emphasized that a careful selection of pertinent assays and application of rigorous controls for accurate unbiased results are critical to maximize the benefit of in vitro testing.

Given the costs of infrastructure, consumables and trained personnel, studies in live animals have to be performed with well defined objectives and meticulously designed protocols. Several factors need to be considered that govern the quality of results and provide recommendations on best practices, both in terms of design parameters and methodologies. Even more than with in vitro studies, on account of the ethical issues associated with animal handling, researchers need to ensure that the selected animal model and assay protocol provide the necessary insight into the functioning of the formulation being tested. Small animal imaging set-ups, while currently expensive and requiring a specialized working environment, can offer high quality open format verifiable results with less animal usage when employed for novel radiopharmaceutical research or as a tool in mainstream drug evaluation. Moreover, techniques such as ex vivo biodistribution and excised tissue autoradiography remain useful in less equipped facilities and for answering specific queries.

Toxicology and dosimetry are important considerations in the assessment of both diagnostic and therapeutic radiopharmaceuticals. In diagnostic radiopharmaceuticals, they are mainly used for assessment of any unintended damage to the living system, either from unnecessarily high radiation dose or from any pharmacologic effect of the carrier selected to carry the radioactive label. The cytotoxic or modulatory effects are, of course, innate elements of therapeutic radiopharmaceuticals, and their preclinical evaluation would be incomplete without a detailed investigation of these effects. While toxicology and dosimetry are specialized fields that benefit from the involvement of trained staff in the study design and execution, the information provided in this publication, along with the references to established recommendations, is aimed at generating at least a baseline understanding of the factors that govern each field, as valid scientific data in these areas make a valuable addition to any proposal for clinical translation of the laboratory research.

When deciding on a proposed dose of radiopharmaceutical — especially therapeutic — for a FIH study, the amount of radioactivity and the ligand have to be chosen with care to minimize the potential harm to the patient. A guided preclinical development process based on the principles outlined in this

publication would be especially useful in this situation, providing data of affinity, distribution, metabolism, targeted and non-targeted effect, ligand toxicity and radiation dosimetry, which may be used as factors in the decision making.

The principles and recommendations related to the management of data and facilities can not only assist individual researchers but also serve as an overarching development roadmap to institutional management.

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ABBREVIATIONS

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care International
ABSL	animal biological safety level
ALARA	as low as reasonably achievable
BBB	blood brain barrier
COR	centre of rotation
cpm	counts per minute
dpm	disintegrations per minute
FDA	US Food and Drug Administration
FDG	2-deoxy-2-[^{18}F]fluoroglucose; fludeoxyglucose
FIH	first in human
FOV	field of view
GLP	good laboratory practice
HED	human equivalent dose
HPLC	high performance liquid chromatography
IACUC	Institutional Animal Care and Use Committee
ICH	International Conference of Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ID	injected dose
IQ	installation qualification
LAF	laminar airflow hood
LOR	line of response
MIRD	medical internal radiation dose
MRI	magnetic resonance imaging
MRSD	maximum recommended safe dose
NOAEL	no observable adverse effect level
OECD	Organisation for Economic Co-operation and Development
OQ	operational qualification
PET	positron emission tomography
PK	pharmacokinetics
PQ	performance qualification
QA	quality assurance
QC	quality control
ROI	region of interest
SPECT	single photon emission computed tomography
SUV	standardized uptake value

TAC	time–activity curve
TLC	thin layer chromatography
VOI	volume of interest

CONTRIBUTORS TO DRAFTING AND REVIEW

Faria, D.	University of São Paulo, Brazil
Herth, M.M.	University of Copenhagen, Denmark
Konijnenberg, M.	Erasmus Medical Centre, Netherlands
Korde, A.	International Atomic Energy Agency
Kuntner-Hannes, C.	Austrian Institute of Technology, Austria
Moresco, R.M.	University of Milan Bicocca, Italy
Scott, P.J.H.	University of Michigan, United States of America
Shalgunov, V.	University of Copenhagen, Denmark
Subramanian, S.	Bhabha Atomic Research Centre, India

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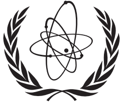

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Preclinical or non-clinical evaluation is an integral part of the development of any drug. The process of developing a new radiopharmaceutical includes rigorous testing before it can be cleared for use in humans. In-depth characterization of its behaviour is necessary to assess its safety and suitability for the intended clinical application. This publication provides a baseline guide for preclinical evaluation of radiopharmaceuticals, a general review of the requirements of a facility and insight into the various scientific activities that constitute this process. The principles and protocols discussed herein provide guidelines for biological assessment of candidate compounds that are consistent with the principles of good laboratory practice to generate valid nonclinical scientific data towards approval for clinical translation. This publication is intended for researchers engaged in radiopharmaceutical development and for Member States planning to set up or upgrade radiopharmaceutical research facilities.

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