GUIDANCE FOR PRECLINICAL STUDIES WITH RADIOPHARMACEUTICALS

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FOREWORD

Preclinical evaluation is an integral part of the development of any drug as well as radiopharmaceuticals. Over the years, progress in related disciplines of biology and chemistry has resulted in a variety of molecules being available for the development of a newer generation of radiopharmaceuticals which aim to specifically deliver the radioisotope 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. Whereas, additional studies involving different in vitro techniques are required today to ascertain the biological properties of radiolabelled molecules for obtaining approval to test them in laboratory animals. Availability of specific disease animal models and technical developments in imaging small animals are adding valuable information for evaluation prior to taking a new drug or radiolabelled molecule for clinical trial.

Preclinical evaluation of some radiopharmaceuticals has been addressed as a part of the work carried out by the participants of International Atomic Energy Agency (IAEA) Coordinated research projects (CRPs) related to radiopharmaceutical developments in the past. Some of these methods are also listed as a part of CRP reports in related IAEA publications. However, a comprehensive publication covering all aspects of preclinical testing has been requested by the researchers in the field. A consultant’s meeting was conducted on April 2016 that recommended the preparation of the guidance publication on preclinical testing of radiopharmaceuticals. This publication is the outcome of the consultants meeting, held from 25 to 28 February 2019 on the same topic. At the meeting, experts discussed various aspects related to in vitro and in vivo preclinical evaluation of radiopharmaceuticals and formulated the outline of the guidance publication. The publication will be useful not only for the researchers engaged in radiopharmaceutical development, but also for the Member States planning to set up or upgrade facilities for radiopharmaceuticals’ research.

The IAEA officer responsible for this publication was Aruna Korde of 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 like 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 developments. In the present era, radiopharmaceuticals are a multi-billion-dollar global market, running the gamut from simple labelled compounds to sophisticated molecular machines. They have the ability to actively localize the disease’ area 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, it is not only nuclear medicine with its increasingly sophisticated list of applications, but also mainline drug development that is benefited by the creation of new radioactive compounds to answer specific needs.

Even though radiopharmaceuticals account for only a fraction of the overall pharmaceutical products, the process of generating new radiopharmaceuticals is subject to many of the logistical and regulatory requirements like 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 must undergo rigorous testing to provide in-depth characterization of its behaviour, both physico-chemical 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 incurred during clinical trials. Additionally, even though most radiopharmaceuticals are applied only in tracer doses in terms of drug content, which usually cause no or only very limited pharmacological effect, currently toxicology testing is required for all radiopharmaceuticals when translating the drug into the clinic. In this respect, it is of vital importance that preclinical testing follows standardized protocols which allow a direct comparison of results from laboratory to laboratory 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 that will give its readers a general review of the requirements of a facility and insight into the various scientific activities that constitute this process. The objective is to provide the general principles and baseline preclinical study protocols to characterize safety, efficacy and quality of intended research 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 will provide guidelines for biological assessment of candidate compounds, which are consistent with the principles of good laboratory practices and generate valid preclinical scientific data towards approval for clinical translation.

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

1.4. STRUCTURE

This publication has been structured in a manner that gives its readers an orderly overview of the various stages of 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, laying the foundation for the contents that follow. Section 2 touches upon the aspects of radiopharmaceutical design that are related to preclinical evaluation. Section 3 details the basic requirements and relevant procedures of assessment of candidate molecules in vitro in cell / tissue / serum matrices outside of living systems to render it an effective screening platform. An effective in vitro assessment testing regimen helps 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 like 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, this section 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 gives an insight into pharmacokinetic modelling and medical internal radiation dose (MIRD) principles of calculations, and discusses various aspects related to the extrapolation of animal dosimetry 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 design of 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 serves as an essential section to this guide, giving detailed methodologies for several of the techniques discussed in the course of this publication. As well as useful example study protocols for some of the commonly performed evaluations 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 broadly spoken its pharmacological profile in said application [2]. Inorganic ions, small organic molecules, peptides, proteins and even particles and polymers can serve as carriers for radionuclides in radiopharmaceuticals [5]. Like with any other drug, alterations made in the carrier molecule can affect parameters like target binding, distribution or metabolism, which in turn 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 must ensure adherence to the following properties for nuclear medicine purposes [1]:

- Rapid uptake and sufficient period of retention in the region of interest;
- Minimal uptake or quick washout from non-target regions;
- Minimal non-selective retention in the target region;
- Adequate in vitro and in vivo stability, with minimal presence of redistributing radio metabolites;
- Minimal unintended toxic or pharmacologic effects;
- 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 is based on 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 like binding affinity, off-target-binding, metabolism and labelling capabilities with a radioisotope possessing nuclear decay properties matching the biological half-life of the radiopharmaceutical (lead optimization);
(d) Development and optimization of a radiolabelling procedure providing sufficient radioactive yield, radiochemical purity and molar activity for preclinical studies (labelling procedure development);
(e) Preclinical assessment (see section 2.2.) (tracer evaluation);
(f) Upscaling of production and ensuring good manufacturing practice (production) and;
(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 becomes crucial – from the point of view of safety and efficacy – that the chosen target can selectively be reached. This is for example possible, 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 programs. 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 originated from the previously known amyloid dye Thioflavin T.

In comparison to ‘standard’ drugs, radiopharmaceuticals need a much higher ‘target to background’ ratios to increase contrast and/or reduce radiation to healthy tissue. Three main factors that determine the possibility of high ‘target to background’ ratio are:

(a) High affinity and selectivity of the radiopharmaceuticals for its target, considering the density of target of interest in the tissue environment;
(b) 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) and;
(c) 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 of radiometabolite analyses in respect with different uptake in various tissues compared to 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 by Pike 2016 [7]. In the case of therapeutic radiopharmaceuticals, 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 directly linking the radionuclide to the carrier ligand or indirectly by means of a bifunctional chelating agent conserving the pharmacological properties of the carrier molecule.
**TABLE 1. SELECTED RADIONUCLIDES USED IN RADIOPHARMACEUTICALS**

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Decay Mode</th>
<th>Half-life</th>
<th>Common route of production</th>
<th>Most common carrier moieties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon-11</td>
<td>β⁻</td>
<td>20.4 min</td>
<td>cyclotron</td>
<td>Small molecules</td>
</tr>
<tr>
<td>Fluorine-18</td>
<td>β⁻</td>
<td>1.83 h</td>
<td>cyclotron</td>
<td>Small molecules, peptides</td>
</tr>
<tr>
<td>Nitrogen-13</td>
<td>β⁻</td>
<td>9.97 min</td>
<td>cyclotron</td>
<td>Simple chemical products (NH₃), small molecules</td>
</tr>
<tr>
<td>Oxygen-15</td>
<td>β⁻</td>
<td>2.04 min</td>
<td>cyclotron</td>
<td>Simple chemical products (H₂O, CO₂, CO, O₂ etc.)</td>
</tr>
<tr>
<td>Copper-64</td>
<td>β⁻</td>
<td>12 h</td>
<td>cyclotron</td>
<td>Peptides, antibodies</td>
</tr>
<tr>
<td>Zirconium-89</td>
<td>β⁻</td>
<td>78.4 h</td>
<td>cyclotron</td>
<td>Peptides, antibodies, cells</td>
</tr>
<tr>
<td>Iodine-124</td>
<td>β⁻</td>
<td>100.2 h</td>
<td>cyclotron</td>
<td>Peptides, small molecules</td>
</tr>
<tr>
<td>Gallium-68</td>
<td>β⁺</td>
<td>68 min</td>
<td>generator or cyclotron</td>
<td>Small molecules, peptides</td>
</tr>
<tr>
<td>Iodine-123</td>
<td>γ</td>
<td>13.22 h</td>
<td>cyclotron</td>
<td>Peptides, small molecules</td>
</tr>
<tr>
<td>Technetium-99m</td>
<td>γ</td>
<td>6.01 h</td>
<td>generator or cyclotron</td>
<td>Small molecules, peptides,</td>
</tr>
<tr>
<td>Indium-111</td>
<td>γ</td>
<td>67.32 h</td>
<td>cyclotron</td>
<td>Peptides, antibodies</td>
</tr>
<tr>
<td>Lutetium-177</td>
<td>β⁻</td>
<td>6.64 d</td>
<td>nuclear reactor</td>
<td>Small molecules, peptides, antibodies</td>
</tr>
<tr>
<td>Yttrium-90</td>
<td>β⁻</td>
<td>2.67 d</td>
<td>nuclear reactor</td>
<td>Small molecules, peptides, antibodies</td>
</tr>
<tr>
<td>Iodine-131</td>
<td>β⁻</td>
<td>8.02 d</td>
<td>nuclear reactor</td>
<td>Radionuclide in ionic form, small molecules, peptides, antibodies</td>
</tr>
<tr>
<td>Actinium-225</td>
<td>α</td>
<td>9.92 d</td>
<td>nuclear reactor, cyclotron</td>
<td>Peptides, antibodies, small molecules</td>
</tr>
<tr>
<td>Astatine-211</td>
<td>α</td>
<td>7.2 h</td>
<td>cyclotron</td>
<td>Small molecules, peptides,</td>
</tr>
<tr>
<td>Radium-223</td>
<td>α</td>
<td>11.43 d</td>
<td>nuclear reactor</td>
<td>Radionuclide in ionic form</td>
</tr>
<tr>
<td>Bismuth-213</td>
<td>α</td>
<td>45.6 min</td>
<td>Linear accelerator, nuclear reactor, generator</td>
<td>Peptides, antibodies, small molecules</td>
</tr>
</tbody>
</table>

**2.1.1. Preclinical evaluation of novel radiopharmaceuticals**

Preliminary in vitro preclinical evaluation studies of a novel radiopharmaceutical are carried out to evaluate if the radiopharmaceutical under development can be used in vivo. Many pharmacokinetic properties as ‘target to background’ ratios or target retention can only be evaluated in vivo. These properties, however, are essential to estimate if the radiopharmaceutical meets the requirement 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 must be run 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 also include in vitro studies on isolated human hepatocytes. The latter should be carried out to determine early if there are species differences in respect to 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 (SA) can influence the biodistribution of the radiopharmaceutical. In general, high SA candidates are preferred to avoid self-blocking effects and thus reduce target binding. The SA of a radiopharmaceutical is defined by 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 $[^{11}\text{C}]\text{CO}_2$ 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 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), solid phase extraction (SPE), etc. 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 are 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 estimation of 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 must 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 (i.e. the agent’s effects on vital organs and signs) evaluation is not necessary since these parameters are normally evaluated either during the ligand induced toxicology study or 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 duration 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, the incorporation of evaluation of clinical vital signs, body weight, haematology and serum chemistry into the biodistribution and dosimetry in order to evaluate the results during the radiation toxicity study is recommended. 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 radionuclide induced toxicity and ligand induced toxicity should be assessed. Once the biodistribution and dosimetry evaluation is completed, the obtained data can be used to evaluate radiation induced toxicity. This can be done through conversion of the measured organ dosimetry to organ toxicity estimates using the methodologies in the existing scientific literature and general scientific knowledge on the relationship on organ specific dosimetry and the respective organ toxicities. The clinical vital signs
and organ evaluation data obtained during the biodistribution may also be used in addition to
dosimetric calculations.

Vector induced toxicity should be evaluated using the cold pharmaceutical similar to the evaluation
of ligand induced toxicity for diagnostic radioactive pharmaceuticals and non-radioactive
pharmaceuticals. The study design, with respect to the number of administration and the dose, should
accurately reflect the planned use in the clinic. The same single species that was used in 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 ligand induced toxicology assessment should be representative of the highest
possible mass planned to be used in a clinical setting. Generally, at least a 100-fold higher mass dose
of human, 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
and to cause biological damage.

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 due to associated radioactive
marker. Such studies accelerate the evaluation process and decrease the costs involved in drug
development. Below are some of the ways 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.
They can e.g. 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 programs. Moreover, established
radiopharmaceuticals can be used to better understand a certain disease. The advantage of molecular
imaging compared to 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
exemplarily 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

<table>
<thead>
<tr>
<th>Radiopharmaceutical</th>
<th>Biological processes</th>
<th>Field</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>[18F]FDG</td>
<td>Glycolysis</td>
<td>Oncology</td>
<td>Tumour staging, therapy monitoring</td>
</tr>
<tr>
<td>[18F]FDG</td>
<td>Glycolysis</td>
<td>Inflammatory diseases</td>
<td>Arthritis</td>
</tr>
<tr>
<td>[18F]FDG</td>
<td>Glycolysis</td>
<td>Cardiology</td>
<td>Assessment of myocardial viability</td>
</tr>
<tr>
<td>[18F]FDG</td>
<td>Glycolysis</td>
<td>Endocrinology</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>[18F]FDG</td>
<td>Glycolysis</td>
<td>Neurology</td>
<td>Brain Disorders</td>
</tr>
<tr>
<td>[18F]FLT</td>
<td>Thymidine Kinase activity</td>
<td>Oncology</td>
<td>Brain tumour monitoring</td>
</tr>
<tr>
<td>[18F]FAZA</td>
<td>Hypoxia</td>
<td>Oncology</td>
<td>Tumour monitoring</td>
</tr>
<tr>
<td>[18F]FET</td>
<td>Amino acid transport</td>
<td>Oncology</td>
<td>Brain tumour viability monitoring</td>
</tr>
<tr>
<td>[18F]FDOPA</td>
<td>Dopaminergic neurons</td>
<td>Neurology</td>
<td>Dopaminergic neuron plasticity</td>
</tr>
<tr>
<td>[11C]Raclopride</td>
<td>D2 dopamine receptors</td>
<td>Neurology, psychiatry</td>
<td>Receptor occupancy dopamine release Intrastratial neuron plasticity</td>
</tr>
<tr>
<td>[11C] /[18F]Choline</td>
<td>Choline kinases</td>
<td>Oncology</td>
<td>Prostate cancer monitoring</td>
</tr>
<tr>
<td>[123I]Ioflupane</td>
<td>Dopamine transporter</td>
<td>Neurology</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>[177Lu]PSMA-617 / [68Ga]/[18F]PSMA</td>
<td>Prostate specific membrane antigen</td>
<td>Oncology</td>
<td>Prostate cancer treatment and imaging</td>
</tr>
<tr>
<td>[177Lu]Lutathera / [68Ga]DOTATOC / [68Ga]DOTATATE</td>
<td>somatostatin receptor</td>
<td>Oncology</td>
<td>Neuroendocrine tumours treatment and imaging</td>
</tr>
<tr>
<td>[15O]H2O</td>
<td>Blood flow</td>
<td>Cardiology</td>
<td>Myocardial blood flow</td>
</tr>
<tr>
<td>[13N]NH3</td>
<td>Blood flow</td>
<td>Cardiology</td>
<td>Myocardial blood flow</td>
</tr>
<tr>
<td>[223Ra]RaCl2</td>
<td>Bone turnover</td>
<td>Oncology</td>
<td>Metastatic bone cancer</td>
</tr>
<tr>
<td>[131I]NaI</td>
<td>Iodine absorption</td>
<td>Oncology</td>
<td>Thyroid cancer</td>
</tr>
</tbody>
</table>

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 cut down the required number of experimental animals for this work. This is particularly useful when the same techniques are applied in actual clinical practice to monitor patient response to treatment. In oncology, efficacy studies may be performed by PET imaging for example with [18F]FDG if the drug under development targets a glycolytic tumour, while other radiopharmaceuticals such as [11C] /[18F]Choline or [68Ga]/[18F]PSMA are suitable to image non glycolytic prostate cancers. In case of drugs for treatment of differentiated neuroendocrine tumours, [68Ga]DOTATOC, [68Ga]DOTATATE or [18F]FDOPA may be used. Moreover, [18F]FDG may permit assessment of the effect of a tested drug on brain inflammation, or to monitor CNS disorders. [11C]PIB or [18F] labelled amyloid targeting radiopharmaceuticals may be used to evaluate the efficacy of drugs acting on amyloid deposition, whereas [123I]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 radiolabelled drug candidate

Drug molecules under evaluation can be labelled with suitable radionuclides. Chemical structure and biological half-life of the drug candidate require consideration while choosing the suitable radionuclides. Short lived PET radionuclides like $^{11}$C and $^{18}$F are commonly used for labelling small organic molecules, whereas radiometal diagnostic nuclides with 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 do in vivo PK/pharmacodynamics modelling to determine the kinetics of drug occupancy in the target organ. Occupancy studies require that the drug under evaluation binds to a target that can be studied with an existing validated radiopharmaceutical. Examples include $^{[11]}$Craclopide for dopamine D2/D3 receptors or $^{[11]}$CDASB for serotonin uptake sites [12].

2.3. SPECIES DIFFERENCES

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

2.4. QUALITY CRITERIA OF RADIOPHARMACEUTICALS FOR PRECLINICAL RESEARCH

Quality control with respect to radiochemical identity and purity, radionuclide identity and SA of the radiopharmaceutical as well as of all possible contaminations, 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 refer to the technique of conducting a given procedure in a controlled environment outside of 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 animals. In contrast to ex vivo studies, at the time of collection radioactivity is not already present in the samples. However, samples will be exposed to radiopharmaceuticals in the course of the 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 / 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. Below, a brief overview of some key characteristics is given, especially with respect to their utility in predicting the ligand’s in vivo performance as a radiopharmaceutical.

(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) or the inhibition constant (K_i), respectively. 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 (B_max) and the ligand affinity (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, and as such the affinity of a radiopharmaceutical, expressed as K_D or K_i, should be approximately 1 nM or lower. A radiopharmaceutical’s affinity can also be used to select which dose should be administered to achieve 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 which 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 point when the equilibrium in 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 in animals / 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 an 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 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 α or β emitting therapeutic radiopharmaceuticals.
Internalization assays for radioactively labelled tracers are a bit 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. Total tracer uptake is a sum of tracer uptake components due to surface binding and internalization.

Uptake studies help to find out, if a proposed tracer is taken up sufficiently at some point in time (or superior to other tracers in development) to function as a useful in-organism tracer.

Care must 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. This is essentially an extended cell uptake study, where after an initial period of cell uptake, the cells are washed with pure cell culture medium and 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 which competes with the tracer for binding to a certain target. Unlabelled ligand is introduced in excess, so the binding of the tracer to the target is reduced to negligible values or blocked.

Tracer selectivity can be tested by making it compete with the ligand that binds to the target other than the intended. If the presence of such 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 bind to specific target(s).

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 actually occupy at least 90%
of the target binding sites (i.e. minimum 10 times KD) and that the incubation time is sufficient for the binding of both the radiopharmaceutical and the blocking ligand to equilibrate.

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 assessment of the selectivity of binding to the intended target in the presence of other targets which may cause off target signal.

3.1.6. Efficacy / functional assays

(a) Functional assays test whether the tracer molecule launches or blocks a certain biochemical pathway after binding to its intended target;
(b) For therapeutic radiopharmaceuticals, in vitro efficacy assays test the ability of the radiopharmaceutical to exert therapeutic action on the target expressing cells at relevant concentration. Usually, the ability of radiopharmaceuticals to destroy cancer cells or bacterial/viral pathogens is tested in such assays;
(c) 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 due to insufficient solubility in the membrane bilayer or due 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 are available, where tracers can be evaluated 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 all in vitro assays to predict a compound’s behaviour in vivo is limited thus far.

A number of physicochemical properties are believed to be important for optimal brain penetration. These include water/octanol distribution coefficients (logP or logD at pH 7.4), polar surface area, hydrogen donor count, acidic/basic properties (pKa) and others. These properties can be calculated using proprietary or open source software packages, and some of them can be measured experimentally. For example, logD is easy to measure by shake flask, HPLC based or NMR based methods [18–20]. In silico scoring systems have been developed, which evaluate the likelihood that a radiopharmaceutical will penetrate the BBB based on a composite of weighted calculated physicochemical parameters. Examples of such scoring systems are the MPO score or the BBB score [21]. Such scores can help to pre-select candidate tracers before in vitro evaluation starts.

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 slide mounted tissue sections with radioligands under carefully defined conditions, washing and drying of 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] GTP\(_{\gamma}\)S 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 e.g. as logD value) 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, it is a prerequisite that the target is not dissociated from the tissue during the incubation with the radiopharmaceutical. Usually, targets such as membrane proteins or insoluble supramolecular aggregates like β amyloid plagues 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 which underwent a certain preservation procedure. For instance, β amyloid tracers based on Thioflavin T structure recognize β amyloid plagues 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 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 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 it is designed to mimic. It also provides insight into the fate of the tracer, especially relating to post-metabolic 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/homogenates must be sufficient for any possible metabolic degradation of the tracer. The selected assay must be able to differentiate between the tracer and its various metabolites. Care must be taken to ensure that metabolites are not generated on account of
microbial contamination or degradation of the tracer by air, light, generic components of the incubation medium etc.

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 period of time (shelf life). It is essential that a radiopharmaceutical formulation retains 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 seriously 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 go out of 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 to assess suitability of a radiopharmaceutical. The incubation time will be dependent 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 obtained data. This requirement exists because each cell line may not be adequately representing cells within patient population. Preferably, the cell lines derived from human tumours should be used as these are better representative for such studies than the transfected cell lines. In addition, it is important to include negative control cell lines (at least one, and preferably more than one), i.e. cell lines from 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 which 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 in humans. Metabolic pathways in humans and test animals, for example rodents, cats, pigs or non-human primates (NHP) 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 as per instructions from the manufacturer or vendor, unless there are justified reasons against this. The typical cell culturing environment would be at a temperature of 37°C with 85% relative humidity and an atmosphere containing 5% CO₂. Alternate buffering systems like HEPES salt can be used if for any reason CO₂-bicarbonate buffering is unavailable or should not be used. It should be noted that HEPES is known to be toxic to a number of cell lines. Fetal bovine serum is one of the most commonly used growth supplements. Certain studies may require the use of special starvation media (for example, folate deficient media to do cell studies with folate receptor targeting ligands). It is important to maintain uniformity in sourcing of all media and supplements (especially fetal bovine serum, 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 specific lots) to maintain a baseline consistency in results obtained from cell studies. The passage number of cell lines should be documented, kept as low as practically possible, and special care should be taken to make comparative studies on cells that are of similar passage number. Storage of frozen cells should be initiated at an early passage number as possible, preferably not exceeding passage number ten.

3.5. GENERAL REMARKS ON IN VITRO METHODS

The bulk of in vitro assessment of radiopharmaceuticals is based on tissue sections and homogenates or mammalian cell cultures, using either primary cell cultures or transformed cell lines. There are already multiple sources in the literature, including books, handbooks and monographs. They 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 volume to delve into those details.

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

(a) Incubators to maintain mammalian cells under normal growth conditions (typically 37°C, 5% CO₂ containing atmosphere);
(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 envisioned;
(g) Personnel must be properly trained in the protocols related to general cell culture activities and the specific functions they are expected to perform. Detailed standard operating procedures should be available for all procedures. Any deviation from standard procedure or expected observation should be noted;
(h) General precautions for cell culture/experiment facility: entry into the cell culture facility must be restricted to authorized personnel. Rules pertaining to the use of sterilized outer garments, interlocking access doors, pass box for transfer of material, and proper segregation and disposal of waste should be rigorously followed. The facility must undergo regular meticulous cleaning with surface disinfectant, and fumigation with a peroxide-based microbicidal aerosol would be advantageous. Periodic validation of the laminar airflow filters must be performed by qualified
agencies. Any issues of microbial contamination must be dealt with immediately to protect the integrity of the facility;

(i) In addition to these general precautions, during radiopharmaceutical evaluation studies, radioactive and non-radioactive work must be performed in separately designated areas to prevent cross-contamination and ensure worker safety. All necessary care must 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 the injection of the radiopharmaceutical into a living laboratory animal (e.g. a mouse or a rat). In vivo refers to experiments using a whole, living organism as opposed to a partial or dead organism.

4.1.1. Ethical considerations

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

— Replacement: animal experiments have to be replaced, where possible, with any other methods, such as computer simulation, mathematical models, in vitro studies, artificial tissues/organs, etc.
— Reduction: only the minimum number of animals necessary to answer the stated research question has to 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.
— Refinement: animal experiments have to be executed 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’s principles are published by professional organizations in the field of laboratory animal science and governmental regulatory agencies (for example, see [27]).

4.2. STUDY DESIGN

Studies with newly developed diagnostic radiopharmaceuticals typically aim to test their PK (e.g. target identification and 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, PK as well as therapeutic effect are of interest. Consequently, different experimental setups 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. Based on this information, a comprehensive study can be designed. Some critical points to consider in the study design are:
— The way PK/biodistribution data are obtained (ex vivo or imaging studies);
— The match between the decay half-life of the radionuclide and the kinetics of the process to be imaged;
— Administered dose (mass and radioactivity) of the radiopharmaceutical;
— Route of administration;
— Imaging time points and type of scan (dynamic or static);
— Animal model, inclusion and exclusion criteria, type of anaesthesia;
— End points and samples to be collected upon termination;
— Selection of the animal model;
— Need for a preliminary pilot study.

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

4.2.1. Design and data analysis considerations

The reproducibility, robustness and translatability of preclinical imaging studies – due to limitations in their experimental design and statistical analysis – is an ongoing topic of conversation. 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 of 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 due to their small size, short generation times, known genetic background and relative ease of procurement, handling and housing. Moreover, due to 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 (syngeneic or xenogeneic, subcutaneous or orthotopic, chemically induced) and the chemically induced models for neuroimaging (e.g. epilepsy, Parkinson’s disease, multiple sclerosis).
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 gene(s) from their genetic code). Typically, human DNA parts are incorporated to create so-called ‘humanized’ mouse models. Examples here are the transgenic models of Alzheimer’s disease.

In the negative model, the disease state does not develop in the animal, so it can be used as a negative control or for the investigation of factors which prevent disease development.

In orphan models, the disease state occurs naturally in non-human species but has 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 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 availability of the disease model;
— The physiology in comparison to human physiology;
— The structure and size of the organ or region of interest compared to the resolution and sensitivity of the imaging system;
— The desired information one wants to obtain;
— The hypothesis (such as biochemical, behavioural or biodistribution) to be tested;
— The number of measurements required (related to the number of animals needed for the study) and;
— The requirements for staff and experimental facilities.

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

Larger species such as pigs or NHPs offer the advantage, because their physiology is closer to human physiology and behaviour, the dosimetry is more closely matched to humans and surgical interventions are simpler. Moreover, it is possible to obtain larger blood samples for analysis and, finally, their larger structure sizes can be resolved with most of the imaging systems. For brain imaging, the closer similarity of pig, primate and human neuroanatomy (compared to 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, are easier to handle, 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 of the imaging systems, surgical interventions are more challenging and finally the 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 into 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 can be violated (see 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 do a power analysis. This analysis establishes a mathematical relation between:

— The magnitude of the effect of interest;
— The expected variability of the effect readout;
— The desired power of the test (the probability that the posited hypothesis will be confirmed if it is true);
— The desired significance level of the test (the probability that the alternative hypothesis will be rejected if it is true);
— An alternative hypothesis (typically the ‘null hypothesis’ in frequentist approaches)
— The sample size.

Software packages which provide free power analysis calculations for simple situations are available. A deeper review regarding design and statistical analysis of animal experiments can be found in references [42–44].

4.3.2.2. Randomization

Randomization is an important method of experimental control which 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:

— Animals should not be separated into treatment groups based on the cages they are housed in. 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;
— 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 paw). Different systemic treatments can be applied to the same animal at different points in time;
— Blinding is another method to reduce bias. Blinding in this context can mean that experimenters who evaluate the treatment effect are unaware of which treatment group each animal belongs to. Blinding can also be implemented in animal allocation into treatment groups, administration of treatments, animal caretaking etc.

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 or 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 as well as blood sampling should be performed using an aseptic technique. The used equipment should be appropriate for the used species. For example, smaller animals require thinner needles (Table 3). However, the viscosity of the administered substance has also to be considered in needle size selection process. Bigger needles are better suited for thick viscous liquids. To minimize distress caused to animals during the administration procedure, test animals should be anaesthetized. Recommended administration volumes differ between species and administration route. Table 3 displays some examples. The volume of any substance administered must be as small as possible and is naturally limited by the size of the used animal. Good practice guidelines for substance administration to laboratory animals are reviewed in [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 only be performed 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 summarized in Table 4 and is dependent on the circulating blood volume in each species.

| 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 (bolus) ml/kg | Needle size (G) | Volume (bolus) ml/kg | Needle size (G) | Volume (bolus) ml/kg | Needle size (G) | Volume (bolus) ml/kg | Needle size (G) | Volume (bolus) ml/kg | Needle size (G) |
| 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 |

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, e.g. the lateral tarsal (saphenous) vein, the marginal ear vein, the sublingual vein, the submandibular vein, the lateral tail vein, or the retrobulbar plexus. For repeated or continuous blood sampling, a 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 this allows quantification of radioactivity concentration in
blood without blood loss [47]. Temporary catheters such as butterfly needles can be used, whereas for long-term use surgical implantation of a biocompatible catheter is required.

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

— The purpose of the blood collection;
— The need for an arterial versus venous sample;
— The duration and frequency of sampling;
— The impact on animal welfare;
— The health status of the animal being bled;
— Whether the sample is taken as part of a terminal procedure;
— The potential for stress induced effects on biochemical and haematological parameters;
— The training and experience of the staff involved.

**TABLE 4. PRACTICAL BLOOD SAMPLE VOLUMES FOR LABORATORY ANIMALS**
(Adapted from [46]).

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference weight (g)</th>
<th>Blood volume (mL/kg)</th>
<th>Total blood volume (TBV), normal adult (mL)</th>
<th>Safe volume for single bleed (mL)</th>
<th>Bleed out volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>18–40</td>
<td>59</td>
<td>Male 1.5–2.4 Female 1.0–2.4</td>
<td>0.1–0.2</td>
<td>Male 0.8–1.4 Female 0.6–1.4</td>
</tr>
<tr>
<td>Rat</td>
<td>250–500</td>
<td>54–70</td>
<td>Male 29–33 Female 16–23</td>
<td>Male 2.9–3.3 Female 1.6–1.9</td>
<td>Male 13–15 Female 7.5–9</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1000–6000</td>
<td>57–65</td>
<td>58.5–335</td>
<td>5–50</td>
<td>31–310</td>
</tr>
<tr>
<td>Rhesus Monkey</td>
<td>5000</td>
<td>55–80</td>
<td>Male 420–770 Female 280–650</td>
<td>Male 42–77 Female 28–63</td>
<td>n.a</td>
</tr>
</tbody>
</table>

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 must be considered in the data analysis and group comparison. Control and experimental group must 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 as such 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 % injected dose (ID)/g [49]. Other parameters influenced by the circadian rhythm are hormone levels and body temperature [48].

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1 More information on blood sampling can be found under [https://www.nc3rs.org.uk/our-resources/blood-sampling](https://www.nc3rs.org.uk/our-resources/blood-sampling).
4.3.5. **Physiological monitoring**

4.3.5.1. **Body temperature**

The 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 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 it is possible. The application of anaesthetics can change these rates and influence as such the outcome of the experiment. Monitoring of these parameters in rodents is done relatively seldom due to technical difficulties. Rodents are small, and have very high respiratory and heart rates, which can only be measured by specific devices. However, in larger species such as pigs, dogs or NHPs, respiratory and heart rates are almost universally monitored to allow the experimenter, for example, to adjust the concentration of applied anaesthetics or intervene in any other 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 $[^{18}\text{F}]$FDG. Glucose levels influence the accumulation profile of $[^{18}\text{F}]$FDG. As such, it is important to have control over this parameter.

4.3.6. **Fasting**

Some experiments require fasting of the experimental animals. It can help to decrease blood glucose level, which may be necessary for imaging studies with $[^{18}\text{F}]$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 body weight loss, decrease in 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 if fasting is needed. 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). In this way, less discomfort to the animal is generated [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 the acquisition of the image so that no image artefacts are caused by motion. The type of anaesthesia should be chosen based on the experiment to be performed, especially in the organ of interest. Neuroimaging in particular is 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 must be monitored during anaesthesia. It can also be useful to directly control these parameters by e.g. using heating mats or introducing artificial lung ventilation in animals being scanned.

Anaesthesia can be induced by injectable agents and also, more commonly, by inhalation agents. Comparing injectable and inhalational anaesthesia, the inhalation is safer, especially in long procedures. Inhalation anaesthesia causes lower cardiovascular depression and also lower impact on the liver and kidney function. Besides, it is much easier to control, i.e. 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 [18F]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 [18F]FDG is highly dependent on whether the animals are conscious or not: for example, awake animals show increased [18F]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 inhalational anaesthetics are reported to increase the binding of agonist tracers to dopamine D2/3 receptors, while no such effect is observed for antagonist tracers [56–58].

The most used anaesthetic 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
(Adapted from the book Principles of Laboratory Animal Science [59])

<table>
<thead>
<tr>
<th>Anaesthetic</th>
<th>Dose and administration route</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse</td>
</tr>
<tr>
<td>Ketamine/xyazine</td>
<td>100 mg/kg / 10 mg/kg</td>
</tr>
<tr>
<td></td>
<td>IP*</td>
</tr>
<tr>
<td>Ketamine/ medetomidine</td>
<td>75 mg/kg / 1 mg/kg</td>
</tr>
<tr>
<td></td>
<td>IP*</td>
</tr>
<tr>
<td>Ketamine/diazepam</td>
<td>100 mg/kg / 5 mg/kg</td>
</tr>
<tr>
<td></td>
<td>IP*</td>
</tr>
<tr>
<td>Pentobarbitone</td>
<td>40-60 mg/kg</td>
</tr>
<tr>
<td>Halothane</td>
<td>Induction concentration 4-5 %</td>
</tr>
<tr>
<td>Sevoflurane</td>
<td>Induction concentration 8%</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>Induction concentration 4%</td>
</tr>
</tbody>
</table>

* IP = Intraperitoneal
4.3.8. Euthanasia

Euthanasia is the practice to end the life of an experimental animal. Several procedures are permitted. The right procedure should be selected for each situation considering the animal’s wellbeing and the research question. The procedure must already be decided during the initial study design and approved by a local ethical committee. During the procedure, the animal should be anaesthetised to reduce animal discomfort. The person(s) responsible for the procedure needs to be well trained. The following are the ways in which euthanasia is practiced:

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

4.3.9. Housing conditions, 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 must be secure and not allow animals to escape. They have to be made of a durable material, allow easy and frequent cleaning (minimal corners and angles). Used bedding material must allow the animals adequate resting and sleeping time, let them conduct typical behaviour like digging and burrowing and also facilitate thermoregulation. The bedding material must absorb urine and faeces.

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

It is extremely important that an identification system is established. The identification system should include a cage identification and an individual animal identification (coloured stains, ear holes or piercing and subcutaneous transponders). Cages must have detailed information about the responsible investigator (name and contact information) and 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 the treatment (if any) or description of the treatment (if any). It is also important that the identification tag of the cage shows alert/warning messages if applicable, like ‘infected animals’ and/or ‘radioactive material’, etc. Further information should be clearly visible such as used radionuclide, injected material, etc.
TABLE 6. RECOMMENDED HOUSING SPACE FOR RODENTS
(adapted from [60])

<table>
<thead>
<tr>
<th>Animals</th>
<th>Weight (g)</th>
<th>Floor area/animal (cm²)</th>
<th>Height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mice</td>
<td>&lt;10</td>
<td>38.7</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>Up to 15</td>
<td>51.6</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>Up to 25</td>
<td>77.4</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>&gt; 25</td>
<td>≥ 96.7</td>
<td>12.7</td>
</tr>
<tr>
<td>Rats</td>
<td>&lt; 100</td>
<td>109.6</td>
<td>17.8</td>
</tr>
<tr>
<td></td>
<td>Up to 200</td>
<td>148.35</td>
<td>17.8</td>
</tr>
<tr>
<td></td>
<td>Up to 300</td>
<td>187.05</td>
<td>17.8</td>
</tr>
<tr>
<td></td>
<td>Up to 400</td>
<td>258</td>
<td>17.8</td>
</tr>
<tr>
<td></td>
<td>Up to 500</td>
<td>387</td>
<td>17.8</td>
</tr>
<tr>
<td></td>
<td>&gt; 500</td>
<td>≥ 451.5</td>
<td>17.8</td>
</tr>
</tbody>
</table>

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 under 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 in the last 20 years and are now commercially available from different vendors. This includes 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 an anatomical framework. Moreover, the CT and MRI information can also be used to perform partial volume, motion, scatter and attenuation correction to improve tracer quantification. However, animal studies can also be performed using human scanners with the disadvantage of a reduced spatial resolution. In addition, clinical γ cameras equipped with pinhole collimators complete the array of imaging modalities.

In most of the 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 time points.

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, so called imaging chambers (or animal beds, 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 that are also compatible with the different scanners. Image registration can then be performed based on 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 during transport, especially when the scanners are far apart.

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

(a) high detection efficiency;
(b) high spatial resolution;
(c) low dead time;
(d) good timing resolution and;
(e) good energy resolution.

These requirements are accomplished in most of the preclinical PET scanners by using advanced scintillator materials such as lutetium oxyorthosilicate (LSO):Cerium (Ce) or Cerium-doped Lutetium Yttrium Orthosilicate (LYSO):Ce crystals either in pixelated or monolithic arrangement attached to position sensitive photodetectors: photomultiplier tubes (PMTs), silicon photomultipliers (SiPMs) 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 PET, however, at the cost of a reduced systems 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 (overlap of projections from different pinholes) whereas other scanners exhibit different arrangements of the pinholes to avoid multiplexing as it complicates the image reconstruction. The detection principle in preclinical SPECT system is mostly based on a scintillator coupled to an array of PMTs or SiPMs 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 are using stationary detectors and translate the bed in x-y-z directions to extend the FOV up to 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 scanning mice only. The axial FOV also varies between the 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 applied collimators, different animal species (mice, rats, or rabbits for example) 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 the literature [61–64].
### TABLE 7. CHARACTERISTICS OF PRECLINICAL PET AND SPECT SCANNERS

<table>
<thead>
<tr>
<th></th>
<th>Preclinical PET</th>
<th>Preclinical SPECT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detector material</td>
<td>LSO:Ce, LYSO:Ce, GSO:Ce</td>
<td>NaI:Tl, CZT</td>
</tr>
<tr>
<td>Axial FOV (mm)</td>
<td>50–150</td>
<td>7–300*</td>
</tr>
<tr>
<td>Radial FOV (mm)</td>
<td>80–120</td>
<td>12–250*</td>
</tr>
<tr>
<td>Energy resolution (%)</td>
<td>~20</td>
<td>5–10</td>
</tr>
<tr>
<td>Peak detection efficiency (%)</td>
<td>4–14</td>
<td>0.05–3*</td>
</tr>
<tr>
<td>Spatial resolution at cFOV (mm)</td>
<td>0.8–2</td>
<td>&lt; 0.5*</td>
</tr>
</tbody>
</table>

*Values are highly dependent on the used collimator

#### 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), the radionuclide, the collimator (SPECT) and the time 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 to 15 min, is recorded. Thus, the reconstructed image represents the average tissue activity concentrations at the recorded period. For dynamic imaging, the scans is started at the time point of radiotracer injection. Typical scan times are 60 to 90 min depending on the used radionuclide and tracer. 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 time points. Determination of the time and duration of a scan strongly depends on the following parameters:

— Radiotracer:

(i) Small molecules are usually labelled with short-lived radioisotopes such as C-11 (T<sub>1/2</sub> = 20.4 min) and F-18 (T<sub>1/2</sub> = 109.8 min). As a rule of thumb, the possible acquisition time is around 2-3 radionuclide half-lives, which translates into reasonable scan times of around 60-90 min for carbon-11 and 60-240 min for fluorine-18;

(ii) Large molecules (e.g. peptides, antibodies) are usually labelled with longer-lived radioisotopes such as Zr-89 (T<sub>1/2</sub> = 3.3 d) or I-124 (T<sub>1/2</sub> = 4.2 d). This is related to the longer circulation time in blood and the slower PK and accumulation rates. With such radionuclides, scans can be performed up to one week after administration.

— 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) Inhalational anaesthesia is typically better tolerated, and thus animals can be scanned multiple (2 to 3) times per day (e.g. directly at 4 and 8 hours after injection).
Animals:

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

The setting of the energy and the coincidence time window will also have an influence on the image quality. Considering a typical energy resolution of 20% for a scintillator used in a preclinical PET scanner, a minimum energy window of 409 to 613 keV (511 keV ± 20%) is recommended. Usually, an energy window of 350 to 650 keV is applied in most of the preclinical PET scanners. When using a larger energy window (e.g. 250 to 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 the random coincidences. For preclinical scanners, typical coincidence time windows are 2 to 6 ns. When using so called ‘dirty positron emitters’ such as $^{124}$I that 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 to 20% wide energy window around the photopeak of the respective radionuclide (e.g. $^{99m}$Tc, 140 keV photopeak, energy window 126 to 154 keV).

In contrast to PET, SPECT also offers the possibility to image multiple radiotracers, simultaneously. By defining appropriate energy windows around the photopeak of the used radionuclide, 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 due 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 was shown by Hijnen et al [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 4.4.7.5 tracer kinetic modelling). 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. Based on these results, simpler imaging protocols can be designed.
4.4.4. Image reconstruction

In image reconstruction, the recorded raw data is transformed to obtain temporally and spatially resolved images. Usually this transformation is separated into two steps. In the first step, the raw data is sorted to generate sinograms. Here, coincidence events in PET are grouped along a certain angle into 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 of the preclinical PET scanners operate in 3D 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 3D data set with conventional 2D reconstruction schemes while maintaining the sensitivity of a 3D sinogram data set. There are two approaches that 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 back projection are computationally very fast and accurate; however, they usually provide lower resolution images as compared to iterative reconstructed images. Iterative image reconstruction techniques such as maximum likelihood expectation maximization is routinely used in preclinical imaging. The disadvantage of iterative reconstruction is that it takes more time as compared to filtered back projection and also often leads to amplification of noise. Advanced literature on PET [66] and SPECT image reconstruction [67] is published. Using commercially available preclinical scanners, the user has to define the used histogram and reconstruction technique. 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 (normalization, decay correction, dead time correction, random correction, calibration constant) and are usually applied in the process of data acquisition or image reconstruction. Others only have to be applied when performing kinetic modelling (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 due to the radioactive decay of the radionuclide. Usually, the tracer activity is corrected to the scan start time (and/or time of injection) based on the used isotope. These corrections are typically implemented in the scanner acquisition program 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 too busy handling a previous coincidence event. Several parts of the system contribute to the dead time and the detector is one of them. 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 period, results in an increase of the 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 Correction

The random count rate is often estimated with the delayed coincidence count rate technique. For each line of response (LOR), the measured random rate is subtracted before image reconstruction, either directly (online) or off-line, where the random information is first stored in a separate sinogram data set. 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, therefore increasing the data load on the coincidence controller. In addition, the low statistics of the random may increase image noise. Other random correction schemes are the profile distribution method and count rate methods. The profile distribution method uses the tails of the projection data outside the object to estimate the random (and scatter) background. Various other corrections have to be manually defined by the user when setting up the reconstruction protocols in most of the preclinical systems. These corrections are then implemented in the reconstruction step and they include normalization, attenuation and scatter correction.

4.4.5.4. Normalization

The normalization should be applied to the measured data to level out unavoidable variation in the LOR efficiencies due to variations in the crystal efficiencies (due to 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 of the preclinical PET scanners normalization is done using the direct normalization method.

4.4.5.5. Attenuation Correction

To obtain quantitative images, the attenuation of the 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 from the phantom have a higher absorption probability as 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, one distinguishes between a so called ‘hot’ and ‘cold’ transmission. The cold scan is done before the radioactive tracer is applied to the subject, and the hot indicates the transmission measurement after the PET scan, when there is still some activity of the tracer remaining in the subject.

There are three methods for determining 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 2D transmission sinogram data set. 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 $\mu$ map from the CT transmission data is upscaled from the used 80 to 120 keV to the 511 keV and the generated attenuation map 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 like 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 2 or more mice are scanned at the same time) or dense materials in imaging chambers (e.g. electronic board attached to some MR imaging coils). For all these examples attenuation and scatter correction is recommended.

4.4.6. Correction factors for SPECT

Besides establishing proper scanner function, several correction factors can be applied to SPECT images to ensure they are fit for purpose and provide the highest quality data possible. They will increase in importance with the current drive to more quantitative SPECT correction factors are highlighted in the following sections, and also discussed in comprehensive detail in the ‘IAEA Quality Control Atlas for Scintillation Camera Systems’ [68].

4.4.6.1. Uniformity correction

Uniformity of SPECT system is the ability to produce a uniform image in response to a uniform source of radiation. Each projection image is corrected for uniformity before being used in image reconstruction. This correction is similar to normalization in PET 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 additional asymmetric energy window setting could also contribute to non-uniformity.

Uniformity correction uses the scanner computer running the acquisition to evaluate the high-count flood as well as mean counts per pixel. A pixel by pixel correction factor is created based on 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

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 first of these, linearity correction, is based on the discovery that scanner variability was not due to issues of photon detection efficiency, but rather 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 photo peak [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 photo peak for each individual spectrum. The map can be used to establish an array of correction factors that can be used to adjust energy peaks from a given energy photon and can be used correct future scans acquired on the same camera.

### 4.4.6.3. Centre of Rotation

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 if COR is acceptable based upon pre-defined 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 the COR is out of specification, service by a qualified person is recommended.

— Attenuation Correction
— Scatter Correction
— Collimator-Detector Response (CDR) correction

### 4.4.7. Image data analysis

In this section, image analysis approaches are discussed, which 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 learning about tracer modelling, one needs to understand the simpler analysis approaches.

#### 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 kBq/cc (or kBq/mL or kBq/g) of tissue. Calibration parameters, needed to recalculate 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. 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 while hiding 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 which correspond to a specific organ (whole organ) or part of the organ (e.g. cortex from brain). VOIs can be drawn by selecting the voxels manually or by various image analysis tools, like region growing or segmentation. 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 supposed to be defined based on anatomical landmarks, it is easier to define them if anatomical imaging data (CT or MR) are available. In these cases, VOI is defined on the anatomical image (CT or MR) 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 data set. Ideally, when fusing images, the PET or SPECT image should be the stationary one and the CT or MR image the transformed one. Transformation usually includes a 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 region of interest 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. Semi-quantitative image analysis

To compare results from different subjects, there are two methods for dose normalization. Percent ID per gram only takes the total injected radioactivity into account and can be calculated as in Eq. (1):

\[
\text{%ID/g} = C_t \times \frac{V_t}{W_t} \times \frac{1}{D_{\text{inj}}} \tag{1}
\]

Where

- \( C_t \) is the tissue concentration (given in activity/volume);
- \( V_t \) is the tissue volume;
- \( W_t \) is the tissue weight;
- \( D_{\text{inj}} \) 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.

\[
SUV = \left( \frac{\% ID}{g} \right) \times W_p
\]  

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, the 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 will influence tracer uptake (i.e., s.c.; i.p., bolus, infusion). For intraperitoneal injection, it might happen that the intestine is 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 further be 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 which are determined by kinetic modelling is the (total) volume of distribution, abbreviated as \( V_D \) or \( V_T \), which represents the ratio between 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 (BP), which is equal to the ratio of 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, 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, like binding potential, are proportional to 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 tracer distribution 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 which are performed on radioactive samples derived from living animals. These samples are obtained after injection of a radiotracer into the animal and euthanasia. 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, 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 to 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 either 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 in accordance 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 region of interest 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 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 up 24 h or more for optimal uptake in the region of interest. The selection of organs/tissues excised for measurement of accumulated radioactivity also depends on the nature of the radiopharmaceutical (and its possible metabolites/degradation products) and the proposed clinical application. Typical considerations for the selection of organs and tissues for excretion are:

— Presence of the target (specific brain regions for brain receptor tracers, implanted tumours for tumour tracers) or complete absence of the target to estimate target/non-target contrast (receptor free regions of the brain, skeletal or cardiac muscle, whole blood or plasma);
— Involvement in the excretion of the radiopharmaceutical (kidneys and bladder for urinary excretion, liver, gall bladder and intestine for hepatobiliary excretion);
— Involvement in the recognition of the radiopharmaceutical by the immune system (liver, spleen, lungs, and lymph nodes, especially relevant for large molecules or nanoparticles);
— Tissues acting as radionuclide sinks, after the radionuclide is cleaved from the radiopharmaceutical by metabolic enzymes (bone for $^{18}$F labelled compounds, liver for $^{64}$Cu labelled compounds, stomach and thyroid gland for radioiodinated compounds);

— If there is a reason to suspect that a non-negligible fraction of the radiopharmaceutical becomes trapped at the injection site (e.g. due to imperfect i.v. injection), then the excision of the injection site (e.g. piece of the tail) can 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 PBS to wash off any surface blood and lightly pat on absorbent material to remove excess surface moisture. Individual organs are weighed, and measurement of associated radioactivity is done on the $\gamma$ counter or $\gamma$ 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 tail vein, for i.v. administered radiopharmaceuticals), after ensuring that this value does not account for more than 5 to 7% of total injected radioactivity. For measurement of total radioactivity associated with blood, muscle and bone, representative samples of each tissue are taken, weighed and counted carefully, and the measured radioactivity is extrapolated to account for their proportion in the whole body, using the assumption that in rodent models 7 to 8% of the total body weight is accounted by blood, about 10% by skeletal tissue and around 40% by muscle tissue [77, 78].

There are two ways how the total ID can be measured during ex vivo biodistribution studies: (a) measurement of activity in the syringe before and after injection will give an estimate of total injected activity. This value needs to be decay corrected to the time at which organs are counted and multiplied by the sensitivity of the $\gamma$ counter; and (b) preparation of a standard solution using a known weight of radiopharmaceutical solution, dispensed in 1 to 2 mL saline/PBS in a 5 to 10 mL vial. In this protocol, each syringe must 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 actual ID for each animal. While this method needs additional work, it removes the need to correct total injected activity for radionuclide decay and $\gamma$ counter sensitivity. The measurements of organ weights and associated radioactivity is 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 tissue (%ID/g). Automated organ counters may have in built 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 per g or total %ID value 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 %ID/g or %ID per organ values are normally plotted with the standard deviation represented as error bars. For tumour targeting radiopharmaceuticals, ratios of %ID per g values of tumour to blood and tumour to muscle provide a measure of specific accumulation in tumour tissue. %ID/g ratios of ‘heart to lung’ and ‘heart to liver’ give information about the usefulness of the tracers for myocardial perfusion imaging.

The simplest type of biodistribution experiment is one in which only the radiopharmaceutical is administered. In addition to this, 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 concomitantly 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 which are not suitable for in vivo imaging. For example, ³H or ¹⁴C labelled analogues can be used instead of ¹¹C labelled compounds, and ¹²⁵I can be used instead of ¹²⁴I. ¹⁴C and tritium (³H), 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 animal euthanasia) 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 all radioactivity has decayed. In general, slices of 30 to 40 µm are recommended for autoradiography and 5 to 20 µm for histochemistry.

Once the slices are ready, they should be dried before putting them in contact with the imaging plate (a helium, nitrogen or air flow can be used to accelerate that). For long lived radionuclides (T½ > 1 day), it can be recommended to fix the slices with paraformaldehyde vapours (incubate 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 ³H or ¹⁴C, which have a short β particle penetration depth.

Exposure time (the time tissue remains in contact with the plate) depends of the radioisotope and the activity contained in the sample. As the exposure time increases by one half-life of the used radioisotope, the obtained image contrast 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 known to be 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. Different kinds of equipment are available for autoradiography, where resolution and sensitivity are different, therefore, these parameters should be known before planning the experiment. Radionuclides and activity contained in the sample are also important factors, and they need to be considered when selecting the incubation/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, and therefore, they need to be quantified 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 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 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 obtained extract. Apart from measuring the amount of
radiometabolites relative to parent tracer, it is important to assess whether they are more or less lipophilic than the parent. Lipophilic metabolites are more capable of penetrating across 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 (RP) HPLC with radioactive detection. In 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 one needs a higher sensitivity 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 is able to differentiate 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 must 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 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 is filtered before being injected into the loop to prevent the clogging of the system by residual plasma proteins. Sample preparation and analysis methods for radiometabolites have recently been reviewed [80].

Another option for 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 homogenised 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/identification of various radioactive metabolites. On the other hand, HPLC analysis has high costs (expensive equipment, large amounts of solvents used), relatively long analysis time per sample and requires highly trained personnel. Alternative techniques for radiometabolite analysis, like thin layer chromatography (TLC), solid phase extraction (SPE) and liquid-liquid extraction (LLE) 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 setup 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 SPE columns, leading to an overestimation of 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, existing knowledge about the nature of radiometabolites as well as the radionuclide used. With long lived radionuclides, one can use lower sample amounts and apply more time consuming sample preparation and analysis methods, and vice versa. The optimal strategy is to start with HPLC, then go for TLC and then, if more simplicity and throughput are needed, develop and validate SPE or LLE based methods.

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 species. Therefore, it is often important to measure the ‘free fraction’ of a tracer, i.e. the percentage of unmetabolized tracer in the plasma which 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 treatment of cancer and infectious diseases. Therefore, in vivo experimental setups 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 on dosimetry of this publication.

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 only discusses toxicology concerns stemming from the biological activity that the radiopharmaceutical possesses as a drug. Radioprotection and radiotoxicity are discussed in the Dosimetry section.

Usually, diagnostic radiopharmaceuticals are administered at tracer levels (μg/kg body weight), i.e. 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 the mentioned dose levels or even lower. For example, the LD$_{50}$ of botulinum toxin in mice ranges from 1 to 5 ng per kg 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 be in compliance with the 3R principles. In some cases, literature data might be available that documents the necessary toxicology parameters. If these data suggest no toxicological concerns, they are usually sufficient to receive permission for human application from the authorities. 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 into the ‘investigational medicinal product dossier’ together with quality specifications, non-clinical pharmacology, efficacy (diagnostic or therapeutic), dosimetry etc. In the European Union, 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 the Regulation (EU) No. 536/2014, “Article 25: Non-clinical information submitted in an application dossier shall be based on data derived from studies complying with Union law on the principles of GLP, 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 a 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 final formulation specified to the GLP laboratory responsible for the conduction of toxicological studies and the preparation of non-clinical safety report.

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 achievement for scientists and industries involved in radiopharmaceutical development. Thanks to a large international debate between regulatory authorities and the scientific community, the concept of tracer and tracer theory, the term ‘microdose’ has been associated with imaging radiopharmaceuticals. The ‘microdose’ concept was originally proposed to simplify the non-clinical
safety dossier requested for a new medicine administered at very low doses and included in the Guideline of the International Conference of Harmonization (ICH): “December 2009 EMA/CPMP/ICH/286/1995: ICH guideline M3(R2) on non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals” [87]. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use aims to standardize regulatory requirement for marketing authorization of drugs under development. These guidelines have regulatory value when adopted by local regulatory authorities. In the ICH M3(R2), diagnostic radiopharmaceuticals are specifically mentioned in section 7 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 the ICH M3(R2) described below, can be used as the base 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 i.v. and mg/m² for oral). Approach 2 is applied when more than one administration is required. The total cumulative dose has to be ≤ 500 μg, maximum 5 administrations are permitted with a washout between doses (6 or more actual or predicted half-lives), each dose has to be lower than ≤ 100 μg and each dose must 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 minimum one species (usually rodents). The ‘extended single dose toxicity study’ should evaluate “haematology, clinical chemistry, necropsy data and histopathology in minimum 10 test animals/sex/group for all groups on day 2. Additional data should be provided for 5 test animals/sex/group on day 14 in the group that is planned to support the clinical dose” [87]. A maximum dose of 1 000 fold of the clinical dose on a mg/kg basis for i.v. can be used. For Approach 2, a 7 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 data set.

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 SA 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 compared to the NOAEL. This means that a dose 100-fold higher than what is intended for use in humans should not cause any adverse effects.

The dose limit for ‘microdose’ studies is defined in μg and 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 that, at the time of writing however, is still under public consultation [86]. Recently (August 2018), the U.S. Food and Drug Administration (FDA) also published a document on non-clinical safety evaluation of diagnostic radiopharmaceuticals: ‘Microdose Radiopharmaceutical Diagnostic Drug: non-clinical studies recommendations. Guidance for Industry’ [88]. This publication is also based on ICH M3(R2). 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 (1) The guidance for industry of the FDA ‘Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers’ [89], (2) the ICH guideline M3(R2) and (3) other ICH guidelines depending on the product and application [90–92]. The guidance ‘Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers’ [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 to:

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

The major elements in assessment of MRSD are the determination of the NOAELs in the tested animal species, conversion of NOAELs to HED (human equivalent dose), 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 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/m² basis. The resulting value is called HED (Table 8).

A safety factor should then be applied to the HED to increase assurance 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 effect level NOEL’.

Species conversion of doses for medicinal studies is also reviewed by Nair and Jacob [93].
TABLE 8. CONVERSION OF ANIMAL DOSES TO HUMAN EQUIVALENT DOSES BASED ON BODY SURFACE AREA

<table>
<thead>
<tr>
<th>Species</th>
<th>To convert animal dose in mg/kg to dose in mg/m², multiply by k_m</th>
<th>To convert animal dose in mg/kg to HED in mg/kg, divide animal dose by Human (60 kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (60 kg)</td>
<td>37</td>
<td>n.a</td>
</tr>
<tr>
<td>Mouse</td>
<td>3</td>
<td>12.3</td>
</tr>
<tr>
<td>Rat</td>
<td>6</td>
<td>6.2</td>
</tr>
<tr>
<td>Rabbit</td>
<td>12</td>
<td>3.1</td>
</tr>
<tr>
<td>Dog</td>
<td>20</td>
<td>1.8</td>
</tr>
</tbody>
</table>

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 time-activity curves 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 to the corresponding expected value in humans. Absorbed dose limits for normal organs are well known for external beam exposures and increasingly also for radionuclide therapy internal exposures. Often, once translation of a radiopharmaceutical occurs, true human dosimetry can be determined in the first healthy volunteers that are scanned.

6.1.1. MIRD principle for dosimetry calculations

The absorbed dose of an organ or tissue expresses a measure of the damage by ionising radiation in that organ or tissue. The absorbed dose is defined as the energy absorbed per unit mass and its dimension is expressed in Gy, corresponding to J/kg. For external exposures the absorbed dose can be measured directly through tissue-equivalent dose meters, like for instance ionisation chambers. For internal dosimetry the absorbed doses are derived from dosimetry models according to the MIRD in Eq. (3) [94]:

\[ D(\text{target organ}) = \sum_{\text{source} i} \tilde{A}(\text{source}_i) \times S(\text{target} \leftarrow \text{source}_i) \] (3)

Where

- \( D \) is the absorbed dose;
- \( \tilde{A} \) the cumulative activity in source organ \( i \)
- \( S \) factor stands for the absorbed dose to the target organ per unit activity in 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):
The time integrated activity $\tilde{A}$ is the sum of the total number of nuclear disintegrations in the source, or more visually 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 disintegration per administered activity (Eq. (5)).

$$A_0 \ \tilde{a} = \frac{\tilde{A}}{A_0}$$

By using $\tilde{a}$ instead of $\tilde{A}$ in Eq. (5) the absorbed dose per administered activity is obtained. Biodistribution data and also SPECT or PET imaging quantification data are usually given activity concentration (in percentage of injected activity %IA, or amount of activity per volume kBq/ml). 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, with the scalable mouse phantom MOBY and 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 $\beta$ particles in humans can be considered to be minimal in relation to the source organ dimensions and usually in human applications $\beta$ radiation is considered to be non-penetrating (i.e. all $\beta$ energy is locally absorbed within the source organ) [94]. In small animals, however, the range of $\beta$ particles can become comparable to the organ dimensions. For instance the $\beta$ particles from $^{90}$Y (with mean energy 933.4 keV and end-point energy 2280.1 keV) have a range in tissue of 4 mm (mean) to 11 mm (maximum) according to the continuous slowing down approach. As electron tracks do not follow a straight line, but a random scattered path, $X_{90}$ forms a better parameter to estimate its range. $X_{90}$ is the radius of the sphere in which 90% of the $\beta$ energy is absorbed [98]. The $X_{90}$ in water for $^{90}$Y is 5.4 mm, while the low energy $\beta$ emitter $^{177}$Lu (mean energy 133.5 keV and end-point energy 498.3 keV) has an $X_{90}$ in water of 0.62 mm. A typical 25 g weight mouse bears a 0.302 g kidney with approximate dimensions of 3.5 x 3.5 x 6 mm$^3$ [96]. Here $^{90}$Y will cause radiation exposure to surrounding tissue as well when it is taken up in the kidneys, whereas $\beta$ radiation from renal uptake of $^{177}$Lu will be mainly absorbed within the kidneys with a much smaller exposure to surrounding tissues from its $\gamma$ ray component.

The range of $\alpha$ particles in tissue is of the order of $\mu$m. For example, the 5.83 MeV $\alpha$ particle emitted by $^{225}$Ac has a continuous slowing down approach range of 48 $\mu$m and the 8.38 MeV $\alpha$-particle emitted by $^{213}$Po has a continuous slowing down approach range of 85 $\mu$m in tissue ($\alpha$ particle ranges determined with the Astar code; https://physics.nist.gov/PhysRefData/Star/Text/ASTAR.html). The high mass of the $\alpha$ particles or He$^{++}$ nuclei leads to straight $\alpha$ particle tracks by conservation of momentum in (electron) scatter events. These ranges only reach neighbouring cells adjacent to the cells with the $\alpha$ emitter uptake. The consequence is a possible non-uniformity of the dose distribution by $\alpha$ particle emitters.

### 6.1.2. Pharmacokinetic modelling of in vivo data to derive TAC

The TAC $A(t)$ in each organ with physiological uptake and in animal models (e.g. transgenic models of neurodegenerative disease, tumour bearing mice etc.) should be derived from either biodistribution

\[ \bar{A}(source) = \int_0^\infty A(source, t) dt \]
data or from quantitative imaging studies and can be used to predict pharmacokinetic modelling (Fig. 2). The timing of the animal euthanasia for biodistribution or the scan moments should be carefully chosen. The PK of the compound under investigation are still being determined, but essentially 4 basic kinetic models can be anticipated [99]:

(a) Instantaneous uptake without biologic clearance, just physical decay ($\lambda_{\text{phys}}$);
(b) Instantaneous uptake with biological clearance and physical decay;
(c) Gradual uptake without biologic clearance, just physical decay;
(d) Gradual uptake with clearance by biological clearance and physical decay.

MIRD pamphlet 16 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 radiopharmaceutical in blood or organ or total body. The effective half-life is calculated by combining both the physical decay and the biologic clearance half-life $T_b$, Eq. (6):

$$T_{eff} = \frac{T_p \times T_b}{T_p + T_b}$$  \hspace{1cm} (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. MIRD 16 recommends one or two data points taken at some fraction of $T_{eff}$, one near $T_{eff}$ and one or two other data points taken at 3-5 times $T_{eff}$. Radiopharmaceuticals following kinetic model 4 may therefore need 6 to 9 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 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 in activity concentration. The concentration TAC can also be integrated to calculate the TAC concentration in the source volume, Eq. (7):
\[ \frac{\hat{A}}{m} \text{ (source)} \]  

(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, likely due 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. Dosimetry phantoms for organ absorbed dose - S-values in small animals - MOBY/ROBY phantom dosimetry models

The use of ‘standardised’ reference dosimetry calculation phantoms has improved the absorbed dose results in preclinical experiments considerably. 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, 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 1.5 – 2. Also, the anatomy of the mice in the experiment might be quite different to the standardised MOBY-anatomy. Relative differences of 10 – 40% have been found between dosimetry codes claiming to use the same MOBY-phantom, mostly due 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 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 3 time points. Inclusion of additional time points (beyond 48 h) can enable a 2-compartment model fitting within the tumour a constant compartment, indicating the specific uptake, and in the kidneys and pancreas a second slower washout exponential. Statistical decision criteria, as the F test or the Akaike information criterion, can be used to decide whether the additional compartment is necessary.
Where:

(a) NanoSPECT/CT images of CA20948-tumour bearing rats, 3 h p.i. of 15 μg \(^{111}\text{In}\)-DOTA,Tyr\(^3\)-octreotate, labelled with 60 MBq of \(^{111}\text{In}\), without or with co-injection of Gelofusine 80 mg/kg (and 400 mg/kg Lys).

(b) Quantification of retained radioactivity in CA20948 tumours of imaged rats using InVivoScope software. Amount of radioactivity was expressed in MBq/ml tumour. Each tumour nodule inside a CA20948 tumour was analysed separately. No significant differences were found.

(c) Residence time of \(^{111}\text{In}\) in kidneys, as determined in NanoSPECT/CT images at 3, 24 and 48 h p.i. of 15 μg \(^{111}\text{In}\)-DOTA,Tyr\(^3\)-octreotate. Washout of \(^{111}\text{In}\) was plotted for three rats: control, with Gelofusine 80 mg/kg alone and combined with 400 mg/kg Lys.

(d) Dose calculation of \(^{111}\text{In}\)-DOTA,Tyr\(^3\)-octreotate for whole kidney or renal cortex only, expressed in mGy/MBq \(^{111}\text{In}\). Renal radiation dose in a control rat is compared with rats receiving Gelofusine 80 mg/kg alone or combined with 400 mg/kg Lys as co-administration.

6.1.6. Voxel based dosimetry models

The use of the 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 \(\beta\) 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 point kernel method for homogeneous media or by Monte Carlo calculations for heterogeneous media. All voxel dosimetry methods however do need a voxelized TAC, which would need reliable image registration of all SPECT or PET images taken over time.
6.1.7. Small scale dosimetry models

Especially for short ranged particle emitters, 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 sub-units 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 models for the kidney have now more detailed standardised dosimetry models. The most simple example is for the renal cortex, using the autoradiography at one time point as source distribution [102]. More detailed dosimetry models for the kidneys now include glomeruli and proximal and distal tubuli 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 indications for absorbed dose threshold values for its induction. Translation of absorbed dose limits found in the preclinical setting to the human patient should be performed as guidance, not only taking the difference in morphology into account but also equivalence of toxicity endpoints in humans and small animals. Also, translation of the 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 derive from preclinical experiments in relevant animal models the organ time-activity curves to be used in estimating the percent administered activity (%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 decide on 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 to total body weight ratio in mouse (m) and human (M), leading to the mass scaling option in Eq. (8):

$$\left(\frac{\%IA}{organ}\right)_{human} = \left(\frac{\%IA}{organ}\right)_{animal} \times M_{animal} \times \left(\frac{m}{M}\right)_{human}$$

(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):

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

(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, in the mass scaling option in Eq. (10):
\[ \tilde{A}_{human}(source) = \left( \frac{\tilde{A}}{m} \right)_{source} \times M_{animal} \times \left( \frac{m}{M} \right)_{human} \] (10)

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

\[ \tilde{A}_{human}(source) = \left( \frac{\tilde{A}}{m} \right)_{source} \times m_{animal} \] (11)

6.1.8.2. Time scaling

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

\[ t_{human} = \left( \frac{M_{human}}{M_{animal}} \right)^{0.25} \times t_{animal} \] (12)

When time scaling is applied together with mass scaling of the uptake the time integrated activity coefficients extrapolation form animal to humans would be as seen in Eq. (13):

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

The extrapolated time integrated activity coefficients value needs to be multiplied by a human S-value for the required source and target combination to reach to an estimate of the human dosimetry form the preclinical animal data. Human S values and the whole dosimetry calculation can be performed with 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 an external 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 article Supporting Information) to make it as straightforward as possible to replicate a preclinical imaging study. Accurate reporting is fundamental for reproducibility and thus reliability of scientific results.

An excellent guideline on reporting animal experiments (the ARRIVE guideline) was published by Kilkenny et al. [108]. Moreover, a guidance for describing methods used in preclinical imaging papers was presented by Stout et al. [109]. It is recommended to strictly follow these two guidelines, as these guidelines help to understand scientific publications, 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 obtained results. Moreover, the information should also be adequate to allow the experiment to be reproduced or built on for future studies.

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 used species (e.g. M for mice, R for rats, non-human primates), tracer used and/or imaging modality, animal type [e.g. wild type, transgenic, tumour bearing], or even the actual 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 ID when included in a study; in that case it would be advisable to rename them but keep the records of the old IDs and the 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. There, 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, also documenting the same parameters.

A database for generating study IDs, animal IDs, and web access, and for entering session-specific information such as injected probe, injection time, reconstruction parameters might be a helpful tool. However, if the documentation is stored in individual or study-specific file folders (either hardware or software) 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 etc.), data on the radiopharmaceutical, acquisition and reconstruction protocol to all the acquired image data. In the archiving criterion it is recommended to define, which data, in what form, where, when and how long it should be archived, as well as, how archives will be backed up. Based on this decision, an archiving system can be established. 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 was performed as a contract research study or even after GLP, the predefined archiving system and duration criteria have to be fulfilled. Such studies often define additional specific archiving requirements (e.g., documents should be stored in a fireproof safe and/or on a secure server for 10 years after completion of the study) that need to be adhered to.

It is now also possible to convert current imaging files from preclinical studies into DICOM formats for archivcal and retrieval from PACS systems [110]. The benefits of utilizing existing PACS solutions for preclinical imaging datasets are the standardized data exchange interface, structured data content search and retrieval functionality, and mature vendor-supported hardware and software solutions.
7.2.3.1. Archiving documents

Ideally, all the obtained and created protocol and documents are archived in a file folder. The folder can be named with the study ID and the protocols, reports and other documents can be archived. For easier retrieval, a subdivision into animal IDs 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 PC, a large disk array storage system (e.g. NAS or SAN arrays), a file server, an external flash drive or a hard drive, burned onto DVDs or, increasingly by utilizing institutional or third part cloud storage. For contract 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. There is the raw data file (e.g. in list mode format), a sinogram data file, and a reconstructed image data file. List mode and sinogram files easily exceed 10-20 GB, whereas image data files are in the order of 6-150 MB depending on whether they are from dynamic or static studies. Based on the available archiving system, it might be possible only to store the image files or also to store all the created files. Storing list mode files offers the possibility to reconstruct images with changed parameters at a later time point (e.g. different algorithm or different time frames). It is recommended to archive the analysed image files together with the analysis method (e.g. the defined volumes of interest) and the obtained quantitative parameters from the analysis.

Paperwork can be scanned, and included into the electronic archiving system.

It is recommended to use the same archiving system as for the hardware, which means the same folder structure and IDs.

7.2.4. Data retrieval

For image data analysis, it should be possible to retrieve the data after acquisition. This might be hours, days or even years after image acquisition. Depending on the policy of each laboratory, data retrieval might be restricted for specific person 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 states that [111]:

(a) “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;
(b) 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 that “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” [111].

Moreover, “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” [111]. 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, 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 and 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 lab supplies (syringes, paper towels etc.) and animal waste (carcasses, bedding etc.) to decay to background, 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 still radioactive should be stored 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/or deterioration.

Lastly, OECD guidelines also prompt for 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, centrifuge, HPLC, TLC, autoradiography system, cryostats/microtomes, cell culture equipment, sterile bench, cell counter, incubators, IVC cages, microscopes, staining facilities, anaesthesia system, physiological monitoring system, automated fraction collector, micro balance, laminar flow cabinets, personal computers, data archiving for images and data etc.

In case 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, requirements are of course 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 to reduce risks for personnel and the intended animals, ensure that operations are conducted in proper way, and reduce errors. Thus, the size of the facility will have 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 (access to anaesthesia etc.). In case radioactive doses are to be prepared ‘on-site’, suitably shielded hot cells should be installed (a detailed discussion of radiopharmaceutical synthesis is outside the scope of this publication; readers are referred to IAEA TecDocs specifically covering production and QC of radiopharmaceuticals [112, 113]).

8.3. STAFFING REQUIREMENTS

The number of professionals and their expertise are dependent on the activities that the laboratory is performing. Some recommended trained people are listed below:

— Radiopharmacist and/or radiochemist: necessary for the radiopharmaceutical production and QC;
— Biologist and/or veterinarian: animal care, in vitro and in vivo essays;
— Physicist: imaging equipment calibration and QC and also radioprotection;
— Biotechnologist: image acquisition;
— Image processing and quantification: several backgrounds are possible, but special training is necessary for expertise in this field;
— Responsible person: in general a scientist with years of experience and deep knowledge in the field;
— Technicians: trained people to help with basic activities, as animal care (food and housing care), laboratory material preparation and cleaning, etc.

8.4. STAFF TRAINING

Training will depend on national requirements. Basic recommendations include at least 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 for laboratory animal manipulation and care, radioactive material manipulation and also biosafety. These training should be official and documented and also periodic applied (at least once a year).
8.5. SAFETY CONSIDERATIONS

8.5.1. Biosafety

Diseases transmitted from animals to humans are called zoonoses and the infectious agents responsible can be viruses, bacteria or parasites. Laboratory animals should be supplied by the reputed commercial breeder to avoid getting a colony with zoonosis and then present risk for the personnel and investigators involved in the use of these animals.

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

Genetically modified organisms are also something that should 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% to 30%) [115].

Working with microorganisms is possible, with considerations for the personnel safety aspects. Following things are basic essential requirements: 1) Knowledge about the infection agent you are working with; 2) Trained personnel and 3) Adequate infrastructure.

A useful guideline for biosafety is the World Health Organization manual [116] concerning Laboratory Biosafety Manual, 3rd ed. 2004, providing basic biosafety concepts and section 6 which is dedicated to laboratory animal facilities. Tables 9 and 10 are adapted from the World Health Organization manual and give important information about the requirements prior to executing a potentially hazardous experiment.

Classification of infective microorganisms by risk group:

(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 spread of infection 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 RISK GROUPS, LABORATORY TYPE, BIOSAFETY LEVELS, PRACTICES AND EQUIPMENT

<table>
<thead>
<tr>
<th>Risk group</th>
<th>Biosafety level</th>
<th>Laboratory type</th>
<th>Laboratory practices</th>
<th>Safety equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Basic – Biosafety Level 1</td>
<td>Basic teaching, research</td>
<td>Good microbiological techniques</td>
<td>None; open bench work</td>
</tr>
<tr>
<td>2</td>
<td>Basic – Biosafety Level 2</td>
<td>Primary health services; diagnostic services, research</td>
<td>Good microbiological techniques plus protective clothing, biohazard sign</td>
<td>Open bench plus Biological safety cabinet for potential aerosols</td>
</tr>
<tr>
<td>3</td>
<td>Containment – Biosafety Level 3</td>
<td>Special diagnosis services, research</td>
<td>As level 2 plus special clothing, controlled access, directional airflow</td>
<td>Biological safety cabinet and/or other primary devices for all activities</td>
</tr>
<tr>
<td>4</td>
<td>Maximum containment – Biosafety Level 4</td>
<td>Dangerous pathogen units</td>
<td>As Level 3 plus airlock entry, shower exit, special waste disposal</td>
<td>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</td>
</tr>
</tbody>
</table>

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

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

ABSL: animal facility biosafety level
BSCs: biological safety cabinets
During facility design, the biosafety concepts should be considered for well planning of the site. The following examples should be considered [114]:

— Research line and scientific questions;
— Type of animals to be used;
— Hazardous material (biological, chemical and radioactive) to be manipulated;
— Equipment necessary for the laboratory;
— Physical infrastructure (heating, ventilation, air conditioning, electrical outlet, bench material, etc);
— Standard operating procedures;
— Staff training (in radioprotection and biosafety).

8.5.2. Radiation safety

8.5.2.1. General concepts

Radiation safety is based upon the ‘as low as reasonably achievable’ (ALARA) principle. ALARA is designed to minimize radiation dose to workers (Table 11) [117] (and members of the public), as well as to release radioactive material (Table 12) [117]. ALARA is predicated on legal dose limits for regulatory compliance and is a requirement for all radiation safety programs [117–120].

**TABLE 11. TYPICAL ALARA LIMITS FOR OCCUPATIONAL EXPOSURE**

<table>
<thead>
<tr>
<th></th>
<th>Regulatory limit</th>
<th>ALARA I</th>
<th>ALARA II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Body</td>
<td>50 mSv in a year</td>
<td>5 mSv</td>
<td>15 mSv</td>
</tr>
<tr>
<td>Extremity</td>
<td>500 mSv in a year</td>
<td>50 mSv</td>
<td>150 mSv</td>
</tr>
</tbody>
</table>

**TABLE 12. ENVIRONMENTAL RELEASE OF RADIOACTIVITY**

<table>
<thead>
<tr>
<th></th>
<th>Regulatory Limit</th>
<th>ALARA I</th>
<th>ALARA II</th>
</tr>
</thead>
<tbody>
<tr>
<td>All radionuclides</td>
<td>Local limits (e.g. 10CFR 20, Appendix B)</td>
<td>20% of local limits</td>
<td>50% of local limits</td>
</tr>
</tbody>
</table>

8.5.2.2. ALARA and time, distance and shielding

The ALARA principle acknowledges that even in small doses, exposure to radioactivity has no direct benefit, and so it should be minimized as much as possible. To accomplish this, there are three basic protective measures in radiation safety:

(a) Time

It considers the amount of time a person spends near a radioactive source. Time near a radioactive source should be the time it takes to perform the work and no 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

Distance takes into account how close a person is to a source of radioactive material. Staff should strive to maximize their distance from a radioactive source as much as they can. This is an easy way to protect themselves due to the inverse relationship between distance and dose. In the case of conducting preclinical experiment, increasing distance could involve moving away from experiment, such as PET scan, once the animal has been injected.

(c) Shielding

Shielding involves putting something between the radiation worker and the radiation source. The most effective shielding will depend on what kind of radiation the source is emitting (e.g., α, β, γ), and keep in mind that many radionuclides emit more than one kind of radiation. For preclinical imaging, doses tend to be quite low (compared to radiopharmaceutical production, clinical imaging, etc.) 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, for example, rodents. 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

Radiation exposure to radiation workers 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 and finger ring dosimeters) measure radiation exposure to workers over a given time period (e.g. weekly, monthly or quarterly). 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. When the dosimeter period is complete, 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 luminesce in an amount 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 radiation safety officer = – allowing for the continual monitoring of radiation exposure to staff during their day to day activities and highlighting employees where action might be required to reduce radiation exposure if possible.

Facilities establish ALARA I and II action limits so that the facility radiation safety officer can intervene before legal limits are exceeded and where a staff member would be required to stop working with radioactive material for the remained period 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.
8.5.3. Animal safety

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

8.5.3.1. Safety for personnel handling experimental Animals

(a) Animals and allergies

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

Symptoms typically develop within 12 months when a worker begins research with animals but can also appear years later. In order to prevent the development of an animal allergy, the following precautions are recommended:

— Animals should be housed, as well as used in experiments, in extremely well-ventilated areas;
— 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. The same personal protective equipment should be used while cleaning and changing cages to prevent exposure to waste and/or animal dander.

(b) Zoonotic diseases

As described above, 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 like 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(s)
of hazardous materials used, (e.g., radiation, biohazards etc.), additional regulatory approval may be required before work can start. Any activities that involve the use of hazardous materials should:

- Be conducted in accordance with established animal use protocols;
- Require use of appropriate personal protective equipment;
- 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 details on animal handling and the type of animal, and the type of chemical and/or hazard(s) that may be encountered during the research being undertaken. In the context of preclinical imaging, radiation safety training will also be required for any animal worker handling and administering radioactive doses.

8.5.3.2. Animal safety

Animal safety and wellbeing should be a high priority in facilities conducting animal research activities and is essential for achieving certification, such as the association for assessment and accreditation of laboratory animal care AAALAC (see 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, available for third party consultation. The veterinary staff should oversee daily observations of all animals in the research facilities to ensure their health and wellbeing. Animals in pain or distress should be treated or, if needed, euthanized using approved protocols.

(b) Institutional animal care and use committee (IACUC)

Facilities should consider establishing an institutional animal care and use committee (IACUC). The IACUC is responsible for oversight of the animal care and use program and its components. It can be considered as the preclinical equivalent of the Institutional Review Board responsible for overseeing clinical research.

(c) Composition

An IACUC must have a minimum of three members, usually appointed by the CEO (or other head) of a research facility. If the IACUC consists of 4 or more members, no more than three members can work in the same group at the institution. IACUC must be composed of a chairperson and at least two other members. Membership requirements should include:

- Member must be qualified to regulate animal care;
- One of the members must be a Doctor of Veterinary Medicine with experience in laboratory animal research;
- One member must have no relationship with the institution except for serving on the IACUC.
(d) Oversight

An IACUC oversight of an institution's animal care and use is accomplished by:

- Review of animal research protocols;
- Inspections of facilities to ensure compliance with legal requirements.

IACUC must have the ability to ensure compliance and correct problems in animal care when discovered. As such, fair treatment of whistle blowers who report animal welfare violations to 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 publication, knowledge of safe handling of radioactivity, biosafety considerations, healthy 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, 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 radiation safety, animal handling, etc. 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, the Nuclear Regulation Commission is responsible for ensuring compliance with radiation safety requirements such ALARA and dosimetry, while AAALAC International and the U.S. Department of Agriculture oversees laboratory animal research activities.

8.6.2. Good laboratory practice

Good laboratory practice (GLP) is a quality management system made up 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 around the following:

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

(b) QA programme
- General;
- Responsibilities of QA personnel.
(c) Facilities
   — Test system facilities;
   — Facilities for handling test and reference items;
   — Archive facilities;
   — Waste disposal.

(d) Apparatus, materials and reagents

(e) Test systems
   — Physical/chemical;
   — Biological.

(f) Test and reference items
   — Receipt, handling, sampling and storage;
   — Characterisation.

(g) Standard operating procedures

(h) Performance of the study
   — Study plan;
   — Content of study plan;
   — Conduct of study.

(i) Reporting of study results
   — General;
   — Content of final report.

(j) Storage and retention of records and materials

The FDA has rules for GLP specified in 21CFR58. 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 21CFR58) might be inadmissible in support of regulatory filings such as the fda New Drug Applications.

Similarly, the European Council had adopted two basic Directives concerning GLP principles: Directive 2004/10/EC and Directive 2004/9/EC. These directives state that Member States designate the authorities responsible for GLP inspections in their territory, and that the OECD Guidance for “compliance monitoring procedures for GLP, and conduct of test facility inspections and study audits” [111], must be followed 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 program for GLP is sponsored by the FDA (instead they inspect facilities on a case by case basis for adherence to 21CFR58), whereas GLP accreditation programs exist in the EU as well as certain countries (e.g. Australia, Canada, Japan and South Africa). The EU also has Mutual Recognition
Agreements with Israel, Japan and Switzerland [121]. However, under this GLP, accreditation for preclinical imaging is not currently available.

8.6.3. AAALAC

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 program, a Program Status Evaluation service, and educational programs. Although not legally binding, the above accreditation is nonetheless a suitable measure of the quality level of the intended preclinical facility.

Animal experiments are regulated in the EU by the Directive 2010/63/EU, which follows the principles of replacement, reduction and refinement, aimed 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” [122]. In the US, “The transportation, care, and use of animals should be in accordance with the Animal Welfare Act (7 U.S.C. 2131 et seq.)” [123]. Other specific legislation acts are currently enforced in countries which are members of the OECD. A list of the major legislation reference is provided in section 10. The above legislations set, among other general provisions, general standards for the use of animals for scientific research, underlying that special care has to be taken in case of endangered species of NHPs, and setting up requirements for facilities, animal handling, breeding, care, anaesthesia, and killing. Although radiopharmaceutical preparation and preclinical testing departments are usually not committed to handle animals, except for short term (typically, no more than a few weeks) housing and storage, nonetheless they must comply with the above rules. Major requisites and constraints will be described in detail. While planning a preclinical testing laboratory, where radioactive compounds are expected to be used and administered to small animals, legislation on radiation protection has to be considered. Major legislation reference in the EU is the Directive 2013/59/Euratom, which updated and repealed most of the previously enforcing directives, and that states the principles of radiation protection for professional operators and for the population. The main operations involving radioactivity that could be carried out in such labs may vary, depending on the available instrumentation and on aims and scope of the research, but generally they may include the following:

(a) Radioactive dose preparation;
(b) Radioactive dose administration to the small animal;
(c) Signal acquisition (scan);
(d) Animal storage after tracer injection;
(e) Animal sacrifice and organs/tissues 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 substances/chemicals/biologials applied to non-clinical health and environmental safety studies required by regulations for the purpose of registering or licensing pharmaceuticals. In case of the latter, GLP principles apply. GLP were originally established by the FDA in 1976, to strengthen controls on the quality of non-clinical studies aimed to provide data to be used for drug registration purposes. FDA regulation was then used as the basis for more general OECD guidelines currently enforced in the EU and in other countries which are members of the organization itself. GLP may be seen as a general quality system for the organization of non-clinical studies, and they define for instance the various degree of responsibilities appointed to the so-called ‘study director’, the principal
investigator (PI) and the operating personnel. QA system is also aimed to guarantee 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 the Directive 2004/10/EC, the Annex 1 of which are the above cited OECD guidelines. From the above description, it is clear that in most preclinical applications of radiopharmaceuticals, the GLP is not of concern, but still there are several situations where GLP apply. For instance, in case of studies aimed to provide data and information on a radiopharmaceutical proposed for marketing authorization (MA), or when radiopharmaceuticals are used during drug development of a medicine that should undergo MA procedure. Case studies will be provided in next sections of this publication.

Other regulations/legislation that may apply to the field of preclinical testing of radiopharmaceuticals are biosafety rules, to be considered for biological material handling, storage and disposal. EU main legislation act in this field is represented by Directive 2000/54/EC, on the protection of workers from risks related to exposure to biological agents at work. Legislation and rules in place in other countries will also be described.

9. QA AND QC

9.1. QA/QC 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 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). IQ/OQ is typically performed by the equipment manufacturer, 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, as outlined in the following sections. Records of any IQ/OQ/PQ, preventive maintenance or other testing should be kept in case 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 in the ‘American society for testing and materials’ class 5 weights, either daily or 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 performing preclinical testing of radiopharmaceuticals. They can be used for a range of activities including pipetting TLC samples (1 µL), endotoxin testing samples (25 µL) etc. Since samples can be used for the quantitative analysis, calibration is of critical importance. If a work according to GLP principles is being conducted in a laboratory, then the calibration should be traceable to internationally acceptable standards such as the National Institute of Standards and Technology and ISO 17025 accredited pipette calibration laboratories should be used to recalibrate pipettes on an annual basis (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 ensuring their proper function. After installation or service of a dose calibrator, its geometry (position and volume) dependent response must be measured as well as volume dependent correction factors relative to the standard volume (e.g., 10 mL) derived. Additionally, constancy and accuracy checks are recommended daily, and (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, ≥ 2 National Institute of Standards and Technology traceable reference sources (e.g. $^{137}$Cs, $^{68}$Ge, $^{57}$Co etc.) 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 and its true current activity should agree within ± 10%.

The quarterly check of linearity involves beginning with a high amount of radioactivity (e.g. 37 GBq of $^{99m}$Tc or $^{18}$F), independently calibrated, and then measuring radioactivity 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 amounts are then graphed against time (using a semi-logarithmic 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 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, analysing samples from biodistribution studies, etc. Generally speaking, $\gamma$ counters are relatively low maintenance, but some general guidelines for routine maintenance include:

- Cleaning the counter area where it is housed in, along with wiping off the counter;
- Cleaning the conveyor belt with a clean cloth;
- Check the background periodically to monitor for contamination, and decontaminating if needed;
- Perform a calibration curve for every used radioisotope by measuring a series of decreasing radioactivity concentrations.
Like all of the instruments being discussed, it is recommended that annual manufacturer preventive maintenance is performed.

9.1.6. Phosphor imagers

Like 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 be kept clean, as well as the phosphor imaging plates. 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, ensure plates are being handled correctly by the instrument and the operators. Plates can be washed with soap and water, and dried while taking care not to scratch them.

9.1.7. Chromatography systems

Chromatography systems, such as GC, HPLC and TLC systems, are used to conduct QC and stability testing of radiopharmaceutical doses, to analyse blood samples for presence of radiometabolites, among others. 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 is completed every time they are used. This is usually accomplished by running two standard injections, and the measured peak area of these two peaks should agree within 5% for automated injections, or within 10% for manual injection. The results are averaged and used with the standard concentration to obtain a calibration factor that 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 also noted in the experiment record (lab book, batch record, etc.).

9.1.8. Laminar airflow hoods

Laminar airflow hoods (LAFs) are the typical class 5 [124] environments available in imaging centres. Air is taken in through a HEPA filter, and then 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, cell culture, etc.

IQ/OQ/PQ should be performed upon installation to verify the laminar airflow (e.g., 28 m/min ± 20%). After the fact, a LAF’s HEPA filter should be inspected and certified every six months. The HEPA filter removes bacteria from the air, and 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 [124] (≤ 3520 particles per m³, 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 mins). 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 (CFUs) per plate and could mandate cleaning and/or recertification of the LAF, and/or retraining of the operator [125].

9.1.9. Incubators

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

— Clean and disinfect regularly;
— Check CO\textsubscript{2} levels, refilling as necessary, and calibrate;
— Check water levels, refilling as required;
— Regular cleaning per manufacturer guidelines;
— Replace the HEPA filter every 6 to 12 months;
— Replace gas inlet filters every 6 to 12 months;
— Heat sterilization (e.g. once per month or once every 6 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 is necessary to ensure safe and consistent operation. It is recommended that preventive maintenance is conducted by the manufacturer.

(a) Cryostats

For routine cleaning after every use, remove debris from the cryostat chamber and place into 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 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 then allow to dry. Replace the microtome when dry and reconnect power.

(b) Microtomes

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

9.2. QA/QC FOR PRECLINICAL IMAGING SCANNERS

Routine QA and QC is important to maintain the proper performance of the imaging equipment. For this reason a paper was published in 2017 [126] which provides guidelines for preclinical imaging
specialists in setting up an appropriate QA/QC program for their facility. This paper included guidelines for PET, SPECT, optical imaging, CT and MRI. In the following sections, the recommendations from the above given publication are reproduced for PET and SPECT scanner QA/QC.

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

9.2.1. QA/QC in PET scanner

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

9.2.1.1. PET scanner daily QC tests

The visual assessment and/or efficiency scans (often called quick scan) describe a basic daily check of PET scanner system function and imaging performance. Here, a long lived source with similar diameter and activity of typical animal scans is placed in the central FOV. A short acquisition (5 to 10 min) is recorded and an image is reconstructed. The image should be examined visually for artefacts and quantitative data from a ROI should be analysed (value for mean and SD). Data should not vary from the baseline by more than 20%. Another method for a daily QC test is to check the response by the 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 properly. The daily QC test also includes a measurement of the consistency of the used dose calibrator. These are usually constancy and linearity measurements, which are specified by the vendors.

9.2.1.2. PET scanner monthly QC tests

The PET calibration constant testing 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 for 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 and reconstruction algorithm). The calibration constant is then calculated by using the radio 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 radioactivity 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 obtained data in counts per second (cps) and the known activity concentration, the γ counter efficiency value can be calculated.

9.2.1.3. PET scanner semi-annual and annual QC tests

The PET system setup 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 lookup tables. After the full system set up a normalization (and blank) scan and measurement of the calibration constant should be performed.

Normalization should also be performed annually. For most of the preclinical PET scanners normalization is done using the direct normalization method. Here, a high-statistical scan is acquired with a homogeneous source that provides a uniform irradiation of the detectors. In this case, each LOR is illuminated with the same amount of activity. For a given sinogram bin, the inverse of the measured number of counts then is proportional to the normalization coefficient. A usual normalization scan is done for 4 to 6 h using a 68Ge cylinder that covers the whole axial FOV of the scanner.

When attenuation data is acquired using a transmission scan in PET, then a blank measurement should also be performed semi-annually. For the blank scans the phantom and the animal bed have to be removed from the FOV. Then a scan only using 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 amount of 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 is then plotted on semi-log graph, and the results are expected to show a linear loss of activity over time.

<p>| TABLE 13. RECOMMENDED QA/QC TESTS AND FREQUENCY FOR A PET SCANNER |
| Adapted from 1999 |</p>
<table>
<thead>
<tr>
<th>Daily</th>
<th>Monthly</th>
<th>Semi-annually / annually</th>
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<tbody>
<tr>
<td>Visual assessment</td>
<td>PET calibration constant</td>
<td>Blank measurement (semi-annually)</td>
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<tr>
<td>Efficiency (quick) scan</td>
<td>Gamma counter calibration</td>
<td>Full PET system setup</td>
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<tr>
<td>Dose calibrator constancy</td>
<td></td>
<td>Normalization</td>
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<td></td>
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<td>Dose calibration linearity</td>
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9.2.2. QA/QC in SPECT scanner

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 are in good working order. Quality control procedures are used to evaluate the proper function of SPECT γ camera [127, 128]. 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:

(a) Uniformity floods;
(b) Evaluation of spatial resolution;
(c) Centre of rotation assessments;
(d) SPECT phantom evaluation.

9.2.2.1. Phantoms for SPECT scanner

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

9.2.2.2. Daily QC tests for SPECT scanner

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 4 500 counts/cm² (but not more than 10 000 counts/s). 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 FOV to ensure a uniform exposure of the detectors. This can be a challenging requirement to meet on closed preclinical γ and SPECT camera scanners.

Afterwards 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/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 (CFOV).

9.2.2.3. Weekly QC tests for SPECT scanner

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 QC tests for SPECT scanner

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 was not possible on a daily basis.

In SPECT cameras which include attenuation and scatter correction, the calibration factor should be determined on a monthly basis for all used isotopes. The workflow is similar as for PET systems. 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, scan time, scatter and attenuation correction and reconstruction algorithm). It is recommended to measure the filled phantom for 1 to 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, and the known activity per unit volume from the dose calibrator.

9.2.2.5. Semi-annual and annual QC tests for SPECT scanner

The quality of the reconstruction image over time should be assessed annually by a spatial resolution measurement. It is recommended to measure the resolution by using a $^{99m}$Tc line source placed in the CFOV 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 fitted with a Gaussian plus to calculate the FWHM. Resolution performance should not vary from the baseline by more than 10%.

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

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

<table>
<thead>
<tr>
<th>TABLE 14. RECOMMENDED QC TESTS AND FREQUENCY FOR SPECT SCANNER</th>
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<td>Adapted from [126]</td>
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<tr>
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<th>Daily</th>
<th>Weekly</th>
<th>Monthly</th>
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<td>Photopeak drift</td>
<td>Collimator stability</td>
<td>Uniformity</td>
<td>Collimator durability</td>
<td>Resolution</td>
</tr>
<tr>
<td>Uniformity</td>
<td>Detector stability</td>
<td>Uniformity (if not done daily)</td>
<td>Calibration constant</td>
<td>Rotational uniformity (semi-annually)</td>
</tr>
</tbody>
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10. PROTOCOLS

10.1. IN VITRO EVALUATION PROTOCOLS

10.1.1. Protocol for cell uptake study

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 follows:
(a) The cells are harvested from culture and dispensed into reaction tubes or well plates in cell suspension media containing 2% fetal bovine serum (or recommended supplement). Typically, a cell number between $10^4$-$10^6$ per reaction may be used. When working with adherent cells in well plates, it is important to adjust the cell number, so you get 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 commencement of 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 used of the tracer can be equal to the affinity ($K_D$). If $K_D$ is not known than as a rule of thumb the experiment can be tried 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 we add a 100 fold concentration of unlabelled ligand to assess non-specific adsorption of the tracer to the surface of the cells or the reaction vessel. It is important to keep tracer volume as low as possible and not to introduce any changes into the cell medium. Reaction volume must be kept constant by addition of buffered saline or medium. While allowing for adequate coverage of the cell mass, it is recommended to minimize the reaction volume to maximize the interface between the tracer and the cells, for example, 500 to 1 000 microliters is adequate suspension volume for a reaction carried out in a 24 well plate for up to 24 h;

(d) The cells are incubated in individual reaction sets (in triplicate) for different time points (suggested 0.25h, 0.5h, 0.75h, 1h, 2h, 4h, 8h). The experiment can be repeated with more refined time points to obtain optimal result;

(e) Binding reactions are typically done 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 to 8°C. In such a case, at the end of the incubation, the cells should be carefully examined under microscope to assess possible damage from exposure to the lower temperature;

(f) At the end of the incubation period, 1 ml 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 it will be required to centrifuge the cell mass (800 to 1 000 g, 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-bound 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/1M 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 out when the incubation time reaches binding equilibrium. The earliest convenient time point in 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

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

(a) The cells are harvested from culture and dispensed into reaction tubes or well plates as described previously for the cell uptake study;
(b) The radiolabelled tracer is prepared under aseptic conditions and diluted with buffered saline or cell suspension media into a series of concentrations (six to ten levels) encompassing at least a 100 fold difference, preferably a 1 000 fold difference. The lowest tracer concentration should be at least 10 times below the affinity $K_D$ (often in the nanomolar range or lower) and the highest concentration should be at least 10 times higher than $K_D$;
(c) The different tracer concentrations are added to the respective reaction wells/tubes in the same manner as for cell uptake assay, 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 we add a 100 fold concentration of unlabelled ligand to assess non-specific adsorption of the tracer to the surface of the cells or the reaction vessel;
(d) The cells are incubated with the tracer for an incubation period tending to equilibrium as determined from the cell uptake assay. To specifically assess binding to membrane receptors, the reaction set may be incubated at 4 to 8 °C to block cellular internalization/tturnover of the tracer;
(e) At the end of the incubation period, the cells are washed with cold culture media or buffered saline as described before. 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/1M sodium hydroxide solution) and radioactivity associated with the cell mass is measured on a suitable radioactivity counter. For measurement of pure $\beta$ 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;
(g) The readings in triplicate obtained for each tested concentration are corrected for background, and the average and standard deviation is calculated. Using the average values, standard curve is plotted of tracer concentration against bound radioactivity, which is expected to show a region of saturation. The curve can be used to obtain a value for dissociation constant ($K_D$) using regular software packages. Alternatively, a Scatchard plot can be created to manually specify $K_D$.

10.1.3. Protocol for competitive binding

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 specificity of the radiotracer towards the target receptor. Competitive binding assay is conducted under reaction conditions analogous to the saturation binding assay and many of the steps will be similar. Given below is a stepwise description of a typical competitive binding assay:

(a) Cells are harvested and dispensed into reaction wells similar to the procedure described for saturation binding;
(b) A single tracer concentration, for example corresponding to 50% of saturation binding value, will be used for the binding reaction. A range of concentrations of unlabelled native biomolecule/receptor targeting ligand is prepared. This can go from 0.1 to 100 times the tracer concentration. It is recommended to have six to ten data points or more;
(c) Different reported protocols give different advice about the sequence of addition of tracer and unlabelled ligand. They may be added immediately after each other or the cells may be pre-
incubated with the unlabelled ligand for some time (0.5 to 2 h) before addition of the tracer. All other conditions must be maintained as for saturation binding;
(d) The cells may be incubated as per optimized reaction conditions. After the incubation period they may be washed, harvested and counted as described for saturation binding;
(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 IC$_{50}$ (inhibitory concentration 50%) of the ligand. The IC$_{50}$ in itself provides a measure of the inhibition of the tracer by the unlabelled ligand, and it can be used to calculate the inhibitory constant (K$_i$);
(f) Competition binding assay can also be used to screen potential new ligand analogues in exploratory testing. Here, 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 described 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 dissociation assay is provided below:

(a) Cells are harvested and seeded into reaction wells / tubes (5 x 10$^4$ to 10$^6$ per well) as previously described;
(b) The tracer is added at a concentration approximately correlating to 10 times the $K_D$ as calculated in saturation binding assay. If the $K_D$ is not known, 10 to 20 nM may be used as a starting point;
(c) The cells are incubated with the tracer till the reaction nears equilibrium, which may take from 0.5 to 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 previously detailed. Care should be taken to avoid excessive washing that may lead to loss of bound tracer;
(e) After washing, add complete culture medium to the wells (same as binding reaction volume) and noting the time of addition as ‘time zero’, allow for dissociation in individual reaction sets (in triplicate) for different intervals (suggested 0, 0.25h, 0.5h, 1h, 2h, 4h, 8h, 16 to 20h);
(f) At the end of the respective dissociation intervals, wash the cells twice or more and harvest them by trypsinization or lysis (using 1N NaOH/dimethyl sulfoxide) as previously described. Measure the cell mass associated radioactivity;
(g) Plot a curve of dissociation time against cell bound radioactivity. 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 in 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 measurement of cell associated radioactivity. The following steps describe an archetype internalization assay:

(a) Cells are harvested and seeded into reaction wells/tubes as per the steps described in the cell uptake assay protocol. All the precautions underlined there must be adhered to in the internalization assay as well;
(b) The tracer is prepared under aseptic conditions and dilute in culture medium. It is recommended to use a tracer concentration approximately 10-fold of the calculated $K_D$. If the $K_D$ is not known, then 10-20 nM concentration of the tracer can be employed as a rule of thumb;
(c) The tracer is added to the cells and incubation is performed in triplicate for a set of time points up to the estimated interval for binding equilibrium (suggested 0h, 0.25h, 0.5h, 0.75h, 1h, 2h);
(d) At the end of the respective incubation periods, the reaction wells may be washed once or twice with ice cold plain culture medium/buffered saline to remove unbound tracer as previously described;
(e) After washing off the unbound tracer, the cells may then be quickly exposed (30-60s) to a stripping buffer (same amount as total binding reaction volume). Several recipes are available for the stripping buffer, a typical one uses 0.2M glycine with 0.15M NaCl (pH 3.0). This step may be performed twice;
(f) After stripping, the cells can be 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 cause release of surface bound tracer without significant damage to cell membrane integrity. However, it would be well to verify these aspects to avoid errors in the results. Prior to doing the assay, unlabelled cells exposed to the stripping regimen and washed, can be examined for membrane integrity by microscopic examination after cell staining. It is more tedious but equally important to confirm that the stripping protocol is effective in removing surface bound tracer, otherwise one will end up with falsely high internalization values even with short binding incubation periods. In such case, it is recommended to vary the stripping conditions (buffer composition, incubation time) to ensure correctness of the measured values.

10.1.6. Protocol for tracer stability

It is important also to establish the innate stability of the tracer over its period of shelf-life. To achieve this the basic cell uptake assay as previously described can be repeated at different intervals post-preparation. This will give insight into how long a tracer can be used for performing 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 proposed clinical application, and can vary from a few hours to some days.

10.1.7. Protocol for plasma/serum stability

Plasma/serum stability is a special case of tracer stability in which one measures in vitro the stability and protein binding of the tracer, as an indication of its behaviour when administered via the bloodstream. A typical protocol for plasma/serum stability is described below:

(a) Plasma/serum is collected aseptically, dispensed into single use (0.5 to 1.0 ml) aliquots and stored at -20°C or lower. It is important that the aliquots should 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 fetal 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 time points;
(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 respective vials are removed and an aliquot of reaction stopping/protein precipitating agent equal to the total reaction volume is added. Methanol/ethanol are commonly used precipitating agents. After centrifugation to separate out the precipitated protein (20,000 g, 10 min), the supernatant may be analysed by TLC/HPLC (same protocol as would have been used to characterize the tracer) to assess stability/metabolism of the tracer in serum/plasma. The radioactivity associated with the precipitated protein fraction gives insight into the plasma/serum protein binding tendency of the tracer for the tested incubation periods.

10.1.8. Protocol for in vivo stability

In vivo stability is necessary to evaluate if a radiopharmaceutical is metabolized in vivo and if radiometabolites distribute in the target organ. An example of protocol for metabolites analysis in 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 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 retention time of the parent compound is suited to the radionuclide half-life;
(b) 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 to 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 (500 μL for rats and 200 μL for mice) of plasma (depending on radioactivity concentration) are extracted with CH3CN (1:1 v/v) and filtered. Tissue samples are homogenized in saline (saline 1:1 v/v) using a Potter-Elvehjem tissue homogenizer (for brain or soft tissue) or a rotating homogenizer (for fibrous tissue like heart or tumour) and processed as described for plasma;
(d) Pellet and CH3CN extracts are counted in a γ counter and the extraction efficiency measured;
(e) Plasma and brain extracts are injected in HPLC. Eluted fractions are collected in tubes with an automatic collector every 30 seconds for a time sufficient to collect the radiolabelled parent compound. Tubes are counted with a γ counter to obtain counts per minute (cpm) corrected for decay and cpm with a retention time corresponding to parent compound divide for the total amount of radioactivity. If an HPLC radiodetector with high sensitivity is present, collection in tubes is not necessary.

For 11C labelled compounds the maximum number of samples per analysis is approximately 4 to 5. 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 performed in the same animal only if the concentration of radioactivity is sufficient to collect 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 last only if are labelled with radionuclides with different half-life). Below is an example of 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 case of soft tissue like brain tissue can be cut with a tissue slicer without freezing procedures. Slices are exposed to a phosphor screen in a dark place for a time of approximately 3 h for $^{18}$F or overnight for $^{11}$C and developed with phosphor imager. Regions of interest (ROI) are drawn on the required areas to obtain density light unit (DLU) per square millimetre values that are then converted into percentage of ID per square millimetre of tissue (% ID/mm$^2$) with a calibration factor previously estimated using the protocol described below.

(c) Increasing radioactive concentrations of the radionuclide used in the studies are placed on different disks of paper with the same diameter (1 cm). Each concentration is prepared in duplicate. One disk is counted with a $\gamma$ counter and the other exposed to the phosphor screen to build a straight line of density light units/mm$^2$ versus radioactivity concentration that will be used to convert density light units values in units of radioactivity.

In case of dual label autoradiography, the radiopharmaceuticals are injected at different times so as to sacrifice the animal at the same time according to their respective kinetic behaviours. Slices are exposed as previously described 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 to the first to obtain the image of distribution of the first radionuclide. In the process of conversion from density light units to radioactivity units, pixel should be also corrected for radionuclide decay.

10.1.10. Protocol for gamma counter calibration

Gamma counter calibration is necessary in a QA system to obtain reliable data. A $\gamma$ counter system is used to measure small amounts of radioactivity like that obtained during plasma counting, metabolite analysis or biodistribution studies. In case of positron emitter radionuclides, a $\gamma$ counter able to garner counts at 511 to 1 022 MeV is required. Absolute $\gamma$ counting units are in Bq but are generally expressed in disintegrations per minute (dpm). Steps for $\gamma$ counting calibration are reported below:

— Calibration test: this procedure allows to estimate $\gamma$ counter efficiency and to convert the counts per second or minute values detected by the instruments into absolute Bq or dpm values. It is performed with a calibrated source of radioactivity and efficiency (E) is calculated as follows: $E=cpm$ measured/dpm present in the calibrated source multiplied per 100;

— Stability: the calibrated source should be measured before each experiment to verify that the system is stable;

— Saturation test and linearity: this test indicates the range linearity between the activity counted and the response of the $\gamma$ counter and should be done. The test is performed by counting different concentration 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 essential information required to be noted before and during a preclinical imaging experiment. It’s recommended to have standard basic protocol approved as per Institutional procedures. The individual experimental variations should be noted for each study.
FIG. 4. Example of PET/SPECT protocol worksheet.
10.2.1.1.  General Considerations

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

10.2.1.2.  Animal preparation

Animals arriving in the vivarium are left at least seven days for acclimation. The conditions of humidity and temperature are controlled in the room and food and water given ad libitum.

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 either 100% oxygen (or air) and then maintained with 2 to 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 (number, weight, experimental group, date, treatment, blood glucose level, fasting, etc.) are recorded in a form.

10.2.1.3.  Brain $^{18}$FFDG study procedure

The $^{18}$FFDG dose is prepared in 0.5/1 mL syringe, as 20 MBq in 0.5 mL. The animal (male Wistar rat of 300 g) is intravenously injected with $^{18}$FFDG in the tail vein. The injection time is noted.

Activity of $^{18}$FFDG is measured in a calibrated dose calibrator before and after injection and the numbers registered in the form with their respective measuring time.

The rat is allowed to wake up after injection, put back in the cage and kept warmed (keeping the animal awake during distribution phase of $^{18}$FFDG increases brain uptake and keeping it warmed decreases muscle and brown adipose tissue uptake).

After a period of 45 min post-injection the animal is anaesthetized again and positioned in a warmed bed with the brain in the centre of the FOV and a static image acquired for 30 min. The animal is taken from the equipment and allowed to wake up in a warmed cage. Image is reconstructed using a OSEM-3D algorithm with 20 iterations and 4 subsets. Images are fused with a MRI template for drawing volume of interest (VOI) in different brain regions and the result 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 $^{18}$FFDG study procedure

The animal should be prepared in the same way as for brain study, however, fasting must be considered in this case.

All described procedures are the same, except for the positioning, where the heart should be in the centre of the FOV.

Using isoflurane is a good option in case of cardiac studies because this anaesthetic agent increases heart uptake.
Image quantification should use VOIs in the different myocardium axes and be presented as a percentage of uptake related to the maximum region and/or SUV. In cardiac studies it is customary to use the 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 like brain and heart studies. An important aspect to consider in tumour models is the location of the tumour. The positioning must consider the right location of the tumour and adjacent organs. For example, tumour should not be too close to bladder. However, if the model presents spontaneous tumour and it is close to the bladder, a hand massage can be given to the animal bladder before putting the animal in the equipment to minimize urine content in the bladder.

The choice of anaesthesia 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 + xylazine can be considered.

During image quantification one may consider manually drawing VOIs in the tumour if it has irregular borders and size. Results are commonly expressed in $SUV_{\text{max}}$ (maximum SUV value inside the VOI) and also tumour/muscle ratio.

10.2.2. In vivo preclinical imaging – Rhesus monkey PET neuroimaging study with $[^{11}C]$dihydrotetrabenazine (DTBZ) to determine % occupancy of vesicular monoamine transporter 2 by different doses of DTBZ

10.2.2.1. General Considerations

The procedure describes a dynamic dual primate PET neuroimaging study with $[^{11}C]$dihydrotetrabenazine ($[^{11}C]$DTBZ) to determine % occupancy of vesicular monoamine transporter 2 (VMAT 2) by a pharmaceutical test article. 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) [129]. 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 be in compliance with local regulations, such as those defined by the US Department of Agriculture (USDA). For example, monkeys should at least be individually housed in steel cages (83.3 cm high × 152.4 cm wide × 78.8 cm deep) equipped with foraging boxes. Monkeys are sociable so, if possible, house them in the same cage. But this can be problematic if aggressive incompatibility is an issue. Cages should be metal, with gridded floors, for radiation safety reasons (radioactive waste is drained through the gridded floor and is easier to clean). Temperature and humidity should be carefully controlled, and monkeys kept on a 12 h light/12 h dark schedule. Monkeys should be fed an appropriate diet approved by concern food authorities and this may be supplemented with fresh fruit and vegetables daily as necessary. Water and enrichment toys (manipulanda 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 i.m.) 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 O\(_2\)/air, and a percutaneous catheter 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 (electronic, water etc.), and 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 \([^{11}C]DTBZ\) (in ~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 [OSEM] for rapid initial convergence and 3D maximum a posteriori [3DMAP] 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 (http://www.fil.ion.ucl.ac.uk/spm/software/spm12/), and can also be registered to MRI scans for the animal if available.

10.2.2.6. **Image analysis**

For quantitative analyses, 3D VOI should be established for brain regions of interest (e.g. striatum), along with the reference region to be used (e.g. occipital cortex), on the average image of both scans on the monkey. VOIs should be defined using either PET scanner software or in-house 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 assures 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 [130] to calculate striatal distribution volume ratios (DVR), from which binding potentials can calculated, as seen in Eq. (14):

\[
BP_{ND-Logan} = DVR - 1
\]  

Finally, VMAT2 % occupancy values can be calculated using the following, in Eq. (15):

\[
\%Occupancy = 100[(BP_{control} - BP_{test}) / BP_{control}]
\]
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 user personnel from β emissions.

10.2.3.2. Animal model preparation

Animals are subcutaneously injected with the sufficient numbers of tumour cells, either based on existing literature or as deduced from animal model standardization experiments. They are monitored 3 times a week with a caliper until the tumour reaches an approximate volume of 1 cm³.

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 (for example, 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, right up to 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/pain (rough coat, unkept appearance, malaise). Animals are monitored until all meet a humane endpoint 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 (OS). Tumour volume is derived from caliper measurements using the formula; \( V = (W^2 \times L)/2 \). The therapeutic effect is expressed in terms of tumour growth inhibition. OS effect is evaluated using the Kaplan Mayer curves [131].

10.2.3.5. FIH dose selection

When selecting a proposed dose of therapeutic radiopharmaceutical for a FIH study, the radioactive administered dose and the mass dose of the pharmaceutical should be considered. With respect to the radiation administered dose, factors such as scientifically available evidence, clinical experience, and the results of the animal dosimetry studies should be used to decide on the initial radiation dose. The available literature describing 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

From simple radiochemicals and the early labelled molecules whose biological profile is a passive reflection of their physico-chemical characteristics, radiopharmaceuticals today have become 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 has provided a useful primer to both existing and upcoming radiopharmaceutical research facilities about the elaborate sequence of activities that come under the collective appellation of ‘preclinical development and evaluation’.

With its multitude of cell based assay recommendations, the section on in vitro assessment detailed the measurement of a new compound’s affinity, avidity and specificity towards 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 in adhering to the 3R principles of laboratory animal research, but also by proving more convenient, time saving 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 must be emphasized that a careful selection of pertinent assays and application of rigorous controls for accurate unbiased results is critical to accrue the greatest benefit from in vitro testing.

Given the costs of infrastructure, consumables and trained manpower, studies in live animals must 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 must ensure that the selected animal model and assay protocol provide the necessary insight into the functioning of the formulation being tested. Small animal imaging setups, 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. At the same time techniques like ex vivo biodistribution and excised tissue autoradiography remain useful in less equipped facilities and to answer specific queries.

Toxicology and dosimetry are important considerations in the assessment of both diagnostic and therapeutic radiopharmaceuticals. In the former case, it is mainly 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. Cytotoxic or modulatory effect is of course an innate element of any therapeutic radiopharmaceutical and their preclinical evaluation would be incomplete without a detailed investigation of these effects. While toxicity and dosimetry are specialized fields which benefit from having trained staff being involved in the study design and execution, the information provided in this publication, along with the references to established recommendations, is hoped to generate at least a baseline understanding of the factors governing
each, as valid scientific data in these areas make a valuable addition to any proposal for clinical translation of the laboratory research.

When deciding upon a proposed dose of radiopharmaceutical – especially therapeutic – for a FIH study, the amount of radioactivity and ligand must 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 will prove 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 data management and facilities requirements can not only assist individual researchers, but also serve as an overarching development roadmap to institutional management.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AAALAC</td>
<td>association for assessment and accreditation of laboratory animal care international</td>
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<tr>
<td>ABSL</td>
<td>animal biosafety level</td>
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<tr>
<td>ALARA</td>
<td>as low as reasonably achievable</td>
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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
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<tr>
<td>cpm</td>
<td>counts per minute</td>
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<td>dpm</td>
<td>disintegrations per minute</td>
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<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
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<tr>
<td>FDG</td>
<td>2-Deoxy-2-[^18]F]fluoroglucose; fludeoxyglucose</td>
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<tr>
<td>FIH</td>
<td>first in human</td>
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<tr>
<td>FOV</td>
<td>field of view</td>
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<tr>
<td>GLP</td>
<td>good laboratory practice</td>
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<tr>
<td>HED</td>
<td>human effective dose</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>IACUC</td>
<td>institutional animal care and use committee</td>
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<td>IAEA</td>
<td>international atomic energy agency</td>
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<td>international conference of harmonization</td>
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<td>ID</td>
<td>injected dose</td>
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<td>LOR</td>
<td>line of response</td>
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<td>MIRD</td>
<td>medical internal radiation dose</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>MRSD</td>
<td>maximum recommended safe dose</td>
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<tr>
<td>NOAEL</td>
<td>no observable adverse effect level</td>
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<tr>
<td>OECD</td>
<td>organisation for economic co-operation</td>
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<td>PET</td>
<td>positron emission tomography</td>
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<td>pharmacokinetics</td>
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<td>specific activity</td>
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<td>SPECT</td>
<td>single photon emission computed tomography</td>
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<td>SUV</td>
<td>standardized uptake value</td>
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<td>time activity curve</td>
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<td>thin layer chromatography</td>
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