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Central Nervous System Radiotracer Development: Bench to Bedside

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CENTRAL NERVOUS SYSTEM RADIOTRACER DEVELOPMENT: BENCH TO BEDSIDE

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INTERNATIONAL ATOMIC ENERGY AGENCY
VIENNA, 2024

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FOREWORD

Neuroimaging, especially functional imaging based on positron emission tomography, provides non-invasive visualization and quantification of biochemical and physiological processes. This contributes substantially to the understanding of the complex mechanisms of the functioning of, and pathophysiological processes in, the human brain. In this way, neuroimaging helps to solve mysteries behind psychiatry disorders, to better understand the neurodegeneration process and to enhance central nervous system drug development. These applications are linked to the availability of specific and avid radiotracers. The success of the development of clinically significant radiotracers depends on many factors such as the selected biological target, the specificity and affinity of the radioligand, and the pharmacokinetics of the radiotracer. The past two decades have witnessed developments of many radioligands for the central nervous system that are evaluated for imaging neuroreceptors, neurotransmitter systems and disease specific misfolded proteins. Despite continuous evolution in the field, challenges remain in the translation of laboratory results into clinical application, as these radiotracers also demand complex kinetic modelling to better understand and define the relationship between positron emission tomography measurements and underlying brain tissue parameters.

This publication provides a baseline guideline for the development of central nervous system radiotracers for clinical application, discussing different aspects and stages of development and consideration for first in human studies. The publication was compiled using inputs from a multidisciplinary team of experts in this area and will be of interest to researchers and scientists engaged in radiopharmaceutical development, neuroscience research and drug development.

CONTENTS

1. INTRODUCTION

1.1. BACKGROUND

Non-invasive neuroimaging with radiotracers can provide functional information at a cellular level and is useful in certain diseases for patient stratification, treatment response monitoring and as an aided technique for drug development. Despite numerous studies and a long history of research, molecular imaging faces several challenges for a better understanding of the complex functioning of the Central Nervous System (CNS) and its association with several disorders. Technological advances in radiotracer development and techniques available today to biomedical sciences, are synergistically applied to meet the demanding criteria for CNS radiotracers for quantitative imaging of specific molecular targets. Some of the driving forces for the development in this field are lifestyle changes, mental health awareness, increased average life expectancy and an aging population. Radiotracers have proved their significant place in the drug discovery and development process, which is more challenging for CNS disorders due to their complex and not fully understood underlying mechanisms. The synthesis of radioligands with promising pharmacophores have been reported in literature, with the aim of producing promising agents for a variety of novel emerging biological targets associated with CNS for diagnostic imaging based on Positron Emission Tomography (PET) and Single Photon Emission Tomography (SPECT).

IAEA had conducted a coordinated research project on the development of agents for imaging CNS receptors based on ^{99m}Tc. The report summarizing the work carried out by different research groups during this coordinated research project, was published as a technical report [1]. This publication provides relevant updates applicable for the development of CNS radiotracers.

1.2. OBJECTIVE

The primary objective of this publication is to provide a base guideline for the development of CNS tracers for clinical applications. The objective is not to provide an extensive literature review, but to give an insight into the various factors involved in the process of designing, testing, and translating the radiolabel molecule from laboratory to clinic. The guidance provided here, represents international expert opinions, but does not constitute recommendations made on the basis of a consensus by Member States.

1.3. SCOPE

The scope of this publication includes important criteria and the strategies applied for the design of CNS tracers at the laboratory stage, during pre-clinical evaluation, and during further development of promising molecules for clinical applications, describing applicable methods and protocols. This publication considers the lessons learned over the years of development of CNS tracers. The field of neuro-oncology is explicitly not included in this publication.

The information provided in the document will be useful to researchers, students, and professionals engaged in the development and deployment of radiotracers for clinical, research or drug development applications.

1.4. STRUCTURE

This publication provides an orderly overview of the various steps of CNS radiotracer development. The contents are divided into 8 sections that cover in depth details from target selection, tracer design to clinical translation and quantification methods for CNS radiotracers. The objective, scope and overall structure of the publication are outlined in Section 1. The importance of selection of valid target molecules for biological processes of interest, criteria for selection of specific brain targets are discussed in Section 2, and emerging targets are also highlighted here. Once the target of interest is defined, the suitable radiotracer needs to be selected. Section 3 describes strategies for lead molecule selection,

designing the potential lead molecules which fulfil physiochemical and pharmacological requirements in accordance with the choice of the radionuclide.

In Section 4, radiosynthesis methods to incorporate suitable radionuclides such as ^{11}C, ^{18}F, ^{123}I and ^{99m}Tc are described. The advances in synthesis methods and strategies of application of radiolabelled building blocks are briefly discussed. The quality control and quality assurance for CNS radiotracers is also included in this section. Newly developed CNS tracers need to be evaluated to assess their suitability for imaging. Appropriate in vitro methods for determination of metabolic stability, lipophilicity and affinity for P-glycoprotein are described in detail in Section 5. The Go/No go criteria for radiotracer for further preclinical development is discussed in this section. Section 6 gives details of the in vivo evaluation in animal models, imaging types, ex vivo studies, micro dialysis, metabolite studies etc. The section also highlights currently available techniques, such as application of increased throughput in preclinical studies. Non-human primate utility in CNS radiotracer development is also discussed in this section. Once a radiotracer is shortlisted for further development for human studies, additional factors which need consideration for clinical translation are toxicity, dosimetry and a GMP compliant production method development etc., as discussed in Section 7. Finally, Section 8 describes the kinetic modelling approach to quantify tracer uptake as to maximally benefit from the advantage of PET and SPECT, as quantitative in vivo imaging methods. Some examples of radiotracers that are most commonly used in clinics are also included, and important tips are given that are useful for the development of CNS radiotracers.

2.   TARGET SELECTION FOR CNS IMAGING

Radiotracers for PET and SPECT imaging of CNS targets implicated in neuropsychiatric disorders, are playing an important role for personalized medicine, drug discovery and development. Diagnostic imaging with CNS radiotracers is finding more applications for personalized medicine possibly through early diagnosis of the disease, differentiation of clinically overlapping disorders, prediction of response to therapy and treatment monitoring. CNS radiotracers are increasingly employed to support various stages of drug discovery efforts by pharmaceutical companies, as imaging CNS targets aid in establishing proof of mechanisms (target engagement), safety (off target binding), dosing (receptor occupancy) and pharmacokinetics. Similarly, preclinical and clinical imaging studies with CNS radiotracers are also helpful to evaluate drug metabolism, efficacy monitoring, treatment monitoring, target expression, patient stratification etc.

Given this number of high impact applications, there is enormous motivation to develop radiotracers for new CNS targets that cannot currently be imaged. These can either be historical targets for which it has been challenging to develop an appropriate imaging agent, or new emerging targets discovered as our understanding of the pathophysiology of neuropsychiatric disorders continues to improve. This section provides a road map for the selection of an appropriate imaging target, while Section 3 will consider the design of a radiotracer for a given imaging target. To date, most CNS imaging agents have been small molecules. However, recent work demonstrates antibodies and larger molecules can enter the CNS and be used for imaging suggest; this is an emerging area of development [2].

2.1. HISTORICAL NEUROIMAGING

The ability to image biochemical and physiological processes at very low radiotracer concentrations (the tracer principle) is the hallmark of diagnostic imaging in nuclear medicine. Since the earliest days of this field, application of PET and SPECT imaging to understand normal and abnormal brain function has been an important area of research for those working at the interface between nuclear medicine and neuroscience. Much of the field's foundation stems from the pioneering work with radioactivity in the first half of the 20th century. However, nuclear medicine truly took off after World War II, when radiochemist Alfred P. Wolf, transitioned from working on the Manhattan Project to more scientific and medical pursuits.

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Building on corresponding discoveries in neuroscience, researchers started to explore imaging to further understand brain function and dysfunction. For example, in the late 1940s Kety had begun using inert gas tracers to measure cerebral blood flow, while by the early 1960s, Lassen was using ^{133}Xe to study human brain function. By the late 1960s, Sokoloff and colleagues were developing ^{14}C labelled glucose analogues to study glucose metabolism. Ultimately, Sokoloff joined forces with Al Wolf, Joanna Fowler, Tatsuo Ido and others to develop of 2-[^{18}F]fluoro-2-deoxy-D- glucose (FDG). Indeed, the first clinical FDG study in the 1970s with Mike Phelps and Dave Kuhl involved imaging glucose activity in CNS.

In the 1970s, early imaging scientists were also considering how to image receptors. For example, Mazière was investigating the in vivo distribution and pharmacokinetics of [^{11}C]nicotine, a high affinity agonist of nicotinic acetylcholine receptors. However, due to the high levels of non-specific distribution in the brain (nicotine is a receptor agonist rather than antagonist), and the need to use radioactive water for quantification, by the early 1980s synthetic efforts were being directed to development of radioligands based on high-affinity antagonists for acetylcholine receptors. In 1984, Eckelman et al. reported the first SPECT imaging of muscarinic acetylcholine receptors in the human brain using [^{125}I](R)-3-quinuclidinyl 4-iodobenzilate ((R)-4-[^{125}I]IQNB [3]. This seminal work, together with PET imaging of the dopamine receptor in the brain by Wagner in 1983, was proof of concept for in vivo brain receptor imaging. The 1980s saw the introduction of radiotracers for imaging a whole host of neurological targets such as Wagner and colleagues at Johns Hopkins imaging mu opioid receptors with [^{11}C]carfentanil [4], Garnett's first human imaging studies of the dopamine system using [^{18}F]fluoro-DOPA [5], and Fowler's seminal work using [^{11}C]deprenyl to image monoamine oxidase [6].

2.2. CURRENT STATE OF THE ART

The innovation continued through the 1990s to the present day, with PET radiotracers being developed for numerous CNS targets including receptors, transporters and enzymes (Table 1). A significant milestone in advancing brain PET came with Mathis and Klunk's pioneering work developing [^{11}C]Pittsburgh Compound B for imaging amyloid plaques in dementia, and the first human studies were reported in 2004 [7]. This work sparked the widespread use of PET imaging as a tool in dementia research, including the development by the U.S. Food and Drug Administration approval and subsequent commercialization of three amyloid imaging agents (amyvid, neuroceq and vizamyl), as well as a radiotracer targeting tau neurofibrillary tangles (tauvid). Application of PET in dementia has also led to the creation of multi-site clinical studies aimed at creating large imaging databases (e.g. the AD Neuroimaging Initiative), as well as the use of PET to support therapeutic trials (e.g. with bapineuzumab, aducanumab, solanezumab and BAN2401). Besides use in clinical trials, the use of PET as a serious tool to support drug discovery efforts began in earnest in the early 2000s with, for example, seminal work from Hargreaves and colleagues at Merck using PET to inform go/no-go decisions on therapeutic assets like Emend [8].

TABLE 1. COMMON RADIOTRACERS FOR CNS TARGETS

Target	System / Disorder	Common Tracers
Receptors		
Nicotinic acetylcholine receptors $\alpha7$	Cholinergic disorders (e.g. Alzheimer's disease)	$[^{18}F]$ASEM, $[^{11}C]$CHIBA1001
Nicotinic acetylcholine receptors $\alpha4\beta2$		$[^{18}F]$Flubatine, $[^{18}F]$nifene, $[^{18}F]$A-85360
Muscarinic acetylcholine receptors		$[^{11}C]$NMPB
Adenosine A_{2A}	Adenosine related disorders	$[^{11}C]$TMSX, $[^{11}C]$SCH442416, $[^{11}C]$Preladenant, $[^{18}F]$MNI444
CB_2	Endocannabinoid system	$[^{11}C]$OMAR, $[^{11}C]$NE40
Dopamine D_1	Dopaminergic disorders (e.g. Parkinson's disease (PD), movement disorders, neuropsychiatric disorders)	$[^{11}C]$SCH 23390
Dopamine D_2		$[^{11}C]$Raclopride, $[^{123}I]$IBZM
Dopamine D_3		$[^{11}C]$PHNO
$GABA_A$	Epilepsy, stroke, cerebral palsy, schizophrenia, autism, Alzheimer's disease, and addictions	$[^{11}C]$FMZ, $[^{18}F]$FMZ, $[^{18}F]$GE40
Histamine H_1	Neuropsychiatric disorders	$[^{11}C]$doxepin
mGluR5	Neuropsychiatric disorders (e.g. Depression, schizophrenia, anxiety, PD)	$[^{18}F]$FPEB,$[^{11}C]$ABP688, $[^{18}F]$PSS232
Opioid δ	Opioid use disorder, major depressive disorder, other opioid disorders	$[^{11}C]$MeNTI
Opioid κ		$[^{11}C]$LY2795050
Opioid μ		$[^{11}C]$Carfentanil
5-HT$_{1A}$ Receptor	Serotoninergic neuropsychiatric disorders (e.g. depression)	$[^{11}C]$WAY, $[^{18}F]$MPPF
5-HT$_{2a}$-receptor	Neurodegeneration, schizophrenia	$[^{11}C]$Cimbi-36
Sigma1	Mood disorders, stroke, neurodegeneration, drug addiction	$[^{11}C]$SA4503, $[^{18}F]$fluspidine
Steroid hormone	Neurooncology	$[^{18}F]$FES
Transporters		
ABC	P-gp functionality at the BBB	$[^{11}C]$Verapamil, $[^{11}C]$dLop, $[^{11}C]$metoclopramide, $[^{18}F]$MC225
DAT	Dopaminergic disorders (e.g. PD, movement disorders, neuropsychiatric disorders)	$[^{11}C]$PE2I, $[^{18}F]$FE-PE2I, $[^{123}I]$PE2I$[^{11}C]$methyl phenidate, $[^{99m}Tc]$TRODAT-1, DaTScan, $[^{18}F]$FP and $[^{123}I]$- β -CIT
Glycine type 1	Schizophrenia	$[^{11}C]$Ro5013853
NET		$[^{11}C]$EPI, $[^{11}C]$MRB
SERT (serotonin transporter)	Serotoninergic neuropsychiatric disorders (e.g. depression)	$[^{11}C]$DASB
VAChT	Cholinergic disorders (e.g. Alzheimer's disease)	$[^{18}F]$FEOBV, $[^{18}F]$VAT, [123I]-IBVM
VMAT2	Dopamine / movement disorders, dementia spectrum	$[^{11}C]$DTBZ, $[^{18}F]$AV133, $[^{18}F]$FP-DTBZ

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TABLE 1. COMMON RADIOTRACERS FOR CNS TARGETS

Target	System / Disorder	Common Tracers
Enzymes		
Hexokinase	Glucose metabolism	[^{18}F]FDG
Acetylcholinesterase	Dementia spectrum	[^{11}C]PMP
Butylcholinesterase	Dementia spectrum	[^{11}C]BMP
Monoamine oxidase	Dementia, neuropsychiatric conditions	[^{11}C]Deprenyl, [^{11}C]COU
Phospodiesterase (PDE)-4	Depression, neurodegeneration	[^{11}C]Rolipram, [^{11}C]IMA107, [^{11}C]Lu
Phospodiesterase (PDE)-10	Depression, neurodegeneration	AE92680, [^{18}F]MNI589
Protein Aggregates		
Amyloid	Dementia spectrum	[^{11}C]PiB, Amyvid, Vizamyl, Neuraceq
Tau	Tauopathies	Tauvid, [^{18}F]MK6240,
Others		
Dopaminergic pathway	Dopaminergic disorders	[^{18}F]FDOPA
TSPO 18 kDa	Neuroinflammation	[^{11}C]PK11195, [^{11}C]PBR28, [^{11}C]/[^{18}F]DAA1106
Blood Flow	Cerebral blood flow	[^{15}O]H$_2$O, [^{11}C]Butanol
Synaptic vesicle glycoprotein 2A (SV2A)	Synaptic density	[^{11}C]/[^{18}F]UCB-J
(De)myelination	Multiple Sclerosis	[^{11}C]MeDAS

2.3. SELECTING A TARGET

When considering a target for which to develop a PET or SPECT imaging agent, there are several important criteria to consider, as outlined in the following subsections below.

2.3.1. General considerations

This publication is focused upon CNS PET imaging, but the considerations of a suitable imaging target are the same whether for imaging in the brain or for other applications such as in cardiology, oncology etc. The properties of a target will be dependent upon the physiological process and/or disease in question. Generally, the target should be expressed in high abundance and be accessible by the radiotracer. For neuroimaging applications, targets are (typically) in the CNS and thus sufficient radiotracer has to be able to penetrate the Blood Brain Barrier (BBB) to engage with the imaging target and give a specific signal that can be quantified. This should be kept in mind when designing a radiotracer and considering lipophilicity, size, charge, H-bond donors, etc. (see Section 3 for more information). Considering whether to use an agonist or an antagonist radiotracer is also an important consideration [9]. Moreover, when designing radiotracers, it is ideal to select a target that is preferentially expressed in the diseased brain vs the normal brain. This is because identifying areas of target reduction involves detecting negative signals in a PET scan. This is inherently more difficult than identifying a positive signal, due to uncertainty stemming from separating low counts in a reduced PET signal from background, particularly in the early stages of a neuropsychiatric disorder.

2.3.2. Is it abundant enough to image and is it relevant?

The abundance of a target required for imaging to be relevant depends on the concentration of the target and both the affinity and specificity of the radiotracer for the target. Normally, radiotracers that have off target binding are not desirable. One way of estimating the suitability of a target for imaging is to estimate the Binding Potential (BP). The BP is a combined measure of the density of 'available' targets

(e.g. receptor B_{max}) and the affinity of a drug for the target (e.g. dissociation constant, K_d). BP can then be estimated as follows:

$$BP \sim B_{max}/K_d$$

Useful radiotracers have a BP that falls within a range of values but, as a rule of thumb, the US National Institutes of Health recommends a BP >5 for CNS applications [10]. An additional point of note is that the lowest abundance targets that have been imaged to date have B_{max} values ~1 nM. Similarly, if the affinity of a radiotracer for its target is too low, sufficient target to background (i.e. signal to noise) ratios will not be obtained.

When considering whether imaging a given target is relevant, in addition to abundance, stability of the target also has to be considered. Any changes in target abundance throughout a disease (e.g. variation, metabolic degradation, shedding from cells etc.) could negatively impact the sensitivity of the imaging technique and is to be carefully considered. When imaging a disease, the PET scan to be conducted is also considered. For example, amyloid PET is used for early diagnosis of Alzheimer's Disease (AD), and thus the increase in amyloid burden that occurs as AD progresses needs to be considered when conducting longitudinal imaging studies.

2.3.3. What is the targets' function and is it specific for the disease of interest?

Just like the need to understand whether a radiotracer is specific for a given target, it is important to understand a target's function and whether it is specific for a given disease. It is important to understand the function of a target in normal physiological conditions, as well as any changes that occur in disease states. There is no right or wrong answer to the question of target specificity, but it does need to be accounted during the development of a new radiopharmaceutical. Indeed, many targets imaged in nuclear medicine are not specific and are expressed in normal tissues (e.g. receptors, enzymes, transporters), but their expression changes in a disease state (e.g. overexpression). As stated above, it is preferred to select a target that increases in the diseased brain vs the normal brain as it is easier to identify a positive signal in a PET scan than a negative signal. The latter is possible, as in the case of identifying areas of hypometabolism with FDG in brains of dementia patients, but generally speaking a positive signal is preferred.

In other instances, a target might be expressed in a disease, but is not selective for only one condition. When considering CNS, the classical example of this is amyloid plaques. While aggregated amyloid is a hallmark neuropathology of dementia, they are not specific to a single condition. Thus, patients with AD will have an amyloid plaque burden that tracks with Braak staging, but amyloid plaques can also be present in dementia with Lewy bodies and cerebral amyloid angiopathy. By conducting large imaging studies like AD Neuroimaging Initiative, it is possible to build databases of scan data so that patterns of amyloid in different conditions can be identified and help improve diagnostic accuracy across the entire dementia spectrum.

2.4. EMERGING TARGETS

Development of new CNS imaging agents remains an extremely active area of research. This includes historical targets for which an imaging agent remains elusive, as well as newly discovered targets for which minimal molecular discovery might yet be undertaken.

2.4.1. Historical targets without an appropriate imaging agent

Imaging of transporters has been extremely successful in many instances, spurring efforts to develop radiopharmaceuticals for quantifying other pre and post synaptic transporters. Notably, to date efforts have been unsuccessful for the g-aminobutyric acid (GABA) transporter type 1 (GAT-1) and the high affinity choline uptake transporter (CHT-1). Radiopharmaceuticals targeting GAT-1 ([125I] tiagibine, CI-966, nipecotic acid) and CHT-1 ([11C/18F] hemicholiniums, [18F]FA-4, [11C]ML352) have been developed

and evaluated but do not have the required brain uptake for CNS applications. Efforts continue to develop brain penetrating tracers for both targets that can be used for neuroimaging.

2.4.2. New targets

In dementia research, the success and high impact of amyloid and tau PET imaging have paved the way for the development of analogous imaging agents for imaging other protein aggregates implicated in neurodegeneration, such as alpha synuclein and TDP-43. Neuroinflammation is also taking on more importance in neuropsychiatric disorders. Historically, neuroinflammation imaging has been achieved using imaging agents targeting the translocator protein (TSPO). However, limitations of TSPO PET have led researchers to explore newer, more cell specific and functionally relevant biomarkers. New targets in this space have been recently reviewed [11]. Lastly, synaptic loss is a consistent pathology of many neuropsychiatric disorders, and PET imaging of synaptic vesicle glycoprotein 2A (SV2A) has emerged as a promising biomarker of synaptic density and an active area of research [12].

3. SELECTION CRITERIA FOR CNS RADIOLIGANDS

In this section, methods are described to maximize the chances of identifying an appropriate CNS tracer once the target of interest and purpose of the study have been determined. This section includes criteria in addition to those used in the guidelines for generic radiopharmaceutical development described in Section 2.1 [13].

In brief, this section describes the development of a new radiopharmaceutical as 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) Lead Identification: Identification of a good lead molecule that shows suitable characteristics, such as affinity, selectivity or lipophilicity, to develop a radiopharmaceutical;
(c) Lead Optimization: Optimization of the chemical structure with respect to properties such as binding affinity, off target binding, metabolism and labelling feasibility and choice of radionuclide to match the biological half-life of the biological process under investigation;
(d) Labelling procedure development: Development and optimization of a radiolabelling procedure to provide sufficient radiochemical yield, purity, and molar activity for preclinical studies;
(e) Tracer Evaluation: preclinical assessment;
(f) Scale up and Tech transfer: Scaling up the production process, validation and GMP implementation;
(g) Clinical translation (clinical phase).

Lead molecules are often identified through standard high throughput screening programmes. Alternatively, candidates may arise from literature reports of biomolecules or synthetic chemical structures known to possess appropriate physicochemical and pharmacological properties (lipophilicity, affinity, selectivity for the target etc).

Radiopharmaceuticals require high 'target to background' ratios to ensure sufficient tissue contrast in target versus non target containing regions and/or to reduce the radiation burden in non-target tissues. The four main factors that determine high 'target to background' ratios are:

(a) The target density (e.g. B_{max});
(b) The affinity and selectivity of the radiopharmaceutical for its target;

(c) Binding to other non-specific cell components, i.e. non-displaceable binding contribution (e.g. binding to membrane phospholipids);

(d) Rapid clearance of the radiopharmaceutical from non-target tissues.

The non-displaceable binding component and clearance from non-target tissues is partly influenced by lipophilicity. Metabolism is another important parameter that influences the 'target to background' ratio. To minimize complications of radiometabolite analyses in respect of 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.

For CNS tracers the following aspects are of additional interest/importance:

(a) Receptor/target occupancy by a non-radioactive endogenous neurotransmitter, or drug. In case the aim of the study is to assess this afore-mentioned process, the tracer is required to bind to the same protein binding pocket as the non-radiolabelled molecule of interest;

(b) Brain permeability: A tracer has to be able to penetrate the BBB in order to bind to the target of interest;

(c) Target density or functionality, and measurement of density changes (as addressed in Section 2);

(d) The administration route can affect the biodistribution of the tracer. Besides the usual intravenous administrations there is the option to intrathecally inject the tracer to circumvent the BBB;

(e) A study of the biological mechanism pathway in relation to brain related disease, disease biomarker and progression, which are often very slow.

The choice of the target depends on the question to be answered or clinical problem to be solved. Section 2 of this publication deals with target selection. The choice for a corresponding CNS tracer depends on the specific clinical or research question.

3.1. LEAD MOLECULE SELECTION

For the selection of the right tracer for the right application, several approaches are possible. Depending on the available information and the fact that tracers may already be available, the timelines to have the tracer ready for use are very varied. In one situation, the appropriate tracer is already described in literature and all compounds needed are commercially available. In another situation, no tracers are available and not much is known about the structure–activity relationships regarding binding of the tracer to the target. The approaches to identify a CNS tracer are listed below and will be discussed in detail:

(a) Evaluation of an existing tracer already published and validated in humans;

(b) Evaluation of published tracers that appear to be promising in preclinical models, but not yet translated to humans;

(c) Evaluation of promising compounds published in the literature;

(d) Development of a lead compound from scratch (e.g. high throughput screening in combination within silico docking, or by structural modifications of existing radiotracers).

3.1.1. Evaluation of existing tracers that are already published and tested in humans

For many clinical and research questions, CNS tracers are already available. Table 1 summarizes CNS tracers that have been applied in clinical studies with respect to specific targets and diseases. As can be seen, several targets have been studied in detail. These target categories are enzymes, receptors, and transporters, with the corresponding tracers being enzyme inhibitors/substrates, agonists, antagonists,

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inhibitors/substrates for transporters. In addition, tracers have also been developed to image protein misfolding and deposition. These tracers bind to, for example, the β sheet structure of a protein aggregate. The molecular structure of these tracers is based on staining agents originally developed for post-mortem pathology studies.

Some of the tracers in Table 1 are being used by multiple institutions and have been validated to image the intended target. Others are used by one or a limited number of institutions and are published as a first in human study showing proof of concept. In the latter case, data needs to be carefully analysed according to the criteria mentioned above, or further pilot studies need to be conducted to determine whether the CNS tracer is appropriate for its intended application.

3.1.2. Evaluation of tracers with published data from preclinical studies

In case no information on CNS tracers for human studies has been published, there are situations whereby published preclinical data on novel tracers may be an attractive option. Such an approach is to be performed with caution as translation of a tracer from animals to humans is a significant hurdle to overcome and is a significant source of failure and requires significant resources. Further aspects on the translation are described in Section 7. The production process also needs to be carefully evaluated regarding availability of the precursor and reference standard, and the robustness of the production method. It is recommended to contact the research group who published the novel tracer to gain more insight and information on the suitability of the tracer for further development. Some tracers have appeared in literature for new targets, but these tracers did not find their way yet for human PET (see Table 2).

TABLE 2. OVERVIEW OF TARGETS THAT ARE UNDER INVESTIGATION WITH RESPECT TO THE DEVELOPMENT OF NUCLEAR MEDICINE TRACERS (Reproduced from Ref. [14])

Target	Related Disease	Tracers (Preclinical Application)	Binding Mechanism
D_3 receptor	Psychosis, neurodegeneration	Mixed D_2/D_3 tracers	Antagonists
Reactive Oxygen Species (ROS)	Neuroinflammation	[11C]hydromethidine	Trapping by oxidation
Sphingosine 1-phophate receptor	MS	[11C]TZ3321	CNS homeostasis
Receptor for advanced glycation end products (RAGE)	AD	[18F]RAGER	Beta-amyloid transport
COX-1	Neuroinflammation	[18F]PS2	Enzyme inhibitor
COX-2	Neuroinflammation	[11C]MC1	Enzyme inhibitor

3.1.3. Evaluation of promising compounds published in medicinal chemistry literature

In many cases no PET CNS tracer information has been published. To develop a tracer from scratch is then the challenging path to follow. Medicinal chemistry literature or search databases/libraries of compounds offer many data on structure–activity relationships. Besides the synthesis of compounds, in vitro data are often reported, such as affinity for the target, selectivity (IC50 or KD values), effectivity (EC50), and lipophilicity (measured or calculated, logP or logD). If the B_{max} of the target is known, it is possible to estimate B_{max}/KD which should ideally be >10. Another important parameter to check is the identification of compounds with a fluorine or N, O, or S-methyl group, as these compounds may be more easily labelled. A challenging aspect is the synthesis of a corresponding precursor.

3.1.4. Find a lead compound from scratch

If a lead compound needs to be identified from scratch, this is a challenging task requiring close collaboration with a medicinal chemistry research group or with industry. With such collaboration it is often possible to identify a lead molecule by high throughput screening in combination with in-silico docking. The pharmaceutical industry has often large libraries of compounds that can be screened using high throughput analytical methods or assays. Screening mostly discloses information on the binding interactions and affinity to the target. Often the focus in these databases is on potency as they are created for drug development and not for radiopharmaceuticals.

Both the pharmaceutical industry and academic medicinal chemistry research groups have experience with in-silico docking studies yielding information on the binding interactions between a compound and the binding site derived from a crystal structure. Knowledge on important binding interactions (hydrogen bridges, hydrophobic interactions) tells us which functional groups in the molecule are part of the crucial pharmacophore and which groups will tolerate modification in order to optimize the structure and where a radiolabel can be introduced without affecting the binding affinity. Based on the available information, a first generation tracer can be developed and evaluated in vitro and in vivo (different technologies and methods are described in following sections). In most cases, the evaluated tracer needs adjustment (e.g. choice of a longer-lived radionuclide to better align with the PK, stabilization to reduce metabolism, etc) resulting in second generation tracers.

3.2. TRACER DESIGN: SELECTION CRITERIA SPECIFIC FOR CNS RADIOLIGANDS

An ideal CNS tracer for brain imaging fulfils the following criteria:

a) A simple automated synthesis procedure suitable for reliable and robust production and low radiation exposure of personnel. For clinical studies, GMP compliance is a prerequisite. The introduction of the radiolabel takes place at the latest stage possible;

b) Appropriate molar activity sufficiently high so that binding of the tracers is minimally affected by nonradioactive counterparts. Especially for targets with low densities, the high molar activity is an important issue. In addition, some compounds are neuro active at very small concentrations requiring ultra high molar activities. For example, carfentanil being a veterinary drug needs to be administered with a very low dose to prevent a pharmacological effect;

c) LogP should be between 1.5 and 4 to passively penetrate the lipophilic BBB enabling high accumulation of target bound radioactivity. There should be low non-specific binding to achieve sufficient signal to noise ratio;

d) High affinity (expressed as B_{max}/K_D) and selectivity to achieve sufficient signal from radioactivity bound at the target and high specificity so that the measured signal represents binding to the target of interest. The ratio between target density and affinity (B_{max}/K_d) should ideally be >10. This usually means that K_D has to be in the low or sub nM range;

e) Metabolic stability to ensure that measured radioactivity represents binding of the administered tracer and not the metabolite. Metabolites should not penetrate the brain;

f) Low affinity for P-glycoprotein since P-glycoprotein can transport tracers out of the brain resulting in low brain uptake;

g) An appropriate radionuclide so that the radioactive half-life is in agreement with the kinetics of the physiological process of interest.

The importance of the selectivity of a CNS PET tracer is demonstrated by two different PET tracers for the dopamine D_2 receptor (D_2R). $[^{11}C]$N-methylspiperone is the first PET ligand to visualize D_2R in the living human brain [15]. $[^{11}C]$N-methylspiperone has high affinity for D_2R; however, it also has significant affinity to the serotine 2A receptor (5-HT$_2$AR) [16]. The in vitro selectivity of $[^{11}C]$N-methylspiperone to D_2R against 5-HT$_2$A is low (4 times). For this reason, $[^{11}C]$N-methylspiperone does not only visualize striatal D_2R but also cortical 5-HT$_2$AR (Fig. 1). In contrast, in vitro selectivity of the currently standard striatal D_2R imaging ligand $[^{11}C]$raclopride against cortical 5-HT$_2$A is very high (500 times) [17] and $[^{11}C]$raclopride selectively localized striatum in vivo (Fig. 1), where the images were obtained using 2-D mode ($[^{11}C]$N-methylspiperone) or 3-D mode ($[^{11}C]$raclopride). Static images were acquired 40–60 min after injection of the tracer and expressed as a Standardized Uptake Value (SUV) with a Gaussian filter (4 mm).

FIG. 1. Representative images of $[^{11}C]$raclopride (left) and $[^{11}C]$N-methylspiperone (right) (Courtesy of J. Toyohara, Tokyo Metropolitan Institute of Gerontology).

The importance of the affinity is exemplified with CNS tracers for the adenosine A_{2A} receptor ($A_{2A}R$), comparing $[^{11}C]$TMSX (low affinity) versus $[^{11}C]$preladenant (high affinity), and for the D_2R raclopride (striatum) versus FLB457 (cortex) have different affinities giving different PET results.

Sometimes in vivo binding (BP$_{ND}$) of a radioligands in PET studies was much lower than its affinity measured by in vitro binding assays. For example, the A$_{2A}$R ligand [^{11}C]TMSX (K_i = 5.9 nM in rat striatum A$_{2A}$R; B_{max}/K_i = 14) gives a relatively low distribution volume DVR (= BP$_{ND}$ + 1) 1.1–1.2 in human striatum. Higher in vivo A$_{2A}$R binding in human striatum (DVR ~5) was obtained with the higher affinity radioligand [^{11}C]preladenant (Ki = 1.1 nM in human striatum A$_{2A}$R; B$_{max}$/K$_i$ = 78), as shown in Fig. 2. This less than expected result can be explained by the relatively low levels of free radioligand in vivo [18]. Because only the free fraction of the radioligand in tissue (f_{ND}) can bind to the receptor, the specific in vivo binding of the ligand can be approximated as BP$_{ND}$ = f_{ND} × (B$_{max}/K_d$). It is difficult to measure f_{ND} in vivo; however, as $K_1/k_2 = f_P/f_{ND}$, where f_P is the plasma free fraction, K_1 is the plasma to brain transport rate constant and k_2 is the brain to plasma out rate constant, f_{ND} can be estimated. f_{ND} of [^{11}C]preladenant was calculated as 0.04 from the mean K_1/k_2 value of 0.68 in kinetic analysis and mean measured f_P value of 0.03. Considering this, measurement in vivo density of A$_{2A}$R regions of human striatum can be estimated more closely to the actual values as BP$_{ND}$ ~3.12 (DVR ~4). In Fig. 2, images were obtained by using 2-D mode ([^{11}C]TMSX) or 3-D mode ([^{11}C]preladenant). Static images were acquired 40–60 min after injection of the tracer and expressed as an SUV with a Gaussian filter (4 mm).
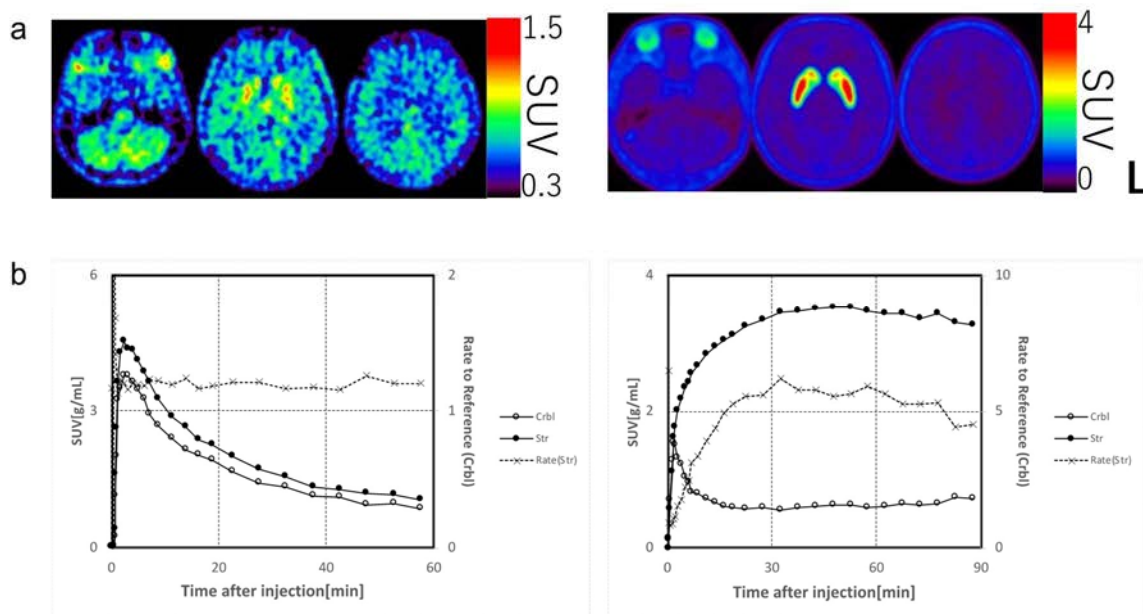
FIG. 2. (a) Representative images of low affinity radioligand [^{11}C]TMSX (left) and high affinity radioligand [^{11}C]preladenant (right). (b) Time–activity curves of striatum (target region) cerebellum (reference region), and striatum to cerebellum ratio (target to non-target ratio) of [^{11}C]TMSX (left) and [^{11}C]preladenant (right) (courtesy of J. Toyohara, Tokyo Metropolitan Institute of Gerontology).

The use of B$_{max}/K_d$ as a guideline is useful for selecting the radioligand for specific target regions of the same receptor ligand especially for lower density sites. The extra striatal density of the dopamine D$_2$ receptor (D$_2$R) is very low (<1 nM) (Table 3). Hence, the most standard radioligand for D$_2$R in striatum [^{11}C]raclopride (K_i = 1.3 nM) cannot visualize extra striatal D$_2$R. To visualize extra striatal D$_2$R higher affinity radioligand like [^{11}C]FLB457 (K_i = 18 pM) is selected (Fig. 3). However, [^{11}C]FLB457 cannot measure the striatal D$_2$R because a too high B$_{max}/K_d$ may reflect blood flow rather than receptor binding and the pseudoequilibrium state is not reached during the imaging time window. In Fig. 3, images were obtained using 3-D mode. Static images were acquired 40–60 min after injection of the tracer and expressed as an SUV with a Gaussian filter (4 mm).

TABLE 3. SELECTION OF SUITABLE K_D OF RADIOLIGAND DEPENDING ON THE RECEPTOR DENSITY (B_{MAX})

Tissue	B_{max} (nM)	B_{max}/K_i	
Human D_2R		Raclopride	FLB457
Putamen	13.3	10	739
Caudate	14.7	11	817
Temporal cortex	0.6	0.5	33
Frontal cortex	0.7	0.5	39
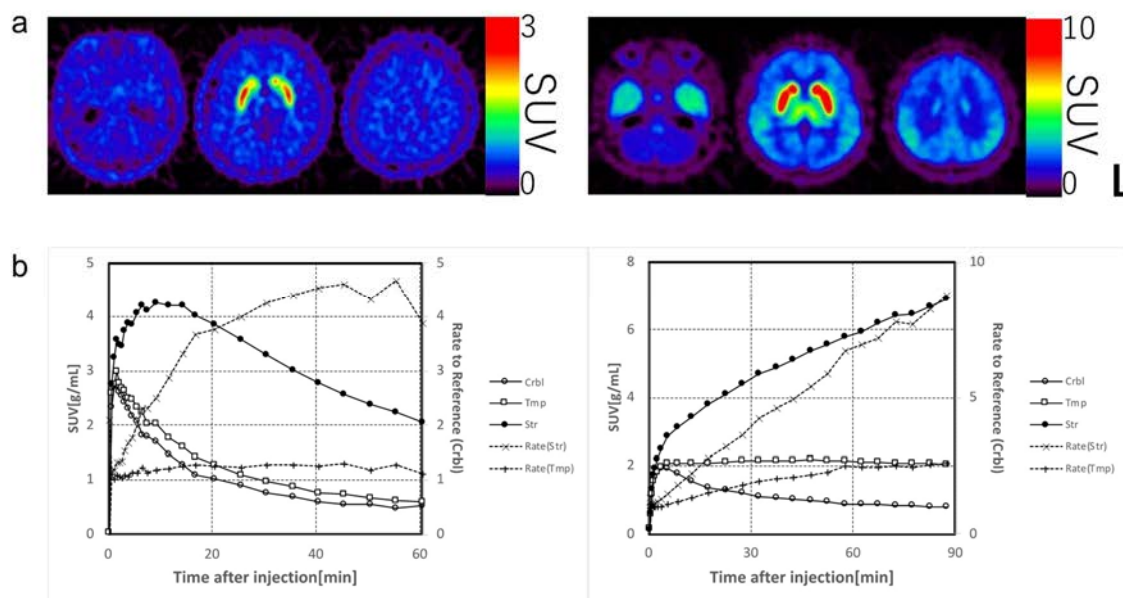
FIG. 3. (a) Representative images of [^{11}C]raclopride (left) and [^{11}C]FLB457 (right). (b) Time–activity curves of striatum (D_2R high density target region), temporal cortex (D_2R low density region), cerebellum (reference region), striatum to cerebellum ratio, and temporal cortex to cerebellum ratio of [^{11}C]raclopride (left) and [^{11}C]FLB457 (right) (Courtesy of J. Toyohara, Tokyo Metropolitan Institute of Gerontology).

For the same reason, the Dopamine Transporter (DAT) ligand [^{11}C]PE2I is more suitable than [^{11}C]CFT because [^{11}C]PE2I reached pseudoequilibrium within the PET imaging time window for a ^{11}C labelled tracer (90 min). In contrast, [^{11}C]CFT could not reach pseudoequilibrium state during 90 min (Fig. 4). In Fig. 4, images were obtained using 3-D mode. Static images were acquired 40–60 min after the injection of the tracer and expressed as an SUV with a Gaussian filter (4 mm).

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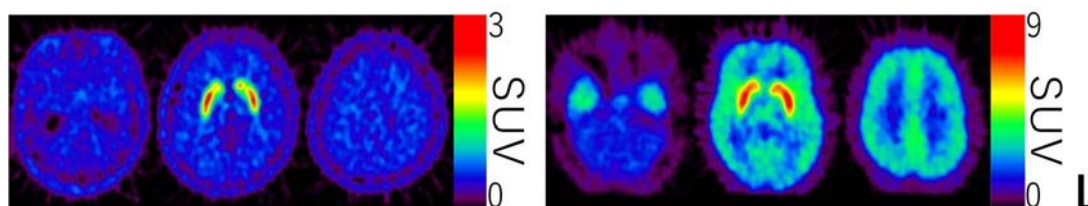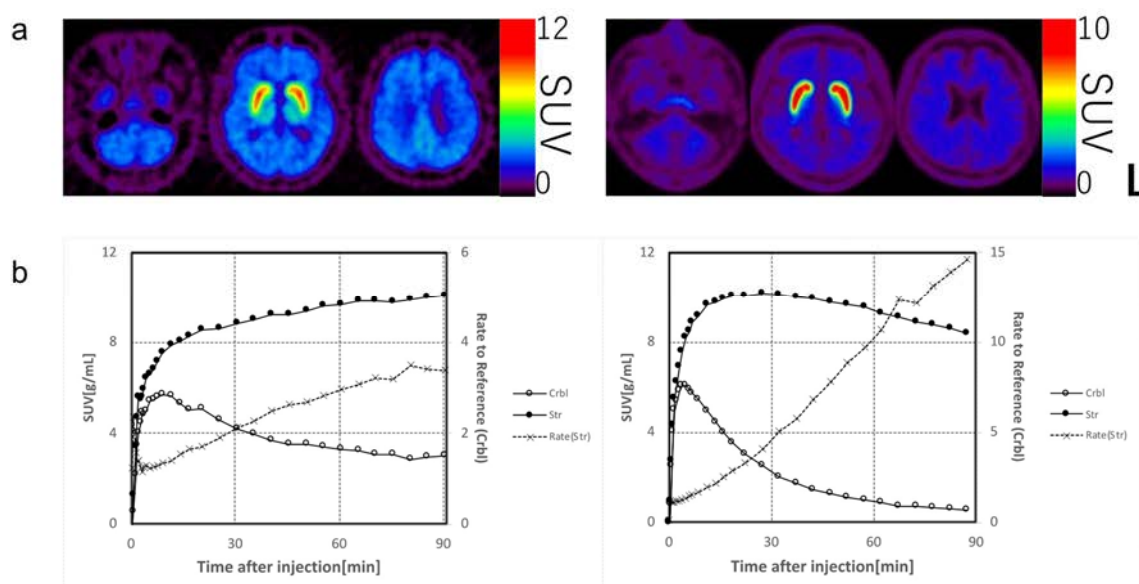*FIG. 4. (a) Representative images of [^{11}C]CFT (left) and [^{11}C]PE2I (right). (b) Time–activity curves of striatum (target region), cerebellum (reference region), and striatum to cerebellum ratio (target to non-target ratio) of [^{11}C]CFT (left) and [^{11}C]PE2I (right) (courtesy of J. Toyohara, Tokyo Metropolitan Institute of Gerontology).*

Increasing the affinity is mandatory for targeting the low density regions. Typically, increasing the affinity K_d (= K_{on}/K_{off}) will decrease the dissociation rate (K_{off}) from the binding pocket meaning a non-displaceable nature of the binding. In this case, radioligands labelled with short half-life radionuclides such as ^{11}C (20 min) are not suitable as tracer kinetics will not reach pseudoequilibrium within the PET measurement time window (90 min). Therefore, selecting a radionuclide with a longer half-life, such as ^{18}F or ^{123}I, is recommended for the radiotracer design targeting low density regions.

Polymorphism can also affect the choice of the radioligand: for instance, PET tracers targeted for TSPO are sensitive to a single nucleotide polymorphism (rs6971-SNP), resulting in low, medium and high affinity binders. Preliminary data indicate that polymorphism may also play a role in P2X7 binding [19].

Lei Zhang described strategies to further streamline the CNS PET ligand discovery process and improve the success rate [20], as follows:

(a) Properties of the target need to be well understood aiming for a B_{max}/K_D >10. Knowing the target B_{max} is crucial to achieve this ratio. Very low B_{max} implies the need for very high affinities and very low dose with impact on non-specific binding (NSB) or pharmacokinetics (PK). An approach to use ^{3}H or ^{125}I radiotracer to assess B_{max} is an option;

(b) PET ligand design parameters: A fraction of unbound tracer in brain (Fu_b) >0.05 leads to a higher success rate (Fig. 5). Passive permeability coefficient P_{app} apical to basolateral (RRCK Ralph Russ Canine Kidney AB > 5x10^{-6} cm/s) and efflux rate (BA/AB < 2.5) are important parameters in this respect;

(c) Multiparameter optimization (MPO) ranges from 0 to 6. MPO includes the following parameters: cLogP, cLogD, MW, tPSA (topic polar surface area), HBD (number of hydrogen bond donors), and pKa (ionization constant of the most basic centre). CNS PET MPO should be >3 to have an increased success rate (Fig. 5);

(d) By using LC-MS quantification of cold ligand in tissue, time consuming radiolabelling procedures (including the radiochemistry development) could be circumvented.

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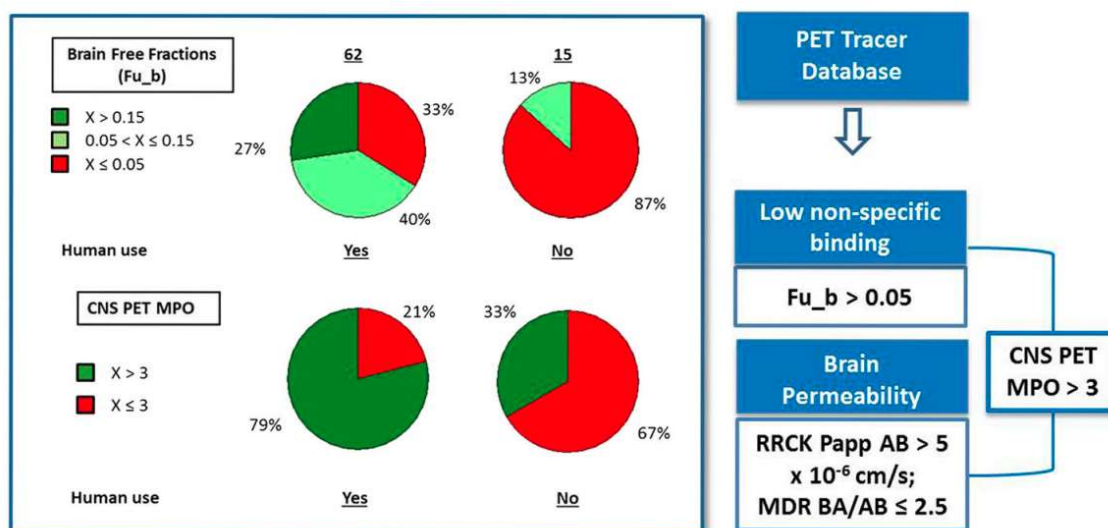
FIG. 5. CNS tracer properties to increase success rate for CNS PET imaging (reproduced from Ref. [20] with permission courtesy of [EJNMMI Radiopharm Chem]).

3.3. CHOICE OF RADIONUCLIDE: ^{11}C, ^{18}F, ^{123}I, ^{99m}Tc

Carbon-11 with a short half-life of 20 min allows labelling of endogenous compounds and many candidates for drug discovery. Several labelling positions may be possible to prevent brain penetrating ^{11}C labelled metabolites. The pharmacokinetics of the tracer should be fast and with ^{11}C tracers, multiple studies within a day are possible.

Fluorine-18 with a practical half-life of 110 min possesses favourable properties such as low positron energy; thus, high spatial resolution, high molar activity, and good availability. In the last decade, a shift is observed from the use of ^{11}C to ^{18}F CNS tracers allowing distribution to multiple sites and multicentre studies.

Iodine-123 with a long half-life of 13.2 h is hardly used. Examples are for IBZM (D$_2$) and beta-CIT/PE2I (DAT). Iodine-123 is expensive and the number of publications is decreasing.

Technetium-99m with a half-life of 6 h is the most widely used radionuclide for organ imaging. However, currently very limited Tc radiopharmaceuticals are available for neuroimaging. Technetium-99m TRODAT for imaging DAT is one successful example. Technetium complexes should be neutral to penetrate the brain.

4. RADIOLABELING AND QUALITY CONTROL OF RADIOPHARMACEUTICALS

The transfer of tracer methods from the basic biological sciences to humans is made possible by the unique nature of the radionuclides such as ^{11}C ($t_{1/2} = 20.4$ min), ^{18}F ($t_{1/2} = 109.77$ min), ^{15}O ($t_{1/2} = 2.04$ min), and ^{13}N ($t_{1/2} = 9.97$ min) used in PET to label compounds. To a considerable extent, the success of a PET radioligand does not depend only on the biological characteristics, but also in an efficient and practical radiolabelling method to enable widespread use. Due to the short half-life, time is the critical parameter for the radiochemistry development. That is why, during the development process, generally the insertion of a radionuclide to a molecule takes place at the last step of the synthesis. In principle, the entire synthesis including the purification, isolation, and formulation of the radioligands should not take more than two half-lives. Taking longer than two half-lives will result in a lowering of the molar activity (A_m) which is often critical for the PET measurements.

Radiopharmaceuticals for CNS imaging applications are predominantly small molecules labelled with ^{11}C or ^{18}F for PET, or radioiodine for SPECT. There are also emerging applications of radiolabelled antibodies for CNS imaging, but these are still in their infancy. In the case of small molecule radiotracers for PET, the decision of which radionuclide to use for labelling is based upon a number of factors:

(a) Synthetic accessibility of precursors;
(b) Suitable method for introduction of radionuclide;
(c) Metabolic stability considerations;
(d) Affinity considerations for radiolabelled molecule;
(e) Dosimetry considerations.

There are also pros and cons to using both ^{11}C and ^{18}F for brain PET applications, often stemming from the differences in half-life (^{11}C: 20 min, ^{18}F: 110 min), as outlined in Table 4.

TABLE 4. COMPARISON OF ^{11}C AND ^{18}F AS A RADIONUCLIDE FOR BRAIN PET IMAGING TRACERS

	^{11}C ($t_{1/2}$ = 20 min)	^{18}F ($t_{1/2}$ = 110 min)
Pros	Multiple studies can be conducted in a single day / hospital visit. Lower radiation dose to patient. Often exact isotopologues of endogenous molecules / known drugs can be prepared (simplifying path to clinic).	Commercialization possible. Synthesis at centralized nuclear pharmacies and distribution to satellite imaging sites feasible. Multi-step syntheses are possible.
	^{11}C ($t_{1/2}$ = 20 min)	^{18}F ($t_{1/2}$ = 110 min)
Cons	Cyclotron needed on site. Complex multi-step labelling not feasible. Distribution is challenging / impossible.	Only single imaging studies can be done per day, necessitating numerous patient visits for multi-scan protocols. Higher radiation dose to patient. Introduction of a fluorine often creates a new chemical entity and associated pharm-tox requirements can complicate clinical translation.

Once an appropriate labelling strategy has been worked out, it is necessary to develop the synthesis and quality oversight required for routine production. The synthesis of CNS radiotracers is typically accomplished using an automated synthesis module (Section 4.2), and frequently employs standard labelling strategies for a given radionuclide. For example, labelling approaches for ^{11}C (Section 4.3), ^{18}F (Section 4.4) ^{99m}Tc (Section 4.5) and radioiodine (Section 4.6) are presented below. Quality Control (QC) testing and Quality Assurance (QA) are important considerations when preparing radiotracers for CNS applications, and both concepts are described in Section 4.7. These topics are highlighted in this section in the context of CNS radiopharmaceuticals. However, many of the issues have also been covered in greater detail in dedicated IAEA publications [21–23].

4.1. SYNTHESIS MODULES FOR RADIOLABELLING

In the 1970s and 1980s, radiopharmaceuticals for the many seminal CNS imaging studies conducted in this period were accomplished manually using small amounts of radioactivity and laboratory set-ups that resembled a typical organic chemistry laboratory. For example, the earliest syntheses of [^{18}F]fluorodeoxyglucose [^{18}F]FDG by Ido. and colleagues for the original brain imaging studies, conducted in the 1970s, were accomplished by using specially designed reaction vessels, generating approximately 700 MBq of the radiopharmaceutical [24].

Manual benchtop syntheses and purifications were appropriate for early studies with low amounts of radioactivity. However, as neuroscience (and also oncology) applications of PET and SPECT imaging

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grew, the need emerged to use larger amounts of starting radioactivity. The radiation safety implications of this meant that by the early 1980s, radiochemists were already considering how to automate radiochemistry using early manually controlled electrical switches, programmable logic controllers, early computers and prototype laboratory robots.

Looking at PET, further increases in utilization of $[^{18}F]FDG$, stemming from marketing and reimbursement approvals entailed the need for even more starting amounts of ^{18}F, increasing radiation safety challenges. Concurrently, there was a need to manufacture PET radiopharmaceuticals according to the current Good Manufacturing Practice (cGMP). These demands created a compelling need for automated radiochemistry synthesis modules, particularly for PET radiopharmaceuticals. Meanwhile, SPECT radiopharmaceuticals are usually compounded, rather than manufactured. This difference in regulatory oversight means that SPECT radionuclides are commercially sourced directly in the case of ^{99m}Tc, from a commercially available Tc/Mo generator. The radiopharmaceutical is then usually compounded using an approved kit as per simple 'shake and bake' methods at hospital or centralized radiopharmacies.

Protype modules for automated PET radiochemistry were introduced in the 1990s, and development has continued since the earliest versions. Several classes of synthesis modules have emerged with each type having pros and cons depending on the intended use. Today, largescale manufacture of PET radiotracers by cGMP for commercial use tends to be conducted on cassette based synthesis modules, by using single use sterile cGMP cassettes. For research on CNS radiotracers, fixed tubed synthesizers offer flexibility for use of more diverse radiochemistry to synthesize new agents, while hybrid synthesis modules offer benefits of both cassette and fixed tube systems for smaller facilities with limited hot cell space. Other systems include dedicated synthesis systems for working with solid targets, and microfluidic systems for conducting chemistry on smaller volumes[1].

4.2. CARBON-11 RADIOCHEMISTRY

In PET radiochemistry, ^{11}C is one of the most useful radionuclides as its introduction into a molecule has minimal effects on the chemical/biological properties. The short half-life of ^{11}C allows for longitudinal in vivo studies with repeated injections on the same experimental day on the same subject. That is why ^{11}C is the most attractive radionuclide for radiolabelling the investigational drug molecule. Generally, ^{11}C is produced via the $^{14}N(p, \alpha)\,^{11}C$ nuclear reaction. The nuclear reaction is performed by high energy proton bombardment of a cyclotron target in the presence of nitrogen gas. In general, a small medical cyclotron produces ^{11}C in two different forms such as $[^{11}C]$carbon dioxide ($^{11}CO_2$) or $[^{11}C]$methane ($^{11}CH_4$), depending on the secondary gas present in the cyclotron target containing nitrogen gas. $^{11}CO_2$ is produced when a small amount of oxygen is present, whereas $^{11}CH_4$ is produced when a small amount of hydrogen is present as secondary gas. These primary precursors ($^{11}CO_2$ or $^{11}CH_4$) are sometimes used directly as labelling agents. More often, these primary precursors are converted into more reactive species (Fig. 6) before being used in ^{11}C labelling reactions [25].

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[1] For further discussion of radiochemistry synthesis modules, see the detailed discussion in Ref. [23].
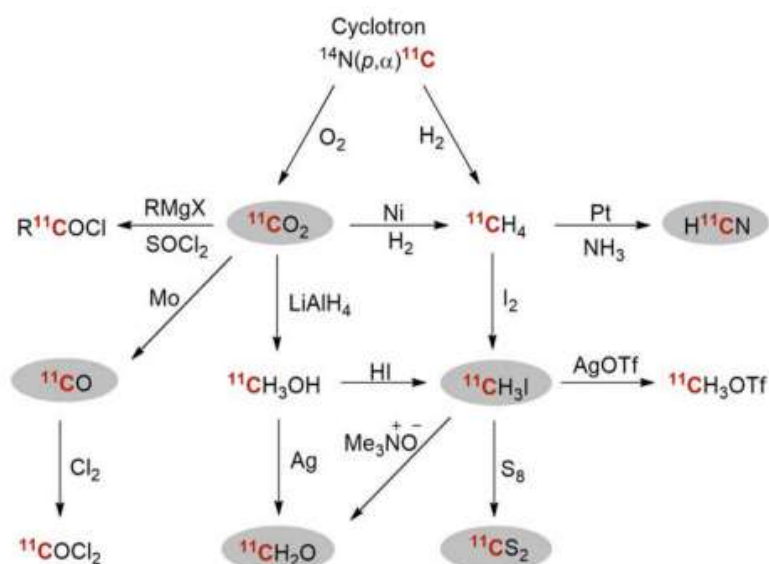
FIG. 6. Common ^{11}C-precursors used in ^{11}C labelling reactions (Courtesy of S. Nag, Karolinska Institute).

Due to the short half-life ($T_{1/2} = 20$ min) of ^{11}C, it is challenging to develop synthetic methods. To optimize the radiochemistry labelling method, these following important steps have always to be considered:

(a) Design the synthesis scheme to introduce ^{11}C radionuclide at the last step;
(b) For a good radiochemical yield and molar activity, the reaction time should be reduced or minimized;
(c) Isotopic dilution should be minimal to achieve maximum molar activities.

To have effective synthesis strategies for ^{11}C based radiochemistry, several methodologies and automation technologies have been adopted.

4.2.1. ^{11}C methylation reaction using $^{11}CH_3I/^{11}CH_3OTf$

Methylation of heteroatoms with $[^{11}C]$methyl iodide ($^{11}CH_3I$) or $[^{11}C]$methyl triflate ($^{11}CH_3OTf$) is the most common method to introduce ^{11}C radionuclide into a molecule. $^{11}CH_3I$ is mainly produced by two different methods such as the so called wet method and gas phase method. In the wet method, cyclotron produced $[^{11}C]$carbon dioxide is reduced to $^{11}CH_3OH$ by using lithium aluminium hydride (LiAlH$_4$) in tetrahydrofuran or diethyl ether (0.5–0.1 M). After the evaporation of the solvent, Hydroiodic Acid (HI) is added into the reaction mixture and then $[^{11}C]$methyl iodide is formed. Produced $^{11}CH_3I$ is distilled off in a stream of neutral gas (nitrogen or helium) through a NaOH/P$_2$O$_5$ trap into a reaction vial where the methylation reaction is carried out [26]. HI is a very corrosive chemical, and its contact can severely irritate and burn the skin and eyes with possible eye damage. For this reason, as an alternative to hydroiodic acid, diphosphorous tetraiodide or triphenylphosphine diiodide were used to produce $^{11}CH_3I$. In terms of radiochemical yield and reproducibility, the wet method is very reliable.

$$^{11}CO_2 \xrightarrow{\text{LiAlH}_4} {}^{11}CH_3OH \xrightarrow{\text{HI or P}_2\text{I}_4 \text{ or PPh}_3\text{I}_2} {}^{11}CH_3I$$

However, using the wet method to produce $^{11}CH_3I$ has some major drawbacks such as:

(a) Using LiAlH$_4$ as a reducing agent is the source cold carbon dioxide in the reaction, which may cause a drastic decrease of molar activity of produced $^{11}CH_3I$;
(b) Deterioration of tubes and valves because of HI used in the system;

(c) The method involves intense and time consuming cleaning and drying procedures.

To overcome the above drawbacks, an alternative method was developed to produce $^{11}CH_3I$ which is known as the gas phase method. In this method $^{11}CH_4$ is converted into $^{11}CH_3I$ by the free radical iodination reaction at 700–750°C in the gas phase (Fig. 7). $^{11}CH_4$ is normally produced in the cyclotron target chamber via the $^{14}N(p, \alpha)^{11}C$ reaction by using about 10% of hydrogen mixed with nitrogen gas. $^{11}CH_4$ can also be produced by reducing $^{11}CO_2$ with hydrogen on a nickel catalyst. The conversion of $^{11}CH_3I$ from $^{11}CH_4$ is very low and to have sufficient conversion of $^{11}CH_3I$, the gas phase iodination is performed in a circulation process. The produced $^{11}CH_3I$ is continuously trapped from the circulation process by using a porous polymer such as Porapak columns. Later, $^{11}CH_3I$ is released by heating the Porapak trap over 160°C.

$$Cyclotron$$

$$\downarrow H_2$$

$$CO_2 \xrightarrow{Ni/H_2} {}^{11}CH_4 \xrightarrow{I_2} {}^{11}CH_3I$$
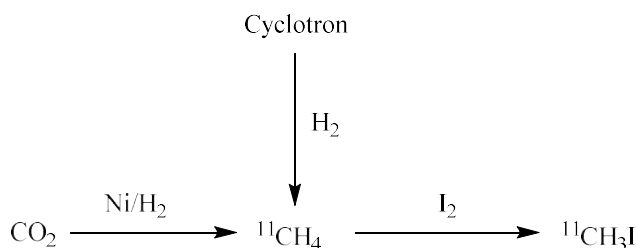
FIG. 7. Synthesis of [^{11}C]methyl iodide ($^{11}CH_3I$) via the gas phase method (Courtesy of S. Nag, Karolinska Institute).

Using [^{11}C]methyl iodide as a methylating agent in heteroatom methylation reactions gives a low radiochemical yield. In order to enhance the reactivity of the methylating agent [^{11}C]methyl iodide can be converted to [^{11}C]methyl triflate ($^{11}CH_3OTf$), as shown below. This conversion is done in an online process where [^{11}C]methyl iodide is passed in a gentle stream of helium or nitrogen through a small column containing silver triflate at 200°C. The conversion of MeOTf is almost quantitative.

$$^{11}CH_3I \xrightarrow[200\,^0C]{AgOTf,\ Heat} {}^{11}CH_3OTf$$
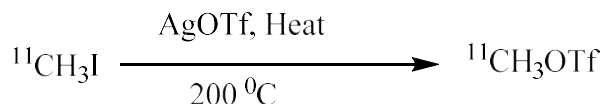
Use of $^{11}CH_3OTf$ over $^{11}CH_3I$ in heteroatom ^{11}C methylation has some advantages such as:

(a) Better reactivity;
(b) Less volatile, and thus more easily trapped in the reaction;
(c) Requires less amount of desmethyl precursor;
(d) Reaction with $^{11}CH_3OTf$ takes place at lower temperature.

Heteroatom [^{11}C]methyl using $^{11}CH_3I/^{11}CH_3OTf$ is generally obtained via N, O and S methylation reactions (Fig. 8). In a typical synthesis of a radioligand via [^{11}C]methyl, $^{11}CH_3I/^{11}CH_3OTf$ is distilled into a vial containing corresponding desmethyl precursor (0.1–2.0 mg) dissolved in an appropriate solvent (0.1–0.6 mL). Commonly used solvents are acetone, acetonitrile, methanol, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), methyl ethyl ketone. Sometimes an appropriate base, such as NaOH, KOH, tetrabutylammonium hydroxide (TBAOH), NaH or K_2CO_3, is required to increase the reactivity of the desmethyl precursor. Different techniques such as conventional vial reaction, Solid Phase Extraction (SPE) cartridge reaction, stainless steel or Teflon in loop reactions are used to carry out the [^{11}C]methyl reactions.
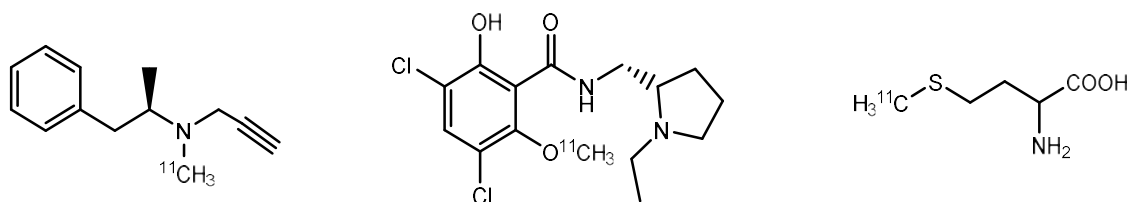
[*N*-methyl-^{11}C]deprenyl   [*O*-methyl-^{11}C]raclopride   [*S*-methyl-^{11}C]methionine

FIG. 8. [11]C labelled radiopharmaceuticals via N, O and S methylation reactions (Courtesy of S. Nag, Karolinska Institute).

[^{11}C]methyl using [11]CH_3I is also done via transition metal mediated reactions. The most used transition metal mediated reactions are Stille and Suzuki coupling reactions where organotin and organoborane compounds act as the starting materials and [11]CH_3I is the coupling part. However, due to the inherent toxicity of the tin, palladium (Pd) mediated cross coupling (Fig. 9) of [11]CH_3I with organozinc reagents has become the main method to synthesize [^{11}C]methyl arenes (Fig. 10).
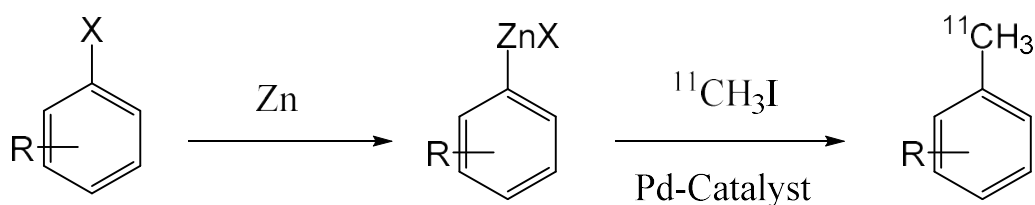
FIG. 9. Pd mediated cross coupling of [11]CH_3I with organozinc reagent (Courtesy of Sangram Nag, Karolinska Institute).

p-methyl-^{11}C-MADAM   ^{11}C-MEP   ^{11}C-A-85380
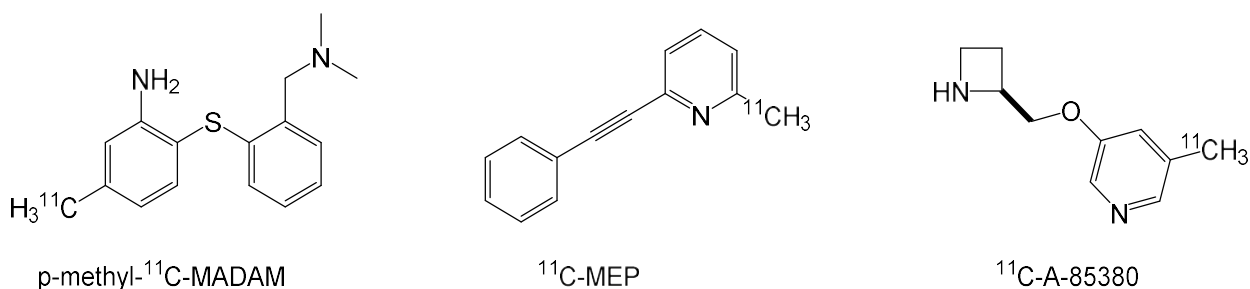
FIG. 10. Radiopharmaceuticals labelled via metal mediated [11]CH_3I methylation (Courtesy of S. Nag, Karolinska Institute).

4.2.2. Carbon-11 labelling using [11]CO_2 and [11]CO

Carbon-11 dioxide is one of the most attractive starting materials for ^{11}C labelling as it is produced directly from cyclotron. However, the direct incorporation of [11]CO_2 into an organic compound is not straightforward due to its low chemical reactivity. In order to enhance the reaction kinetics with [11]CO_2 high pressures, high temperatures or metal catalysts are commonly required. The most traditional method of [11]CO_2 insertion into an organic molecule is via Grignard or organolithium catalysed reactions (Figs 11–12) where the conversion of an alkyl or aryl magnesium halide to [^{11}C]carboxylic acid takes place. Carbon-11 labelled fatty acids, such as acetic, propionic, butyric and palmitic acids are synthesized by following this method. In addition to [^{11}C]carboxylic acids, ^{11}C labelled esters, amides, amines as well as ^{11}C labelled alkyl halides can also be prepared by the Grignard reaction.

$$RMgX \text{ or } RLi \xrightarrow{^{11}CO_2} R^{11}COOMgX \text{ or } R^{11}COOLi \xrightarrow{[H^+]} R^{11}COOH$$

$$RMgX \xrightarrow{^{11}CO_2} R^{11}COOMgX$$

- $\xrightarrow{R_1R_2NH/THF, 5min} R^{11}CONR_1R_2$
- $\xrightarrow{R_1R_2NH/NaBH_4, 25min} R^{11}CH_2NR_1R_2$
- $\xrightarrow{SOCl_2} R^{11}COCl$
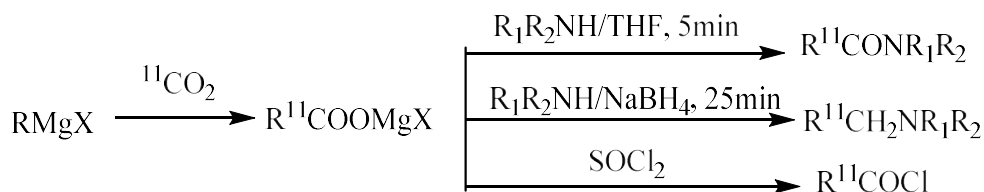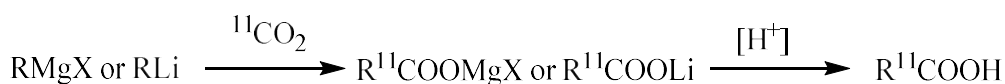
FIG. 11. Insertion of $^{11}CO_2$ using Grignard or organolithium reagent (Courtesy of S. Nag, Karolinska Institute).

[^{11}C]SL25.1188   [^{11}C]AR-A014418   [^{11}C]CURB   [^{11}C]Bexaroten
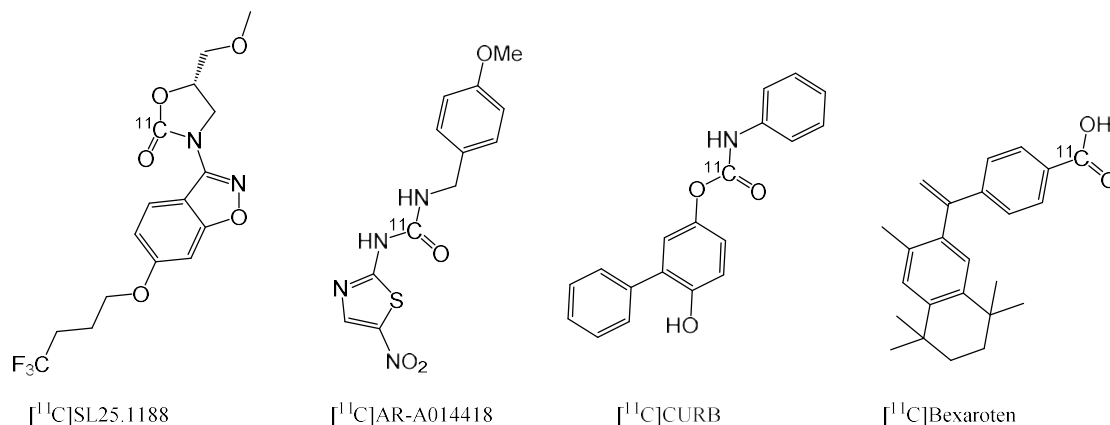
FIG. 12. Radiopharmaceuticals labelled via $^{11}CO_2$ fixation (Courtesy of S. Nag, Karolinska Institute).

However, Grignard and organolithium reagents are reactive species and require great care, especially the exclusion of atmospheric moisture and CO_2 during storage and manipulation. To overcome these challenges a novel strategy was developed in 2012 to synthesize ^{11}C labelled carboxylic acids and derivatives boronic esters in the presence of copper as a catalyst [27]. Organic amines fixation base such as DBU or BEMP can be used as organomediators to also increase the reactivity of $^{11}CO_2$. In this way, ^{11}C labelled carbamates, urea derivatives and oxazolidinones are synthesized with a higher yield (Fig. 13).

Base Q

^{11}C-Carbamate or ^{11}C-Urea
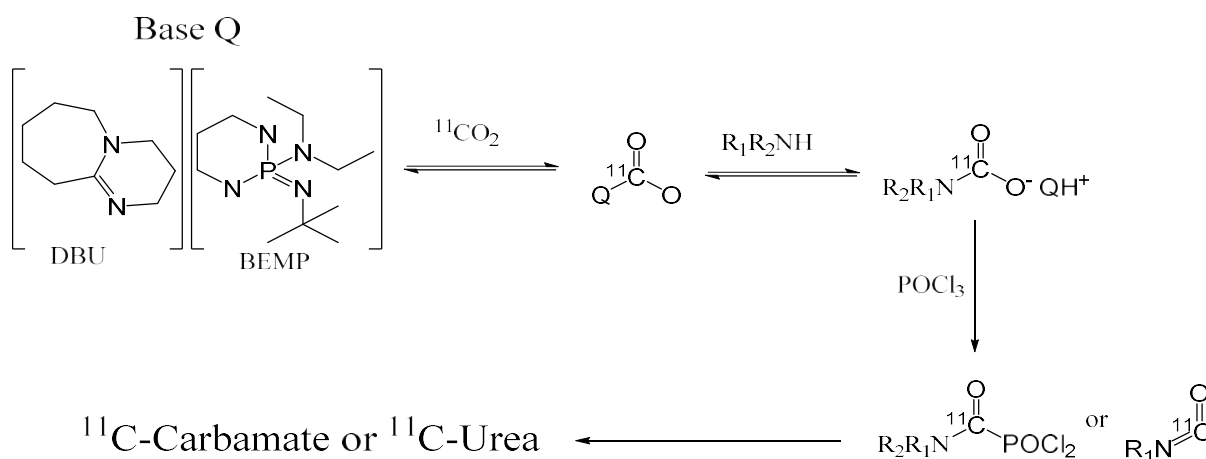
FIG. 13. Synthesis of ^{11}C labelled urea and carbamates via $^{11}CO_2$ fixation (Courtesy of S. Nag, Karolinska Institute).

The Carbonyl (CO) group is one of the most common functional groups in bioactive organic molecules. Carbon-11 monoxide has been found to be a useful building block for the synthesis of ^{11}C-carbonyl labelled carboxylic acids, esters, amides, ketones, and aldehydes (Fig. 14).
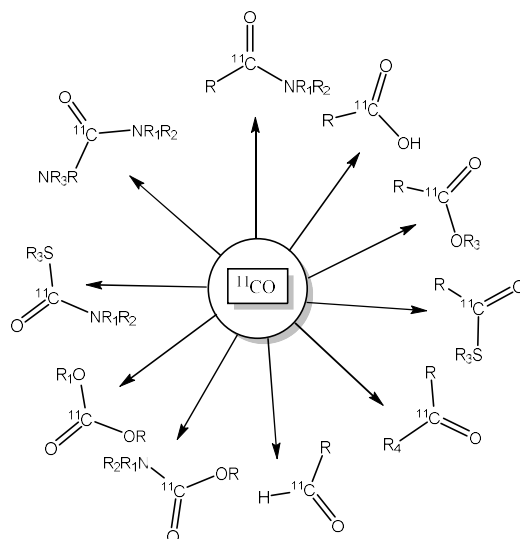
FIG. 14. Functional groups synthesized from [11]CO (Courtesy of S. Nag, Karolinska Institute).

Carbon-11 monoxide is typically produced by the reduction of no carrier added $^{11}CO_2$ produced from a cyclotron. Generally, an automated ^{11}CO synthesizer is used for the production of ^{11}CO. At the end of bombardment of the cyclotron target, the target content is delivered to the ^{11}CO synthesizer where the $^{11}CO_2$ is trapped on a silica gel subsequently removed by a sodium hydroxide coated silica trap. $^{11}CO_2$ is reduced online to ^{11}CO using a pre-heated (850°C) quartz glass column charged with molybdenum powder or using a pre-heated (400°C) quartz glass column filled with metallic zinc powder. Unreacted $^{11}CO_2$ is subsequently removed by a sodium hydroxide coated silica and the ^{11}CO is concentrated on a silica gel trap immersed in liquid nitrogen. After the completed entrapment, the trap is heated to release the ^{11}CO into a closed reaction vial containing the precursor molecule where ^{11}CO is inserted via a metal catalysed reaction. Typical metal catalysed cross-coupling reactions (Pd, Ni, Pt) involve oxidative addition of zero valent metal with appropriate organic halides followed by insertion of ^{11}CO (Fig. 15), followed by the nucleophilic attack by the desired nucleophile, and finally, by the reductive elimination to yield the target molecule (Fig. 16) while freeing the metal for the next cycle.
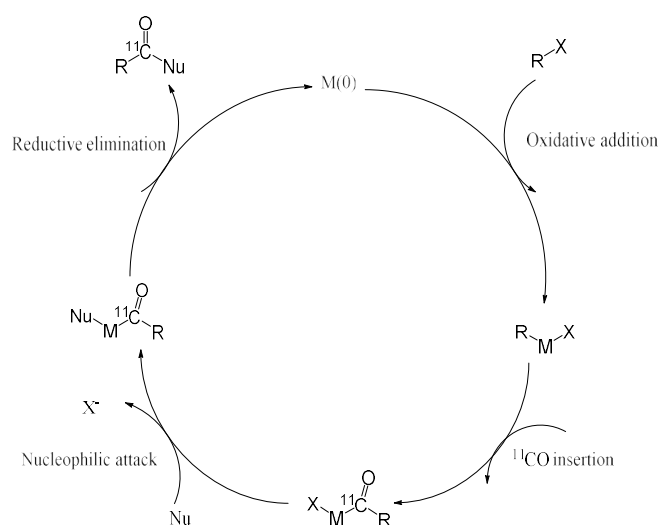
FIG. 15. Catalytic cycle for transition metal mediated ^{11}CO carbonylation (Courtesy of S. Nag, Karolinska Institute).
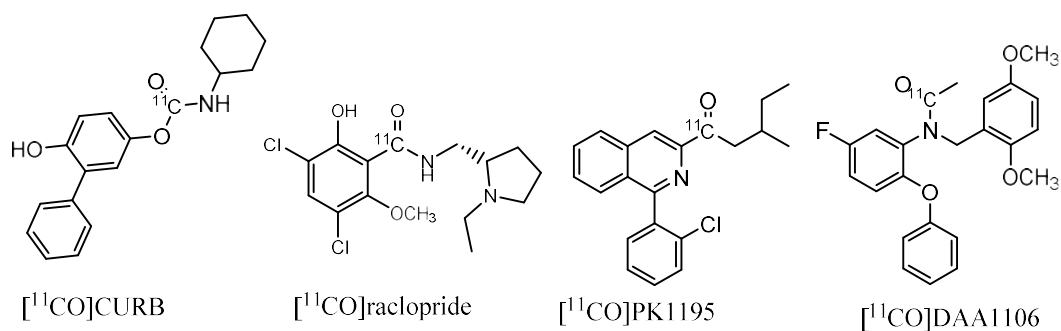
FIG. 16. PET tracers prepared by palladium mediated ^{11}CO carbonylation (Courtesy of S. Nag, Karolinska Institute).

4.2.3. Carbon-11 labelling using ^{11}CN

Both aromatic and aliphatic cyanides are very common scaffolds/pharmacophores in drug molecules. CNs are most commonly used as the intermediates in the preparation of carboxylic acids, amides, amines, and related derivatives. This principle is applicable for ^{11}C labelling chemistry and $H^{11}CN$ is a versatile precursor for the development of PET radiopharmaceuticals. Typically, $^{11}CH_4$ is produced in target via the $^{14}N(p,\alpha)^{11}C$ reaction on nitrogen mixed with 10% of hydrogen. $[^{11}C]CH_4$ together with NH_3 gas is passed through a platinum (Pt) oven (990°C) and converted to $NH_4{}^{11}CN$, which is immediately bubbled through H_2SO_4 (50%) at 65°C to produce $H^{11}CN$. Carbon-11 labelled alkyl cyanides are mostly synthesized by nucleophilic substitution or addition reactions with $H^{11}CN$ as the reagent. The most frequent derivatization is to generate ^{11}C-carboxylic acids or derivatives (Fig. 17).
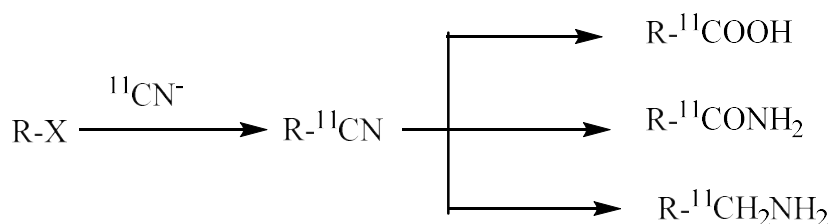
FIG. 17. Synthesis of alkyl ^{11}CN and its derivatives (Courtesy of S. Nag, Karolinska Institute).

Metal mediated cross coupling cyanation of aryl halide is well documented for ^{11}C labelling (Fig. 18).
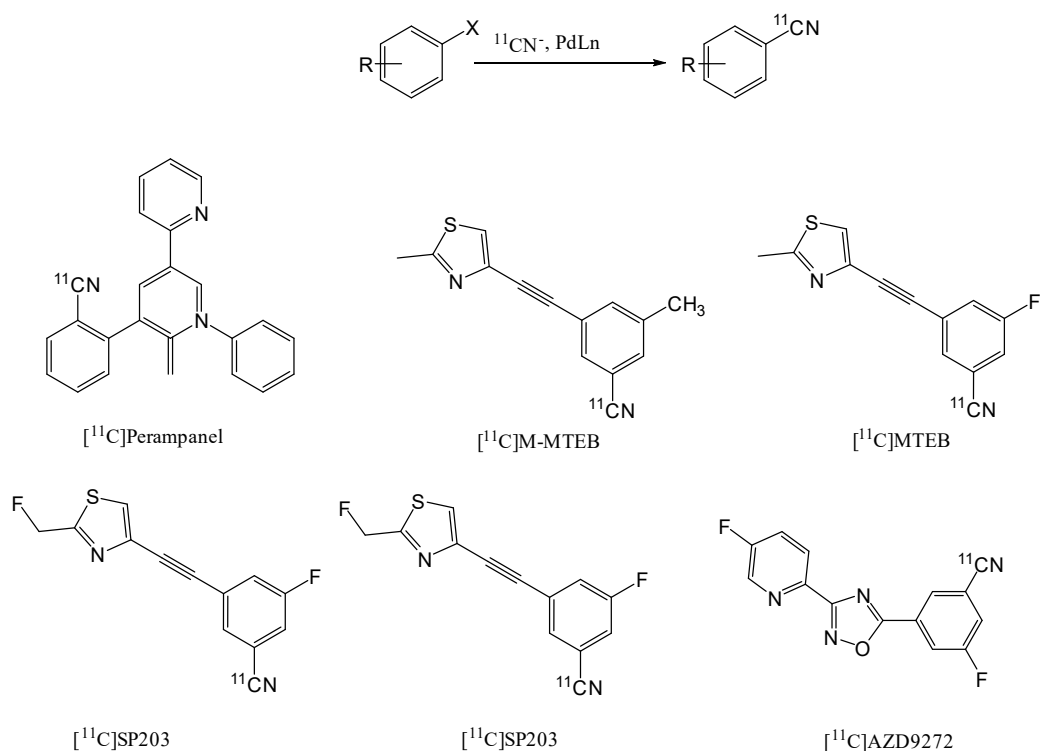
FIG. 18. Synthesis of metal mediated aryl ^{11}CN (Courtesy of S. Nag, Karolinska Institute).

4.3. FLUORINE-18 RADIOCHEMISTRY

Fluorine has been widely introduced in pharmaceutical compounds despite an unusual natural occurrence in biological molecules [28]. Fluorine substitution is common in drug development and at present, fluorine containing pharmaceuticals represent about 20% of all marketed drugs. Although the introduction of a fluorine atom in a molecule generally modifies its physical and biological properties [29], a large number of molecules exist where hydrogen was replaced by fluorine and the new molecules retained comparable properties [30]. Therefore, despite the difference in properties, F is empirically a good mimic of H replacement in a drug molecule. Fluorine-18 is one of the most widely used radionuclides in PET imaging. Fluorine-18 can be introduced to the radioligand by different ways (Fig. 19) such as nucleophilic and electrophilic substitution.
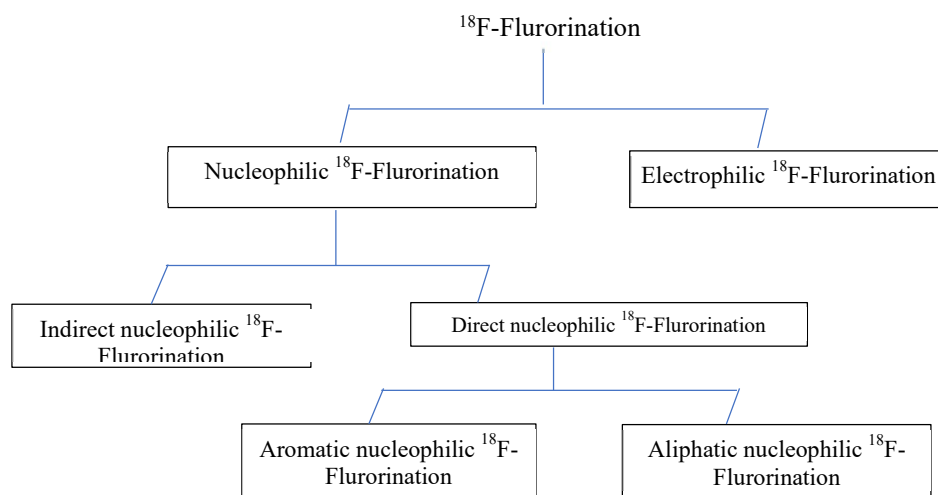
FIG. 19. [^{18}F]fluorination strategies (Courtesy of S. Nag, Karolinska Institute).

4.3.1. Nucleophilic ^{18}F-fluorination

The [^{18}F]fluoride ion is generally produced by the nuclear reaction $^{18}O(p,n)^{18}F$ in an aqueous environment. In an aqueous environment, negatively charged ions has to be accompanied by positively charged counter ions. [^{18}F]fluoride is washed out from the cyclotron target with traces of metal ions (counter ions) from the surface of the target. Nucleophilic $^{18}F^-$fluorination is also very sensitive to the presence of metal-ion traces formed by irradiation of the target. Therefore, [^{18}F]fluoride ion is isolated by passing through an anion exchange column where [^{18}F]fluoride is retained by an ion exchange reaction, and the ^{18}O water as well as the metal ions are allowed to flow through. The retained [^{18}F]fluoride ion is then eluted with an acetonitrile solution of counter ion. Potassium carbonate and commercially available cryptands are commonly used as a counter ion and a phase transfer catalyst.

Fluorine has a high hydration energy, and the fluoride ion is a poor nucleophile in aqueous media. Therefore, nucleophilic substitution reactions involving [^{18}F]fluoride ion require strict exclusion of water. The advantage of using acetonitrile as the eluting solvent is that it forms an azeotropic mixture with water. Evaporation of the acetonitrile in a nitrogen atmosphere will remove any residual ^{18}O water simultaneously. The classical rule is that increased dryness will increase the reactivity of the [^{18}F]fluoride ion. There are, however, numerous aliphatic reactions that withstand relatively large amounts of water without influencing the radiochemical yield. Nucleophilic substitution with [^{18}F]fluoride generally occurs by heating the dried residue of the K^+[^{18}F]fluoride-cryptand (Kryptofix-222) complex with the precursor in a polar aprotic solvent such as acetonitrile, DMSO and DMF [30].

In general, nucleophilic substitution with [^{18}F]fluoride is divided into aliphatic and aromatic substitution reactions. Aliphatic substitution reactions proceed via a S_N2 mechanism which involves the substitution of a suitable leaving group which is liable to displacement by [^{18}F]fluoride (Fig. 20). The most common leaving groups include sulfonic acid esters (e.g. triflates, tosylates, mesylates or sulphamidate) or halides (Cl, Br or I). The choice of a leaving group depends mainly on the stability of the precursors, the purification of the ^{18}F labelled product and the potential for formation of by-products.
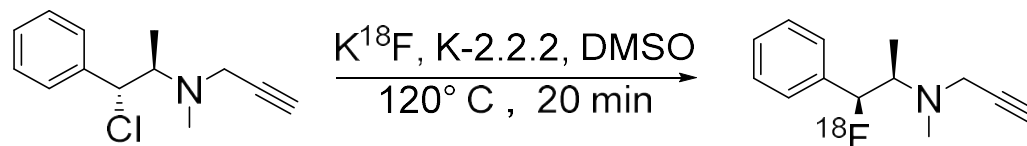
FIG. 20. Aliphatic [^{18}F]fluorination substitution (Courtesy of S. Nag, Karolinska Institute).

Nucleophilic aromatic substitution with [^{18}F]fluoride results in the formation of aryl [^{18}F]fluorides. A prerequisite is that the aromatic ring needs to be activated by the presence of one or more electron withdrawing groups such as nitro, cyano, carbonyl, positioned at ortho or para, to the leaving group (Fig. 21). There are several possible alternatives for leaving groups but mostly a halogen, nitro or trialkylammonium group is substituted (Fig. 22).
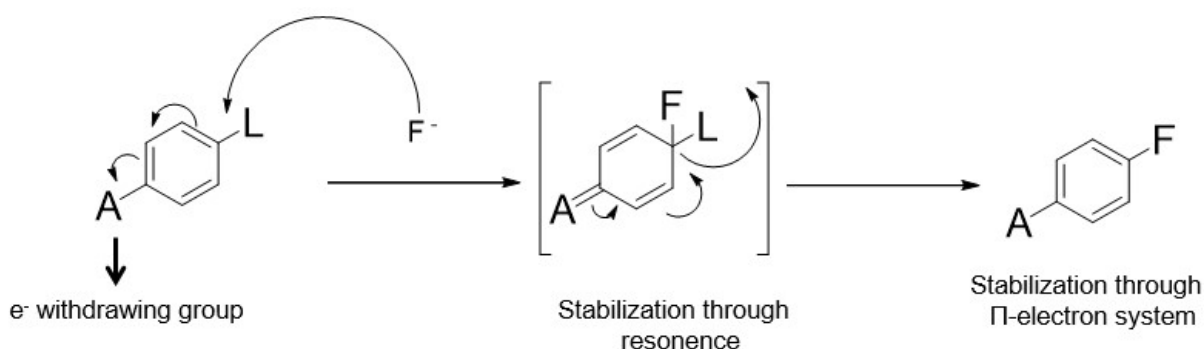
FIG. 21. Mechanism of aromatic [^{18}F]fluorination substitution (Courtesy of S. Nag, Karolinska Institute).

FIG. 22. Example of aromatic [^{18}F]fluorination substitution (Courtesy of S. Nag, Karolinska Institute).

Fluorine-18 radiolabelling of long chain molecules such as proteins, peptides are performed via indirect fluorination. In this case, a prosthetic group (Fig. 23) is first labelled with ^{18}F via nucleophilic fluorination followed by the addition of the prosthetic group to the proteins/peptides (Fig. 24).

FIG. 23. Commonly used ^{18}F prosthetic groups (Courtesy of S. Nag, Karolinska Institute).

FIG. 24. Radiosynthesis of ^{18}F peptides using prosthetic group [^{18}F]SFB [31] (Courtesy of P. Elsinga, University Medical Center Groningen).

4.3.2. Electrophilic [^{18}F]fluorination

For electrophilic radiofluorination reactions, [^{18}F]fluoride gas ([^{18}F]F$_2$) is used as an electrophile. [^{18}F]F$_2$ gas is produced via the nuclear reaction ^{20}Ne(d,α)^{18}F and is liberated from the cyclotron with [^{19}F]F$_2$. Therefore, the molar activity of the electrophilic [^{18}F]fluorine is in general low and not optimal for synthesizing high molar activity ^{18}F labelled radiopharmaceuticals. When [^{18}F]F$_2$ is used for electrophilic substitution, the maximum achievable radiochemical yield is 50% because only one of the fluorine atoms in [^{18}F]F$_2$ is labelled with [^{18}F]F (Fig. 25).
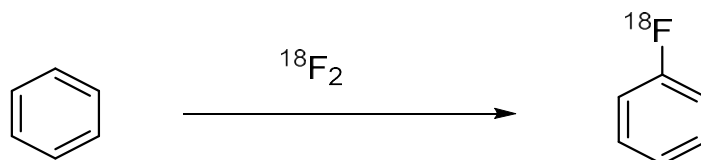
FIG. 25. Electrophilic ^{18}F-fluorination (Courtesy of S. Nag, Karolinska Institute).

However, in 1997, a method was developed to generate [^{18}F]F$_2$ with a higher molar activity (up to 55 GBq/µmol) than was common for [^{18}F]F$_2$ (which is up to 2 GBq/µmol) [32]. In this approach, ^{18}F labelled methyl fluoride ([^{18}F]CH$_3$F) was synthesized from aqueous [^{18}F]F$^-$ (Fig. 26). Applying this method, recently, Gouverneur succeeded in synthesizing [^{18}F]F-TEDA, an electrophilic [^{18}F]fluorination reagent

27

more useful and selective than $[^{18}F]F_2$ [33]. Generally, $[^{18}F]F_2$ is converted into less reactive and more selective $[^{18}F]$fluorination agents [34,35].

$$[^{18}F]F^- \text{ (aq.)} \xrightarrow[\text{Kryptofix}_{222}]{K_2CO_3} K_{222}K^{+18}F^- \xrightarrow[\text{CH}_3\text{CN}]{CH_3I} [^{18}F]CH_3F \xrightarrow[\substack{\text{Electric} \\ \text{discharge}}]{F_2 \text{ (1 }\mu\text{mol)}} [^{18}F]F_2$$
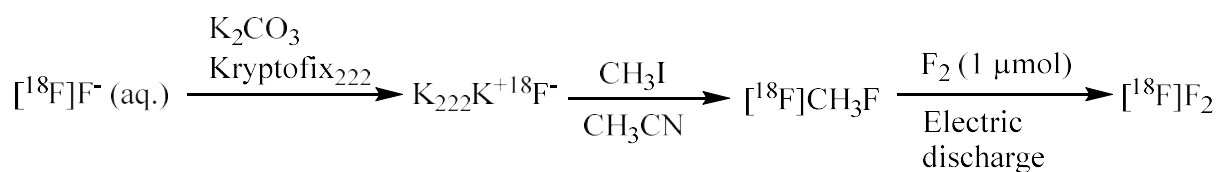
FIG. 26. Synthesis $[^{18}F]F_2$ via $[^{18}F]CH_3F$ (Courtesy of S. Nag, Karolinska Institute).

Historically, the earliest ^{18}F labelled tracers were synthesized via the electrophilic pathway such as $[^{18}F]FDG$, and then nucleophilic pathways were developed to obtain higher radiochemical yield with a higher specific activity. Despite the lower radiochemical yield and lower specific activity, electrophilic fluorination is still used today in the production of PET radioligands, e.g. $[^{18}F]F$-DOPA [34].

4.3.3. Transition metal mediated aromatic $[^{18}F]$fluorination

In recent years, significant development has been done in the aromatic $[^{18}F]$fluorination using transition metals, such as Pd(IV), Ni(II), Cu(ll), Ag(l), with enhanced reactivity, selectivity and tolerance towards functional groups (Fig. 27).
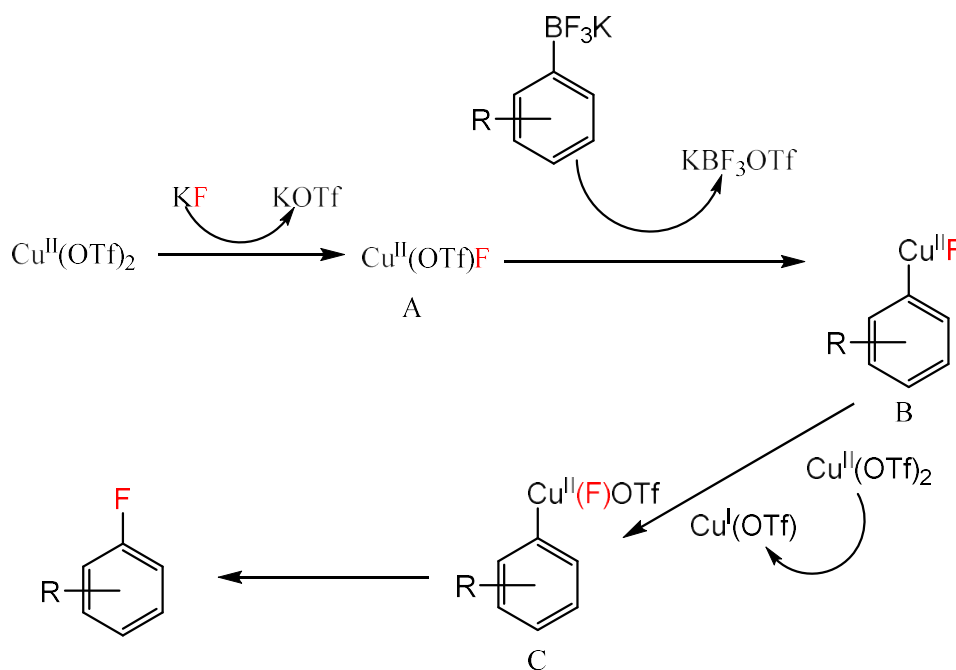
FIG. 27. Example of Cu(ll)-mediated aromatic ^{18}F-fluorination (Courtesy of S. Nag, Karolinska Institute).

4.4. TECHNETIUM-99m FOR CNS RADIOTRACERS

Technetium-99m, the daughter of ^{99}Mo, is the most used radionuclide for SPECT imaging procedures due to its favourable physical properties and convenient availability. It is estimated that 80% of nuclear medicine imaging procedures utilize ^{99m}Tc labelled radiopharmaceuticals. ^{99m}Tc is mostly obtained from $^{99}Mo/^{99m}Tc$ generators. ^{99}Mo has historically been produced in nuclear reactors through the fission reaction, $^{235}U(n,fission)$ ^{99}Mo. However, the unscheduled downtime of ageing worldwide nuclear reactors has led to ^{99}Mo shortages and, as such, alternative supplies have been investigated. For example, cyclotron accelerators have been equipped with targets that enable accelerator production of ^{99}Mo from ^{100}Mo via the $^{100}Mo(p,pn)$ ^{99}Mo reaction. Investigators have also developed methods for the

direct production of ^{99m}Tc using cyclotrons. These topics have been covered extensively in prior IAEA publications [35, 36].

Commercial ^{99}Mo/^{99m}Tc generators consist of ^{99}Mo adsorbed onto a suitable column (e.g. aluminium oxide). Molybdenum-99 (T$_{1/2}$ = 67 h) decays to ^{99m}Tc (T$_{1/2}$ = 6 h) according to a transient equilibrium, and equilibrium occurs within 4 half-lives. Technetium-99m is eluted from the alumina cartridge (as sodium pertechnetate ([^{99m}Tc]NaTcO$_4^-$) using sterile saline. The [^{99}Mo]MoO$_4$ $^{-2}$ is retained on the alumina, where it continues to undergo radioactive decay. The solution of [^{99m}Tc]sodium pertechnetate in saline is passed through a 0.22 µm sterilizing filter and is either used directly for ^{99m}Tc scans or it can be incorporated into radiopharmaceuticals using the strategies described below.

4.4.1. Radiolabelling with ^{99m}Tc

The preparation of ^{99m}Tc labelled radiopharmaceuticals has also been covered extensively in previous IAEA publications [37, 38] and the contents are not replicated here. Rather, for completeness, a short overview of radiolabelling approaches using ^{99m}Tc is provided.

Technetium-99m sodium pertechnetate can be used directly for neuroimaging applications (e.g. for thyroid imaging). However, it is more commonly used as a starting point for the preparation of ^{99m}Tc labelled radiopharmaceuticals for CNS applications.

Technetium can exist in many different oxidation states. Therefore, typically the first step is the reduction of [^{99m}Tc] sodium pertechnetate followed by the complexation with an appropriate ligand. Thus, when designing a new ^{99m}Tc labelled radiopharmaceutical, radiochemists need to consider both the bioactive molecule that will target the site to be imaged, and how a suitable coordinating ligand can be incorporated into the scaffold without a detrimental effect upon the pharmacological and pharmacokinetic profiles of the radiopharmaceutical. Additionally, a reducing agent needs to be selected that reduces the technetium ion to the desired oxidation state, but which does not result in any side reactions with other functionalities in the bioactive molecule to be labelled, or that can form complexes with Tc or compete with it for the coordinating ligand. Thus, transition metal based reducing agents are typically avoided, along with reducing agents like hydrazines and hydroxylamines that can complex Tc. Tin (II) is the preferred reducing agent when working with technetium. The oxidation state of technetium in pertechnetate (^{99m}TcO$_4^-$) is +7. It is common to reduce technetium to another oxidate state (e.g.+5) with SnII, followed by mixing it with a bioactive molecule functionalized with a suitable chelating group.

Previous IAEA publications [37, 39] have defined general criteria for radiochemists to consider when developing ^{99m}Tc labelled radiopharmaceuticals that might eventually be translated into clinical use:

    a) Labelling yield has to be close to quantitative, but at least better than 95%;
    b) Labelling chemistry may not include purification processes;
    c) Labelling chemistry has to be highly efficient at low biomolecule concentration;
    d) Potentially toxic or unstable ingredients are not desirable;
    e) No bio-incompatible organic solvents may be used;
    f) The complex label has to be well defined, if possible, not only by High Performance Liquid Chromatography (HPLC) but also by classical chemical analysis;
    g) No unspecific labelling of the biomolecule should occur;
    h) Transfer of the process to rhenium (for therapy) is desirable;
    i) In vivo stability is necessary.

The radiosynthesis that adheres to these criteria is easily adaptable to a single use kit. Such single use 'shake and bake' kits are the industry standard for preparation of ^{99m}Tc labelled radiopharmaceuticals for clinical use, as it is straightforward to prepare labelled products that are sterile, pyrogen free, and have long shelf lives. They usually contain all the reagents needed for labelling, including a reducing

agent and the bioactive molecule bearing the ^{99m}Tc-chelating group. Radiolabelling is then completed on the day of use and involves simple addition of Na^{99m}TcO$_4^-$ to the kit. Reference [38] has also suggested additional criteria for transitioning radiochemistry into kit form, as follows:

a) The procedure is to be concentrated in an instant kit formulation;
b) The kits are lyophilized, thus, the ingredients are not to be volatile or decomposed during this process;
c) After labelling, the radiopharmaceutical needs to be ready to be administered;
d) Very precise labelling conditions (e.g. 12.56 min at 94.3°C) are not desirable;
e) A reasonable radiochemical purity test is feasible in a short time.

Following the radiosynthesis of a ^{99m}Tc labelled radiopharmaceutical, it is necessary to conduct QC testing as outlined in Section 4.7 to ensure that the product is suitable for clinical use.

4.5. RADIOIODINE RADIOCHEMISTRY

Numerous radioactive isotopes of iodine have applications in molecular imaging and radiotherapy (Table 5).

TABLE 5. ISOTOPES OF IODINE EMPLOYED IN NUCLEAR MEDICINE

Isotope	Half-Life	Gamma Energy	Application
I-123	13.2 h	159 keV	SPECT / Therapy
I-124	4.18 d	511, 602, 722 keV	PET
I-125	59.9 d	35.5 keV	SPECT / Therapy
I-131	8.02 d	364 keV	SPECT / Therapy

The subject of radioiodine production is covered in other IAEA publications [40,41]. Briefly, ^{125}I and ^{131}I are typically produced in nuclear reactors, while ^{123}I and ^{124}I are produced using cyclotrons.

Iodine-123 is produced in a cyclotron by proton irradiation of xenon in a capsule. Xenon-124 absorbs a proton and immediately loses a neutron and proton to form ^{123}Xe, or else loses two neutrons to form ^{123}Cs, which then decays to ^{123}Xe. It then decays to ^{123}I and is trapped on the inner wall of the irradiation capsule under refrigeration before it is eluted (as NaI) with sodium hydroxide.

$$^{124}Xe(p,pn)\ ^{123}Xe \rightarrow\ ^{123}I$$

$$^{124}Xe(p,2n)\ ^{123}Cs \rightarrow\ ^{123}Xe \rightarrow\ ^{123}I$$
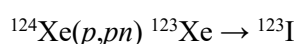
Iodine-124 is produced by using a cyclotron, and routes involving either proton or deuteron irradiation of ^{124}Te targets are typically used:

$$^{124}Te(p,n)^{124}I$$

$$^{124}Te(d,2n)^{124}I$$
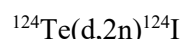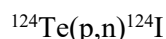
Iodine-125 is produced in nuclear reactors and is available in large quantities. Its production follows these two reactions:

$$^{124}Xe\ (n,\gamma) \rightarrow\ ^{125m}Xe \rightarrow\ ^{125}I$$

$$^{124}Xe\ (n,\gamma) \rightarrow\ ^{125g}Xe \rightarrow\ ^{125}I$$
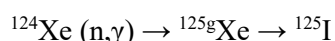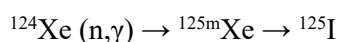
Iodine-131 is mostly produced from neutron irradiation of a natural tellurium target in a nuclear reactor. Irradiation of natural tellurium produces almost entirely ^{131}I as the only radionuclide with a half-life longer than a few hours. [130]Te (34% of natural tellurium) absorbs a neutron to become [131]Te, which beta decays to [131]I (t1/2 = 25 min).

$$^{130}\text{Te}(n,\gamma)^{131}\text{Te} \rightarrow {}^{131}\text{I}$$
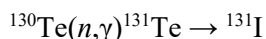
4.5.1. Labelling with radioiodine

The relatively long half-life of iodine isotopes outlined in Table 5 allow the production and distribution of both the radionuclides and radiopharmaceuticals from centralized manufacturing sites, particularly for clinical use. This is advantageous because working with large quantities of radioiodine requires specialized facilities due to the potential for the formation of long lived volatile iodinated species. For clinical production, such facilities also need to be cGMP certified. Local radiochemistry facilities might often handle small amounts of radioiodine for research and preclinical use of iodine radionuclides.

Given the importance of radioiodine to nuclear medicine over the years, many methods for labelling with isotopes of iodine have been developed. These have been the subject of books and recent review articles [42–44] as well as IAEA publications [23, 45].

Labelling with radioiodine can be accomplished using both nucleophilic and electrophilic methods. When synthesizing iodine labelled radiopharmaceuticals, a common strategy is to generate aryl or vinyl iodides because the C–I bond strength is high and is usually quite stable. In contrast, generation of aliphatic iodides is usually avoided because such C–I bonds are weak and deiodination is common.

4.5.2. Nucleophilic iodination

Radiolabelling of aromatic rings with radioiodine can be accomplished by using no carrier added iodide via nucleophilic aromatic substitution (SNAr) (Fig. 28); but this is typically a slow and cumbersome process. SNAr reactions with radioiodine are subject to typical directing effects, and this can be improved by activating the aromatic ring through installation of Electron Withdrawing Groups (EWG) in the ortho or para positions. The leaving group (X) can be iodine, in which case it is an isotopic exchange reaction, but this limits the molar activity that can be achieved since radioiodinated products are inseparable from the iodinated precursor. Alternatively, other leaving groups are used (e.g. X = Br or Cl), and the iodinated product can be separated from the precursor, then higher molar activity products can be prepared. For example, this approach has been used to synthesize meta-iodobenzylguanidine (MIBG) (Fig. 28), a radiopharmaceutical used for diagnosis and treatment in neuroblastoma or pheochromocytoma.
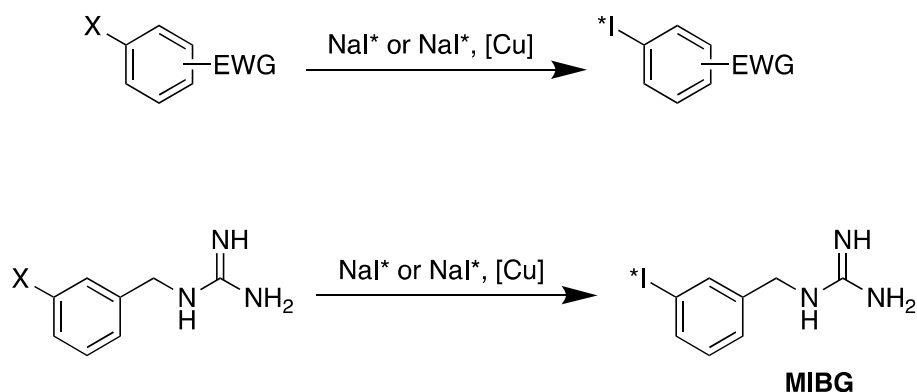
FIG. 28. Nucleophilic radioiodination of aromatic compounds including MIBG (Courtesy of S. Nag, Karolinska Institute).

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4.5.3. Electrophilic Iodination

It is common and often more efficient to prepare iodinated radiopharmaceuticals via electrophilic reactions. These reactions do necessitate preformation of an electrophilic source of radioiodine (*I). Elemental iodine (I_2) is not usually used because of low reactivity as well as challenges associated with volatility. Furthermore, it is by definition carrier added, which negatively impacts the molar activity of the resulting products. Thus, is it more typical to use the radioiodide described above to generate electrophilic species, typically via treatment with an oxidizing agent, which can then be used in electrophilic aromatic substitution reactions. Examples include *ICl, *IF, *IOH, H_2OI* and N-iodosuccinimide. Oxidants include chloramine-T, iodogen, and N-chlorosuccinimide. In some instances, harsh oxidizing agents can cause unwanted side reactions. To overcome this issue, polymer supported oxidants are used (e.g. Iodobeads or polymer supported chloramine-T). Polymer supported oxidants reduce the concentration of the oxidizing agent in solution, and also enable simple filtration to rapidly remove the oxidant after the reaction.

When radiolabelling with the preformed iodinating agents, it then proceeds via a standard electrophilic aromatic substitution reaction. At its simplest, this can involve iodination of a C-H bond (Fig. 29, Y = H). However, this tends to be restricted to activated arenes and the formation of multiple regioisomers is possible, which can complicate subsequent product purification. A common strategy to address this issue is the use of organometallic precursors (e.g. Y = SnR3, BX2, HgCl, etc.) to direct radioiodination to the desired position. The carbon–metal bond has a lower binding energy than carbon–hydrogen bonds at competing sites, favouring iodo–demetallation rather than direct electrophilic aromatic substitution. This is the method of choice for synthesizing DaTscan (^{123}I-ioflupane), a labelled phenyltropane used for striatal DAT visualization using SPECT imaging. DaTscan is prepared via an iodo-destannylation reaction (Fig. 29).
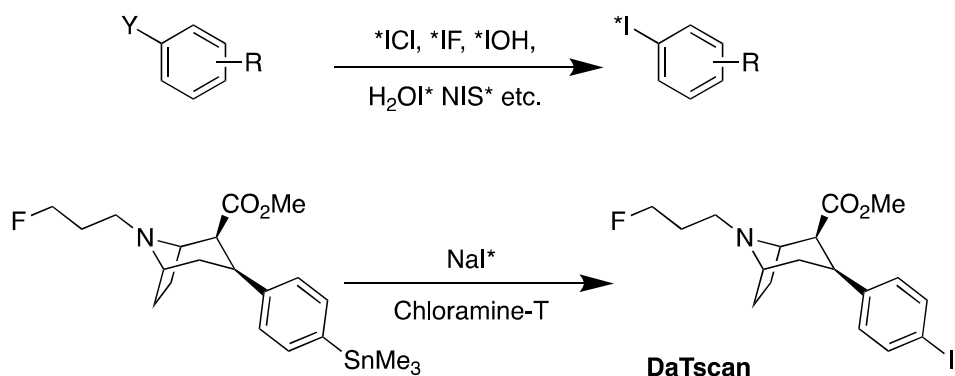
FIG. 29. Electrophilic radioiodination (Courtesy of S. Nag, Karolinska Institute).

4.6. QUALITY CONTROL AND QUALITY ASSURANCE FOR CNS RADIOTRACERS

QC and QA are necessary components of a current cGMP programme, which are of critical importance when developing and translating new CNS radiotracers for use in preclinical PET and SPECT imaging studies. QA and QC are related aspects of quality management. QA is the process by which product quality is ensured at a radiotracer manufacturing facility. QC consists of the methods used to verify the quality of the radiotracers prepared at a manufacturing facility. These concepts have been covered at length in the IAEA TECDOCs [21-22]. As such, an overview is provided here for the sake of brevity.

There are many similarities in cGMP regulations between jurisdictions, but there can also be regional differences that might need to be considered. In general, the manufacture of CNS radiotracers per cGMP will need to involve the control of the following:

(a) Personnel (sufficient numbers with appropriate training);
(b) Quality system (see below);

(c) Facilities and equipment (i.e. which need to be clean and fit for purpose);

(d) Components, containers and closures (control of materials as well as sterile vials used in manufacture of PET and SPECT radiotracers);

(e) Production and process controls (written standard operating procedures and master batch records);

(f) Laboratory controls (QC testing procedures and validation of analytical methods);

(g) Stability testing (stability testing to establish expiration);

(h) Finished tracer controls and acceptance criteria (written specifications).

4.6.1. Quality control

QC testing consists of a series of standard tests intended to establish that a batch of a CNS radiotracer is appropriate for its intended application. These tests confirm identity, purity, sterility and other characteristics that are critical for safe and effective preclinical use of a CNS radiotracer. This section describes special considerations when conducting QC on CNS radiotracers, as well as the full spectrum of QC tests to be conducted on CNS radiotracers made by cGMP, or which are ultimately intended for clinical use. Reduced QC testing might be appropriate for early preclinical studies, or in certain production scenarios (e.g. radiopharmaceutical compounding vs. drug manufacturing). All QC tests are to be passed before a radiotracer is released for clinical use (except for any tests conducted post-release, such as sterility testing).

Historically, QC testing has been conducted using separate pieces of analytical equipment. More recently, there have been efforts to develop next generation analytical equipment to simplify QC testing. These developments range from new chromatography systems (e.g. ultrafast performance liquid chromatography) to completely automated QC devices capable of performing numerous QC tests associated with PET and SPECT radiotracers.

4.6.1.1. Special Considerations for QC of CNS radiotracers

Generally speaking, QC testing of radiotracers is the same regardless of the intended application (e.g. oncology vs CNS imaging). However, there are certain characteristics that need to be carefully considered when manufacturing a CNS radiotracer. In particular, molar activity (A_m) can be particularly important for certain CNS imaging applications (e.g. receptor or transporter imaging), but less significant for others (e.g. metabolic trapping of [^{18}F]FDG). The influence of molar activity on other CNS imaging (e.g. protein aggregates in dementia) has not been widely reported. Generally speaking, high molar activity radiotracers (e.g. >30 GBq/μmol) are to be targeted when possible. High molar activity radiotracers ensure low occupancy of biological targets, therefore avoiding pharmacologic effects (Fig. 30).
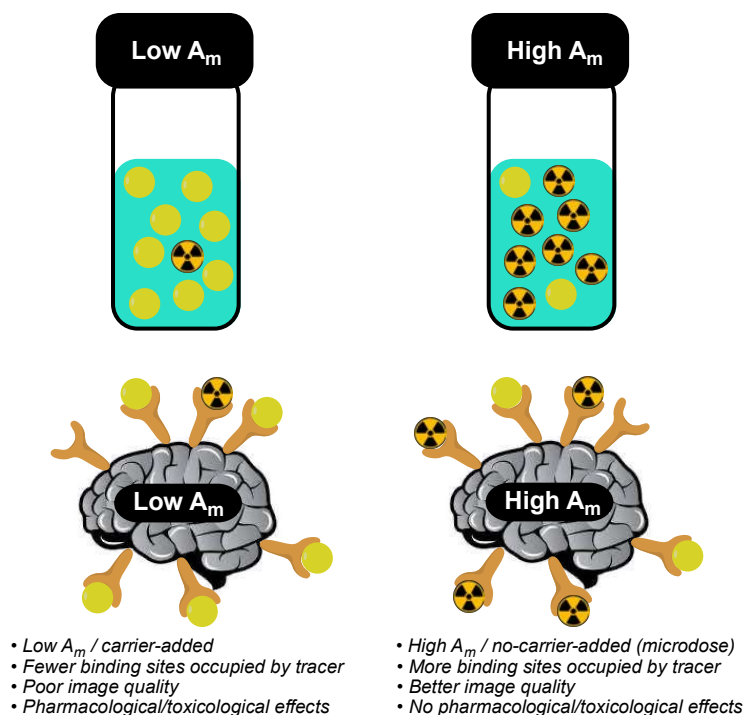
FIG. 30. Impact of Molar Activity on CNS Imaging (Courtesy of P. Scott, University of Michigan).

4.6.1.2. Classical approaches to QC of CNS radiotracers

Final product acceptance criteria, including criteria for release are established for CNS radiotracers and documented in a written procedures The release of the product is performed by a responsible person. Below are the typical QC tests for CNS radiotracers.

Visual inspection

Conduct a visual inspection of the QC sample to ensure the dose is clear, colourless and free of particulate matter.

pH (pH paper)

Analyse the pH of the formulated radiotracer by applying a small amount of the dose to a pH indicator strip and compare it to the scale provided. The dose pH is typically required to be between 4.5 and 7.5.

Residual phase transfer catalyst (^{18}F radiotracers only, thin layer chromatography (TLC)

Determine residual levels of any phase transfer catalysts used to synthesize CNS radiotracers (e.g. tetrabutylammonium (TBA^+) or kryptofix 2.2.2) using the established TLC tests and confirm that they are below the required limits (typically <0.26 mg/mL TBA^+ and < 50μg/mL for kryptofix 2.2.2).

Radiochemical purity (HPLC or TLC)

Analyse the CNS radiotracer by analytical HPLC to determine the identity, and the radiochemical and chemical purity. The radiochemical purity should typically be >90–95% and the chemical purity should confirm tracer mass and that any impurities are below established limits. The identity is confirmed by comparing the retention time of the radiolabelled product with that of the corresponding unlabelled reference standard.

Molar activity (HPLC)

Analytical HPLC data is used to calculate molar activity. Molar activity (A_m) is calculated by using Eq. 1.

$$A_m = A/n \qquad\qquad (1)$$

Where: A is the activity of the radiotracer (expressed in Bq or Ci)
n is the molar amount of non-radioactive compound
A_m is expressed in radioactivity per mol, and needs to exceed the established level for a given radiotracer (e.g. >18.5 TBq/mmol or >500 Ci/mmol).

Residual solvent analysis

Analyse the residual solvents' levels in radiotracer batches using a gas chromatograph system equipped with a flame ionization detector (or equivalent). Limits of residual solvents are usually based upon the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines [46].

Radionuclide identity (half-life)

Confirm radionuclide identity by determining the half-life of the radiotracer and compare it to the known half-life of the radionuclide in question. Measure radioactivity at two time points using a dose calibrator and determine half-life ($T_{1/2}$ = -ln2 (Time Difference /ln (ending activity/starting activity))). The calculated half-life is to be within ± 10%.

Radionuclide purity (gamma spectroscopy)

Determine the radionuclide purity by using gamma spectroscopy. The radionuclide purity is to be >99.5% (i.e. no long-lived radioactive contaminants present).

Sterile filter integrity (bubble point)

Determine integrity of the sterile filter using the bubble point test. The filter from the dose (with the needle still attached) is connected to a nitrogen supply via a regulator. The needle is then submerged in water and the nitrogen pressure is gradually increased. If the pressure can be raised above the filter acceptance pressure (e.g. 50 psi/3.5 bar for 0.9% saline) without seeing a stream of bubbles, the filter is considered intact and the test passes.

Bacterial endotoxins (limulus amebocyte lysate assay)

Determine the bacterial endotoxin content in a batch of radiotracer via the limulus amebocyte lysate assay using an Endosafe PTS (or equivalent) and according to pharmacopeial recommendations. Batches need to contain ≤175 endotoxin units/batch (i.e. ≤17.5 EU/mL for a 10 mL batch).

Sterility testing

Sterility testing is conducted post-release for short lived radiotracers. Within 30 h of end of synthesis, add   1 mL samples of the radiotracer to 20 mL culture tubes of fluid thioglycollate medium (FTM) and Tryptose Soy Broth (TSB) media under aseptic conditions. After adding the samples, the tubes are inverted two or three times to mix and are incubated (along with positive and negative controls) for 14 days. FTM is incubated at 30–35ºC and used to test for anaerobes, aerobes and microaerophiles, while

TSB tubes are incubated at 22°C and used to test for non-fastidious and fastidious microorganisms. The culture tubes are visually inspected on the 3rd, 7th and 14th days of the test period and tubes containing the radiotracer sample are compared to the positive and negative controls. Positive standards need to show growth (turbidity) in the tubes, while tracer samples and negative controls need to show no culture growth after 14 days to be indicative of sterility (Table 6).

TABLE 6. SUMMARY OF STERILITY TESTS AFER PRODUCTION OF A TRACER

Test Article	Growth Media	Sample To Be Added to Tube	Required Result to Be Indicative Of Sterility
Sample 1	FTM	Radiotracer	No growth
Negative Control 1	FTM	Sterile water for injection	No growth
Positive Control 1*	FTM	BioBall® microorganism sample	Growth
Sample 2	TSB	Radiotracer	No growth
Negative Control 2	TSB	Sterile water for injection	No growth
Positive Control 2*	TSB	BioBall® microorganism sample	Growth

Positive controls can be run during sterility testing or in a separate growth promotion experiment.

4.7. NEW APPROACHES TO QC OF CNS RADIOTRACERS

There are ongoing efforts to simplify the time and effort it takes to complete radiotracer QC testing. The first of these is capitalizing on improvements in mainstream analytical equipment such as new developments in chromatography systems. For example, ultrafast performance liquid chromatography operates at higher pressures (up to 15 000 psi) and allows for lower particle sizes in columns (cf. HPLC operating pressures of typically up to 6 000 psi). The higher pressures allow for shorter run times, lower solvent consumption and improved sensitivity and resolution.

A second approach to simplifying QC is the introduction of 'all in one' automated QC systems developed specifically for radiotracers. Historically, the radiotracer QC tests have been conducted using separate pieces of analytical equipment, which can be expensive to purchase and maintain. To simplify QC, minimize waste and reduce lab space requirements, there has been a push for the development of miniaturized 'all in one' QC systems. The positives of such systems include simplified QC testing (e.g. staff need to be trained on one system instead of many), while drawbacks include the limited number of common radiotracers for which consumables have been developed to date. This is acceptable for labs only making common radiotracers (e.g. [18F]FDG), but could mean additional analytical equipment is required to test experimental CNS radiotracers for which no QC consumable has been developed yet.

5. IN VITRO TESTS TO EVALUATE CNS TRACERS FOR PET AND SPECT

Nuclear molecular imaging techniques, like PET need radiolabelled tracers to study in vivo processes. The tracer uptake reflects pathophysiological or physiological processes or drug transport, dynamics and kinetics [47]. However, adequate selection criteria as discussed in Section 3, are needed to select the most appropriate tracer to support in vivo imaging studies. In general, to select the ideal tracer for each application its properties need to be compared with those of an ideal golden standard tracer. Amongst other criteria applicable for tracers and described in [23], an ideal CNS tracer fulfils the following criteria:

a) Its rate of metabolism is low and labelled metabolites do not cross the BBB;
b) Its lipophilicity is high enough for a sufficiently high first pass extraction, yet it is low enough to guarantee low non-specific binding;

c) It has a low affinity to efflux transporters like p glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP).

In this section, the rationale behind and the impact of these criteria for the kinetics of the tracers are described. Besides this, several in vitro assay protocols are described on how to perform these tests in practice. Finally, go/no-go criteria to move further to in vivo is discussed.

5.1. SHELF LIFE, STABILITY AND METABOLISM OF A TRACER

The shelf life of a tracer is the duration of storage time that is allowed without it becoming unfit for its application. The storage time, the matrix and the temperature can influence the quality and the stability of the tracer. Therefore, it is required to test the influence of these parameters and define the shelf life of a tracer. For instance, a test to determine the radiochemical purity should check on the sterility of the end product, and also the stickiness of the tracer to the rubber stopper of the vial or the syringe.

It is evident that the in vivo stability of the tracer and the formed radioactive metabolites can influence the quantification of PET data because they can disturb the specific target signal and create noise. The formation of radioactive metabolites needs to be included in a mathematical model that describes the kinetics of the tracer in a correct way.

Tracers for CNS applications are classified as lipophilic and are therefore, in general, metabolized in the liver via cytochrome P450 enzymes [48]. In general, two metabolic routes can be distinguished, as follows:

(a) Phase I Metabolism: Via oxidation (via cytochrome P450), reduction, and hydrolysis reactions: Phase I reactions convert a lipophilic parent compound to more polar (water soluble) active metabolites by unmasking or inserting a polar functional group (-OH, -SH, -NH2). Drugs metabolized via phase I reactions have longer biological half-lives. This metabolic route mainly takes place in the liver.
(b) Phase II Metabolism: Glucuronidation, acetylation, and sulfation reactions: these conjugation reactions increase water solubility of the drug with a polar moiety glucuronate, acetate, and sulfate, respectively. Phase II reactions convert a parent drug to more polar (water soluble) inactive metabolites by the conjugation of subgroups to -OH, -SH, -NH$_2$ functional groups on the drug. Drugs metabolized via phase II reactions are renally excreted.

The formation of radioactive metabolites can be measured in plasma. Which radioactive metabolites are formed depend on the position of the radionuclide in the molecule. Preferably, the tracer is designed in such a way that the formed radioactive metabolites do not interact with the target molecule, have a fast clearance from plasma, and do not pass the BBB. To investigate the presence and the impact of labelled metabolites of a tracer in plasma and brain tissue, it is preferable to use a dedicated analytical method, for instance HPLC combined with radioactivity detectors [49].

A classic example that reflects the impact of the position of the radionuclide ^{11}C in the molecule is clearly demonstrated in studies using the tracer [^{11}C]WAY100635 (Fig. 31). The tracer [O-methyl-^{11}C]WAY100635 for imaging 5HT1A receptor was designed in the 1990s and evaluated using PET in the investigation of psychiatric disorders [50]. Unfortunately, fast plasma metabolism of this tracer, labelled with ^{11}C at the methoxy group, was observed and the formed radioactive metabolites were also able to penetrate the BBB. It is evident that this phenomenon complicates the quantification of the PET data. To tackle this problem, the radiosynthesis to produce [^{11}C]WAY100635 was redesigned and labelled at the carbonyl position. This new strategy reduced the formation of labelled radioactive metabolites and made the tracer more suitable for quantitative PET studies in patients with psychiatric disorders [51].
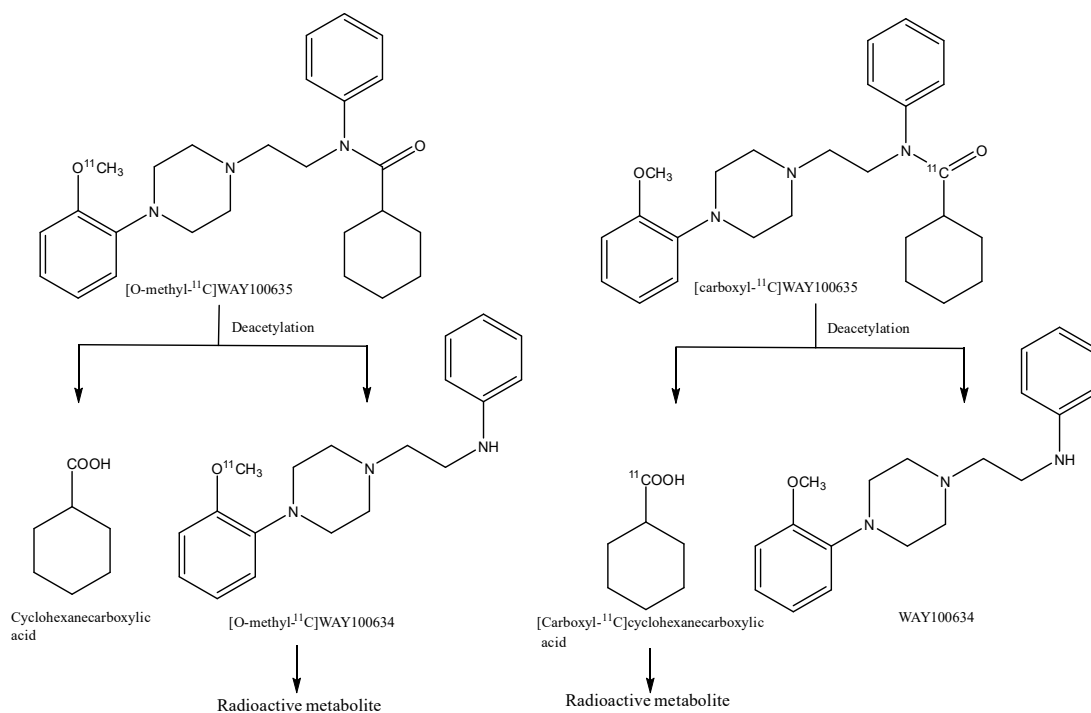
FIG. 31. Pathway deduced for the metabolism of [O-methyl-^{11}C]WAY100635 and [carboxyl-^{11}C]WAY100635 in cynomolgus monkeys and humans (Courtesy of P. Elsinga, University Medical Center Groningen).

More recent work is reported by Raaphorst al [52]. The researchers designed verapamil analogues with the [^{18}F]fluoroethyl moiety on different positions in the molecule. The tracers (R)-N-[^{18}F] fluoroethylverapamil ([^{18}F]1) and (R)-O-[^{18}F] fluoroethylnorverapamil ([^{18}F]2) were defined both as substrate for P-glycoprotein (P-gp). However, both the kinetics and the plasma metabolism were different as shown in Table 7 and Figs 32–33.
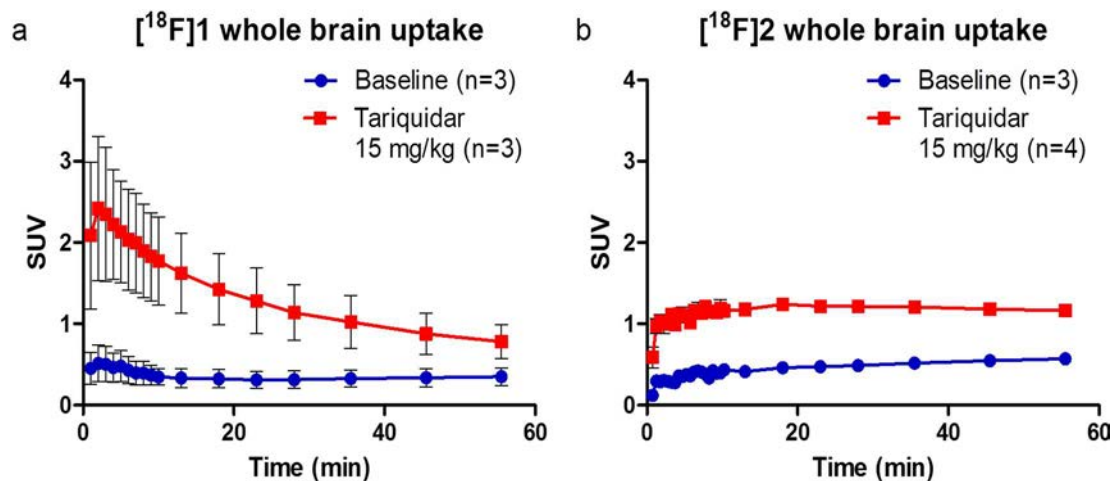
FIG. 32. Whole brain time–activity curves of [^{18}F]1 and [^{18}F]2 in Wistar rats at baseline and after treatment with tariquidar (15 mg/kg) (Reproduced from Ref. [52] with permission courtesy of [ACS Chem Neurosci]).

38
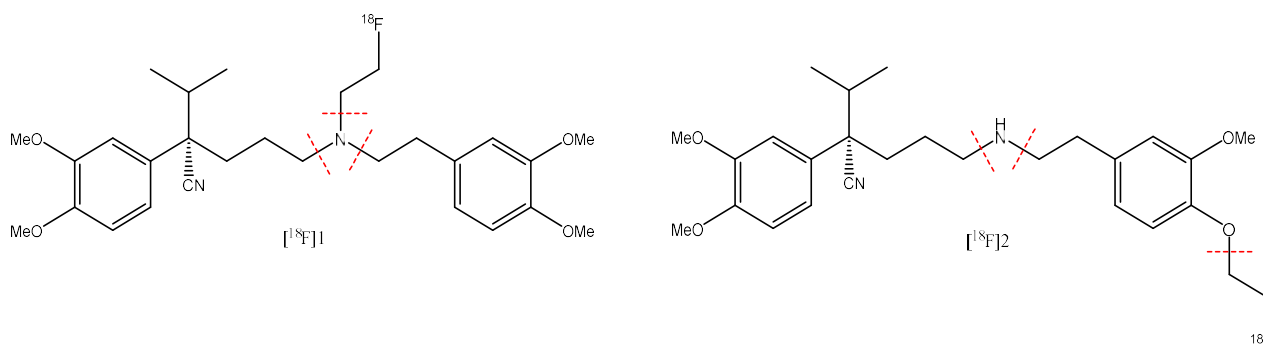
FIG. 33. Structures of ¹⁸F-fluoroethyl verapamil derivatives, their kinetics (with permission reproduced form [52] with permission courtesy of [ACS Chem Neurosci]).

TABLE 7. EXAMPLE OF DIFFERENT METABOLISM FOR TWO DIFFERENT ^{18}F-VERAPAMIL ANALOGS (Reproduced with permission from [51] with permission courtesy of [ACS Chem Neurosci])

	Min	[^{18}F]1		[^{18}F]2	
		Plasma	Brain	Plasma	Brain
Parent Tracer	5	46 ± 14%	41 ± 10%	20 ± 3%	26 ± 6%
	15	19 ± 2%	14 ± 2%	8 ± 3%	17± 7%
	60	3 ± 1%	2 ± 0.3%	4 ± 1%	6 ± 1%
Non-polar metabolites	5	5 ± 2%		5 ± 3%	
	15	9 ± 3%		5 ± 1%	
	60	5 ± 1%		3 ± 1%	
Polar Metabolites	5	49 ± 11%		75 ± 3%	
	15	71 ± 2%		87 ± 1%	
	60	92 ± 1%		93 ± 2%	
Brain Metabolites	5		59 ± 10%		74 ± 6%
	15		86 ± 2%		83 ± 7%
	60		98 ± 0.3%		94 ± 1%

From the practical examples given above, it is evident that the choice and position of the radionuclide in the molecule is important. This information is needed before setting up the labelling procedure of a tracer to perform molecular PET studies. To obtain this information, in vitro testing of stability of the tracer and the formation of metabolites is required.

A practical example is given using the compound 2-[2-(2-fluoroethyl-(18F)-5-methoxyphenyl) oxazol-4-ylmethyl]-6,7-dimethoxy-1,2,3,4,-tetrahydroisoquinoline ([^{18}F-7] incubated in hepatocytes (Fig. 34). The metabolism pathway and the potential formed metabolites were identified using Liquid Chromatography Mass Spectrometry (LC-MS). This information supports the decision for selection of the most appropriate labelling position within a molecule [53].
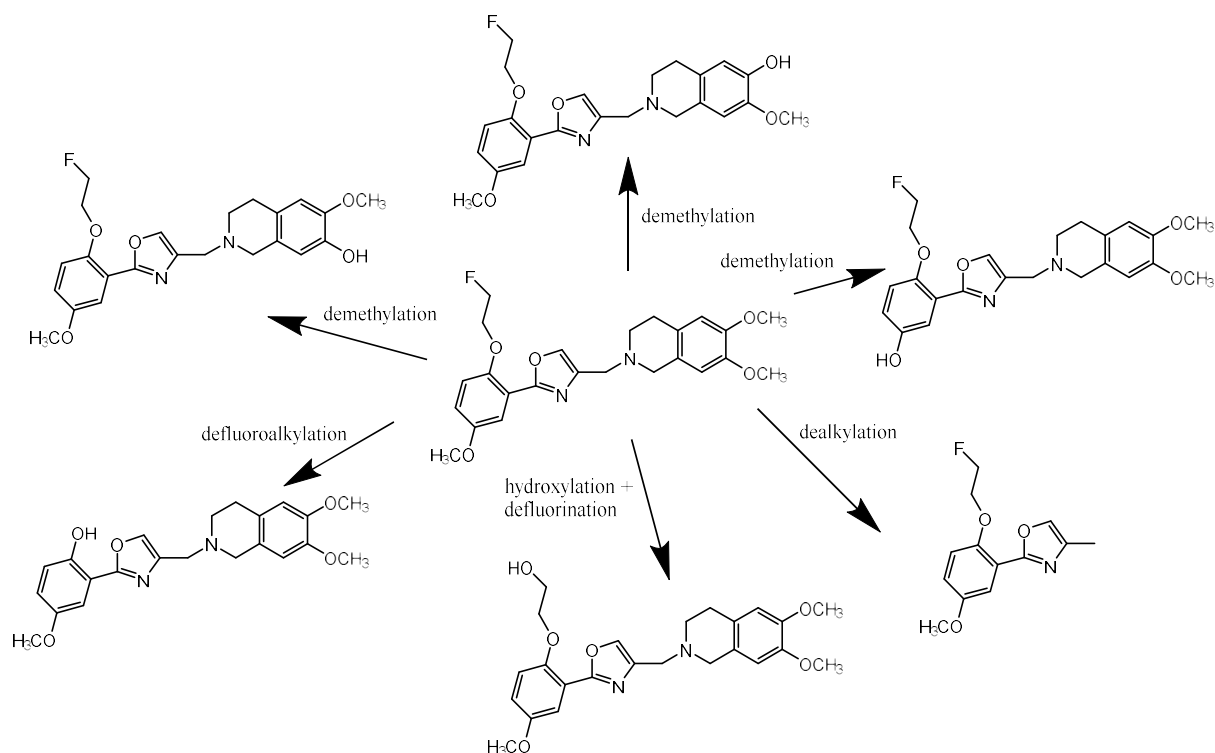
FIG. 34. Different metabolic routes identified for ^{18}F labelled compound 7 using liquid chromatography–mass spectrometry/MS (Courtesy of P. Elsinga, University Medical Center Groningen).

5.2. LIPOPHILICITY

Lipophilicity of a CNS tracer is a critical parameter because it determines the ability to pass the BBB, and thus the entrance into brain tissue. Also, it affects the plasma free fraction and its level of nonspecific binding. The lipophilicity of tracers needs to be selected in such a way that it can easily cross the BBB via passive diffusion and the level of nonspecific binding in tissue is relatively low (i.e. protein binding).

In a case of high lipophilicity, it could lead to high binding for plasma proteins, resulting in a small free fraction of CNS tracer in plasma. Only this small free fraction in plasma crosses the BBB, resulting in low brain uptake of the tracer. In general, the ideal log P for tracers used for brain imaging is in the range 1.5–4. In this case, the tracer has adequate brain penetration, acceptable plasma binding and an optimum target to non-target ratio. The impact of lipophilicity for tracers to cross the BBB is clearly described by Pike et al. [54].

If tracers do not fulfil this criterion, it is possible to redesign the molecule into a more polar molecule. This could be achieved by chemical modification e.g. using hydroxylation reactions. It should be noted that the modified molecule may behave differently in vivo than the original molecule and therefore the re-designed tracer needs to be checked for its kinetics, affinity, and specificity to the target.

5.2.1. In vitro testing of lipophilicity

The most common in vitro assay test for determination of the lipophilicity of compounds is the traditional 1-octanol/water or Phosphate Buffered Saline (PBS) shake flask technique [55]. The most practical execution of this test is using a radioactive label. e.g. 3H or 14C or using gamma and positron emitters. Log D reflects the lipophilicity of a tracer under physiological conditions. The procedure is identical compared with Log P but with the difference that instead of water, PBS buffer pH7.2–7.4 is used [56]. A protocol for determining the logP or logD is described in the Section 5.4.1.

5.2.2. In vitro test of the free fraction or plasma binding in plasma

There are several methods available to determinate the free fraction of the tracer in plasma. Besides predicting the free fraction using software [57], the most common method is via ultrafiltration [58]. Because of the short half-life of the tracer, the execution of the test has to be fast. A commonly used protocol to determine the free fraction of the tracer in plasma is described in Section 5.4.

5.3. AFFINITY FOR BBB EFFLUX TRANSPORTERS

The BBB is a dynamic barrier that limits the transport of molecules from plasma to tissue. The barrier contains influx and efflux transporters and regulates the transport of biological or drug transport across the BBB. There are several categories of influx and efflux transporters that are mainly ATP driven. The brain uptake of the CNS tracer will be reduced when the tracer has affinity for the efflux transporter, for instance with the P-glycoprotein transporter (P-gp). The affinity for P-gp is difficult to predict according to the molecular structure and a whole variety of different molecules could have affinity for P-gp [59].

Because of the impact of this phenomenon, it is normal to screen candidate tracers for substrate affinity, in particular P-gp, before further development. Several methods using cell lines are available for in vitro testing to predict membrane permeability, oral absorption of the drug and interaction with the efflux transporter P-gp.

5.3.1. In vitro testing on P-gp mediated transport of a tracers

Most tracers used for brain imaging interact with efflux transporters at the BBB. Among others, P-glycoprotein (P-gp or multi-drug resistance 1 (MDR1) protein), BCRP and organic anion transporting polypeptide 1B1/1B3 (OATP1B1/OATP1B3), are important active transporters located in the BBB. The most important and best characterized transporter is P-gp. An assay with trans well plates coated with cells overexpressing human P-gp is one of the most common procedures. Most suitable cell lines that can be used are Madin-Darby canine kidney (MDCKII) and porcine renal epithelial cells (LLC-PK1) transfected with the human MDR1-gene. Human epithelial colon adenocarcinoma (Caco-2) cells are also often used [60].

To measure transporter mediated cell interaction, the concentration in the apical (A) and basolateral (B) direction is measured. The efflux ratio can be calculated as apparent permeability from B to A and divided by the apparent permeability from A to B (Fig. 35).
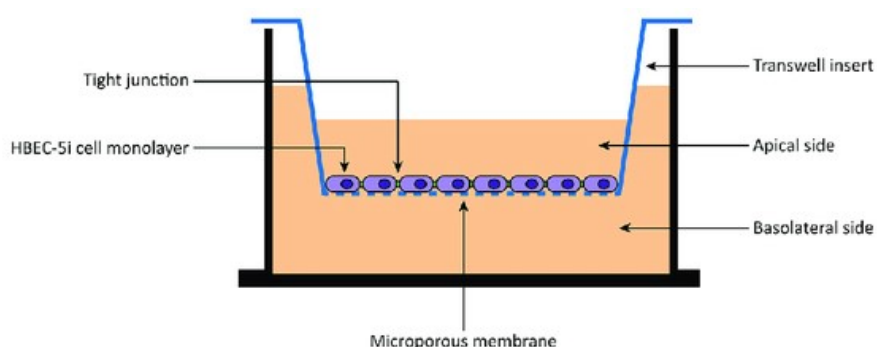
FIG. 35. Schematic representation of measurement of transporter-mediated cell interaction to assess affinity for P-gp is BBB (Reproduced from Ref. [61] with permission courtesy of [Front Bioeng Biotechnol]).

To investigate the transporter interaction of a new compound or tracer, studies are needed to determine the efflux ratio of known P-gp or BCRP substrate in Caco-2, P-gp- or BCRP-overexpressed cells and determine the drug's inhibition potency expressed in ER, IC50 or Ki. After the assay is 'calibrated' with known compounds, new tracers can be tested in the assay to indicate whether a new CNS tracer is a P-gp substrate or inhibitor. Several cut off values can be used being expressed as IC50, Ki or ER to classify substrates and inhibitors. Depending on the used assay a specific parameter can be used.

5.4. GO/NO-GO CRITERIA TO MOVE FURTHER TO IN VIVO

It is evident that an ideal tracer for CNS application has a low rate of metabolism and the labelled metabolites that could be formed do not cross the BBB. Besides this, its lipophilicity should be high enough for a sufficiently high first pass extraction and low enough to guarantee low non-specific binding, so a log P range of 1.5–4 is recommended. Moreover, the tracer should have low affinity to efflux transporters like P-gp and BCRP. In this section, the rationale behind these criteria is clarified. The in vitro assays as described are aimed to study these parameters. Although these in vitro assays will help in screening of molecules to select a potential CNS tracer, and in making decisions to move further to the next step in vivo, it is necessary to be cautious that these tests do not reflect the in vivo situation.

It is particularly important how these in vitro assays are performed and at which concentration level. For example, tracers for molecular imaging are produced with high molar activity. This means, when a tracer is dosed at 200 MBq, less than 10 ug mass will be administered. Therefore, for in vitro assays to screen the behaviour, the procedure needs be performed at the same low tracer level concentrations. Also, the use of 3-D cell systems will reflect a more precise in vivo situation. Furthermore, new developments in screening assays using 'organ on chip' systems will reflect a better in vivo situation and support in making the right decisions for selection of the appropriate compounds to go in vivo.

5.4.1. Protocols for in vitro testing

Three different in vitro tests are described below.

5.4.1.1. In vitro testing of the stability of the tracer using microsomes

To investigate the stability and the metabolic pattern of a molecule to select the most appropriate labelling position, in vitro testing using hepatocytes in combination with LC-MS, can be performed according to the following protocol:

Phase I metabolite protocol

Per incubation reagents:

  (a) 2 μl test compound (4 mM);
  (b) 368 μl PBS;
  (c) 10 μl 20 mg/ml microsomes;
  (d) 20 μl 20 mM nicotinamide adenine dinucleotide phosphate (NADPH) (made up in PBS);
  (e) 800 μl ACN, 0.1% formic acid to stop the reaction.

Steps:

  (a) Set water bath/incubator at 37°C;
  (b) Label all tubes ready for experiment;
  (c) Prepare test compound in (ideally) PBS or DMSO;
  (d) Remove microsomes from freezer;
  (e) Add 2 μl of 4 mM test compound to tube;
  (f) Add 368 μl PBS to each tube;
  (g) Add 10 μl microsomes to each tube;
  (h) Pre-incubate at 37°C for 5 min whilst gently shaking (leave lids open);
  (i) Prepare 20 mM NADPH in PBS;
  (j) After 5 min incubation, add 20 μl of 20 mM NADPH to each tube except to the control;
  (k) Add 20 μl PBS to control tube;
  (l) Incubate for up to 120 min at 37°C with lids open;
  (m) After incubation (or at timepoints throughout incubation) stop the reaction by adding 800 μl

ACN, 0.1% formic acid to each tube;

(n) Vortex and transfer to Eppendorf (if incubation was not performed in Eppendorfs) for centrifugation (5 minutes, 3 000 rpm);

(o) Transfer supernatant to a vial for LC-MS analysis (dilute 1 in 10 with aqueous).

Phase II metabolite protocol

Per incubation reagents:

(a) 2 µL test compound (4 mM);
(b) 286 µL PBS;
(c) 10 µL 20 mg/mL microsomes;
(d) 40 µL 50 mM GSH (γ-l-glutamyl-l-cysteinyl-glycine/glutathione);
(e) 40 µL 50 mM UDPGA (uridine diphosphate glucuronic acid);
(f) 2 µL 2.5 mM (in MeOH) alamethicin;
(g) 20 µL 20 mM NADPH (made up in PBS);
(h) 800 µL ACN, 0.1% formic acid to halt the reaction.

Steps:

(a) Set water bath/incubator at 37°C;
(b) Label all tubes ready for experiment;
(c) Prepare test compound in (ideally) PBS or DMSO;
(d) Remove microsomes from freezer;
(e) Add 2 µL test compound to tube;
(f) Add 286 µL PBS to each tube;
(g) Add 40µL 50 mM GSH and 2 µL 2.5 mM alamethicin to each tube;
(h) Add 10 µL microsomes to each tube;
(i) Pre-incubate at 37°C for 5 min whilst gently shaking (leave lids open);
(j) Prepare 20 mM NADPH in PBS and 50 mM UDPGA in PBS;
(k) After 5 min incubation add 40 µL 50 mM UDPGA and 20 µL of 20 mM NADPH to each tube except to the control;
(l) Add 40 µL 50 mM UDPGA and 20µL PBS to control tube;
(m) Incubate for up to 120 min at 37°C with lids open;
(n) After incubation (or at timepoints throughout incubation) stop the reaction by adding 800 µL ACN, 0.1% formic acid to each tube;
(o) Vortex and transfer to Eppendorf (if incubation was not performed in Eppendorf) for centrifugation (5 min @ 3000 rpm);
(p) Transfer supernatant to a vial for LC-MS analysis (dilute 1 in 10 with aqueous).

Determination of logP or logD

(a) Pipette n-Octanol (0.5 mL) and PBS, pH = 7.2, 0.5 mL in 1:1 ratio into Eppendorf tubes;
(b) Add the radiopharmaceutical solution (~1 MBq, 100 µL);
(c) Vortex tubes vortexed for 1 min and centrifuge for 5 mins at 6 000 rpm;
(d) Collect samples (100 µL) of octanol and PBS layer and count on a γ counter;
(e) Calculate the distribution coefficient Log D as log ($A_{octanol}/A_{PBS}$). Measurements are in triplicate.

5.4.1.2. In vitro plasma binding protocol

Steps:

(a) Take blood samples (0.4ml) at 10 min, 30 min and 60 min during the PET scan;
(b) Centrifuge the whole blood samples for 5 min (3700 rpm), to separate the plasma from the red cells;
(c) Add 0.2 ml plasma with a pipette on the ultrafilter;
(d) Centrifuge the sample at 2000 g for 30 min;
(e) Perform the procedure in duplicate;
(f) Measure the supernatant (clear ultrafiltrate) and the filter in the gamma counter.

5.4.1.3. Study Protocol to measure bidirectional transport- P-gp interaction

A protocol using [3H]labelled novel tracers is described by Raaphorst [62]:

(a)   Prepare a stock solution of digoxin (0.1 mM) in DMSO;
(b)   Mix [3H]digoxin with a test new non-radiolabelled compound producing a final concentration of 0.05 μM digoxin in Hanks' Balanced Salt Solution (HBSS)/ (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in the transwells, and a radioactivity concentration of 10 kBq/mL;
(c)   Prepare stock solutions of test compounds in DMSO with different concentrations (0.01 mM, 1 mM and 100 mM);
(d)   Mix with the tritium labelled tracers and diluted with HBSS/HEPES to final concentrations of 0.01 mM, 1 mM and 50 μM, with radioactive tracer concentration of 10 kBq/mL in the transwells;
(e)   After the integrity assessment, remove the culture medium from the filter inserts (apical compartment (A));
(f)   Wash the inserts once with warm (37°C) HBSS, transfer to new 12 well plates that were preincubated with HBSS/HEPES for 18 h at 37°C, to reduce non-specific binding to the plastic, and then wash with warm HBSS;
(g)   The MDR1-mediated apical to basolateral (A → B) transport in MDCKII-MDR1 cell monolayers is determined by addition of 0.65 mL of a tritiated compound in concentrations of 0.01, 1 and 50 μM to the apical compartment in triplicate;
(h)   Add 1.8 mL of HBSS/HEPES to the basolateral compartment;
(i)   To determine basolateral to apical (B → A) transport, add 1.95mL of a tritiated compound at concentrations of 0.01, 1 and 50 μM to the basolateral compartment and 0.5 ml of HBSS/HEPES to the apical compartment;
(j)   A 150 μL aliquot of the apical (A → B) or basolateral (B → A) compartment is taken at the start of the experiment to measure the initial concentration and to calculate the recovery of radioactivity;
(k)   Incubations are performed on a rocker platform (rotation approximately 60 rpm) at 37°C for 2 h in a humidified incubator containing approximately 95% air/5% CO_2;
(l)   Mix aliquots of the apical and basolateral samples (150 and 1600 μL for A → B and 400 and 150 μL for B → A, respectively) and samples of the initial concentration with 10 mL of liquid scintillant.

6.   PRECLINICAL IN VIVO TESTING

Molecular imaging can be used to study a drug's in vivo behaviour, i.e. to determine its pharmacokinetic or pharmacodynamic behaviour. Preclinical molecular imaging is usually used to acquire surrogate information in animals which can be used to predict the drug´s behaviour in humans [63, 64]. Preclinical data are required to translate radiopharmaceuticals into the clinic by the U.S. Food and Drug Administration or the European Medicines Agency. Within drug development, preclinical imaging is

often used to test parameters that cannot accurately be predicted from in vitro studies [65, 66]. The non-displaceable binding component, metabolism and the BBB permeability of a drug are, for example, parameters which are still difficult to predict [67]. Successful tracers can be further used to study CNS diseases and brain function in vivo. The role of different receptor systems in the behaviour or brain diseases, and their interaction with each other, can as such be visualized, quantified, and correlated. Intervention studies combined with molecular imaging allow to investigate the influence of a drug or a task on brain physiology on a molecular level. This leads to the possibility of connecting molecular pathways with behaviour, function, or disease mechanisms. Finally, preclinical testing is important to monitor treatment progress of radiopharmaceuticals and determine efficacy before translating the treatment into the clinic [66]. For example, molecular probes can be used to determine the efficacy to clear amyloid beta from the brain or to monitor radiation therapy progress.

6.1. ANIMAL SPECIES AND DISEASE MODELS

Preclinical experiments are usually carried out in rodents (mice and rats) due to general availability of Genetically Engineered (GE) animals and disease models, ethical considerations, expenses and throughput aspects [68]. However, preclinical testing is also carried out in larger animals such as pigs, dogs or Non-Human Primates (NHPs) depending on the specific research question raised and the necessity to use animals that represent human physiology as closely as possible [69, 70]. GE animals can recapitulate the pathology of a certain human disease, or simply comprise convenient model organisms to assess the specificity of the candidate tracer binding [71]. Knockout (KO) animals, in whom a certain gene has been artificially deactivated, are especially valuable when it is necessary to confirm that a certain radiopharmaceutical does not specifically bind to any other targets except the one that is knocked out [72]. In general, GE models, including KO strains, are developed in mice because genetic manipulations are more established in this species as compared to rats [73]. However, genetic toolboxes exist for rats and larger species as well [74]. Apart from genetic manipulations, disease models are generated by the selective breeding of animals for certain traits (e.g. spontaneous hypertension), cell inoculations (tumours), pharmacological interventions (e.g. exposure to toxins or hormones), surgery (e.g. artery occlusion) or interventions into the animal's environment (e.g. social defeat or chronic mild stress). It is important that target expression should ideally be in the same range as it would be expected in human disease. Genetic models may induce target expression that is unrealistically high. Table 8 displays most used animal models for a number of relevant pathological states.

TABLE 8. SELECTION OF AVAILABLE ANIMAL MODELS OF CNS DISEASES (Reproduced from Ref. [75] with permission courtesy of [Biochem Pharmacol])

Disease	Disease Model Examples	Species	Pathological Hallmarks	Imaging Examples
AD	GE (transgenic Tg2576, TgArcSwe, 5XFAD mice, TgF344-AD, McGill-R-Thy1-APP rats) Pharmacological (scopolamine, IC beta-amyloid) Surgery (fimbria fornix transection)	Rats, mice, pigs, sheep	Progressive memory loss Extracellular accumulation of beta-amyloid and tau fibrils	Beta-amyloid and tau imaging in transgenic AD rats
PD	GE (transgenic LRRK2^{R1441G} mice, IC lentiviral vectors in rats and monkeys) Pharmacological (6-OHDA, MPTP, IC alpha-synuclein, IC 6-OHDA)	Rats, mice, monkeys	Gait abnormalities, Impaired balance, Degeneration of dopaminergic neurons in substantia nigra, Accumulation of alpha-synuclein fibrils in neurons	Alpha-synuclein imaging in alpha-synuclein injected rats

TABLE 8. SELECTION OF AVAILABLE ANIMAL MODELS OF CNS DISEASES (cont.)

Disease	Disease Model Examples	Species	Pathological Hallmarks	Imaging Examples
Stroke	Surgery (middle cerebral artery occlusion (MCAo)) Pharmacological (IC endothelin-I) Breeding (spontaneously hypertensive rats (SHR))	Rats, mice, rabbits, dogs (rarely)	Penumbral zone formation Tissue hypoxia	Tissue hypoxia imaging in SHR with MCAo
Brain tumours	Human tumour xenografts in immunodeficient rodents (U-87 MG line in athymic rats) Syngeneic tumours in rodents (GS9L line in F344 rats) Spontaneous tumours in GE mice (Nf1/p53 KO mice) Spontaneous tumours in dogs	Rats, mice, dogs	Tumours in the brain	Intracranial tumour imaging with an X_C- tracer
Huntington's disease	Pharmacological (IC quinolinic acid) GE (R6/1 mice, BACHD mice/rats, IC lentiviral vectors in monkeys)	rats, mice, monkeys	Gait abnormalities, impaired balance Expression of mutated HTT in the brain	$D_{2/3}R$ imaging in BACHD rats
Amyotrophic lateral sclerosis	Pharmacological (bisphenol A, BSSG*) GE (TDP43, SOD1 transgenic mice	Mice, zebrafish	Motor neuron loss, paralysis Cytoplasmic aggregation of TDP43 protein	mGluR5 imaging in SOD1 transgenic mice
Depression	Environment (chronic mild stress, etc.) Breeding (congenital learned helplessness line etc.) GE (5-HTR / SERT KO rodents)	Rats, mice	Increased immobility in forced swim test, lower preference for sweet foods	DAT imaging in CB1R KO mice
Anxiety	Environment (foot shocks etc.) GE (5-HTR KO, GABA system mutants)	Rats, mice, guinea pigs	Increased aggression Increased avoidance behaviour	TSPO imaging in rats after chronic mild stress
Schizophrenia	Pharmacological (MAM, PCP, amphetamine) GE (neuregulin1, ErbB4 KO)	Rats, mice, monkeys	Reduced prefrontal cortex / hippocampus size, hypersensitivity to amphetamine, deficits in cognitive tests	DAT and $D_{2/3}R$ imaging in PCP-treated monkeys
Pain	Surgery (chronic constriction injury, spinal nerve ligation) Pharmacological (formalin, capsaicin, carrageenan)	Rats, mice, dogs	Vocalizations, licking, avoidance of limb use	MOR imaging in rats with spared nerve injury

Abbreviations: BSSG - β-sitosterol-β-d-glucoside, GABA - γ-aminobutyric acid, 5-HTR – serotonin receptors, DAT, $D_{2/3}R$ – dopamine D2/3 receptors, ErbB4 – neuregulin receptor, HTT – huntingtin, IC – intracerebral administration, MAM -

methylazoxymethanol, mGluR5 – metabotropic type 5 glutamate receptor, MOR - μ-opioid receptor, MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, Nf1 – neurofibromin, 6-OHDA – 6-hydroxydopamine, p53 – phosphoprotein p53, PCP – phencyclidine, SERT – SERT, SOD1 – superoxide dismutase 1, X_{C-} - sodium-independent cystine-glutamate exchange transport system. * BSSG in rats leads to PD-like rather than ALS-like symptoms

It should be noted that not all CNS diseases and disease models have their pathological hallmarks well characterized on a molecular level. Therefore, novel tracers for targets which represent specific biomarkers for such diseases can be directly evaluated in the respective disease models [76]. However, not all models have a good face validity: for example, transgenic mouse models of PD which overexpress α synuclein in the brain do not prominently or consistently exhibit neurodegeneration, which is why rat models of PD are much more popular. For disorders like depression or schizophrenia, concrete neurochemical alterations underlying them are still being elucidated. Therefore, models of these disorders are rarely used for the evaluation of novel tracers. Instead, previously validated tracers are used to assess the face validity of those models.

While selecting the appropriate animal model, it is important to remember that species differences can influence the outcome of the experiment. Therefore, it is often advisable to study the radiopharmaceutical in two animal models before translating it into the clinic, at least for imaging or safety evaluation purposes. One prominent example of species differences within CNS tracer development is the increased brain expression of the efflux transporter, P-glycoprotein (P-gp), both in absolute terms and relative to other drug efflux transporters in rodents. If radiopharmaceuticals are evaluated in rodents and show low brain uptake, they could be rejected for further translation, even though they could have worked in higher species, including humans [77]. Therefore, it may be beneficial to test for the in vivo P-gp dependency of a radiopharmaceutical before rejecting it. This is possible, for example, by blocking P-gp with elacridar or cyclosporin-A. Differences in metabolic rate and even metabolic pathway are also well known. In general, the metabolic rate decreases in larger animals [78] Metabolic pathway differences, are for example, described for [^{11}C]WAY100635, a PET ligand used to image 5-HT$_{1A}$ receptors [79]. In rats, the tracer is metabolized by demethylation of the ^{11}CH$_3$-moiety, whereas in humans the amide bond is primarily hydrolyzed (Fig. 36). This different metabolic pathway resulted in a substantially lower ratio between receptor rich and receptor poor regions in humans compared to rats as the formed metabolite was able to cross the BBB and additionally showed binding to α$_1$-adrenergic receptors in the human brain.
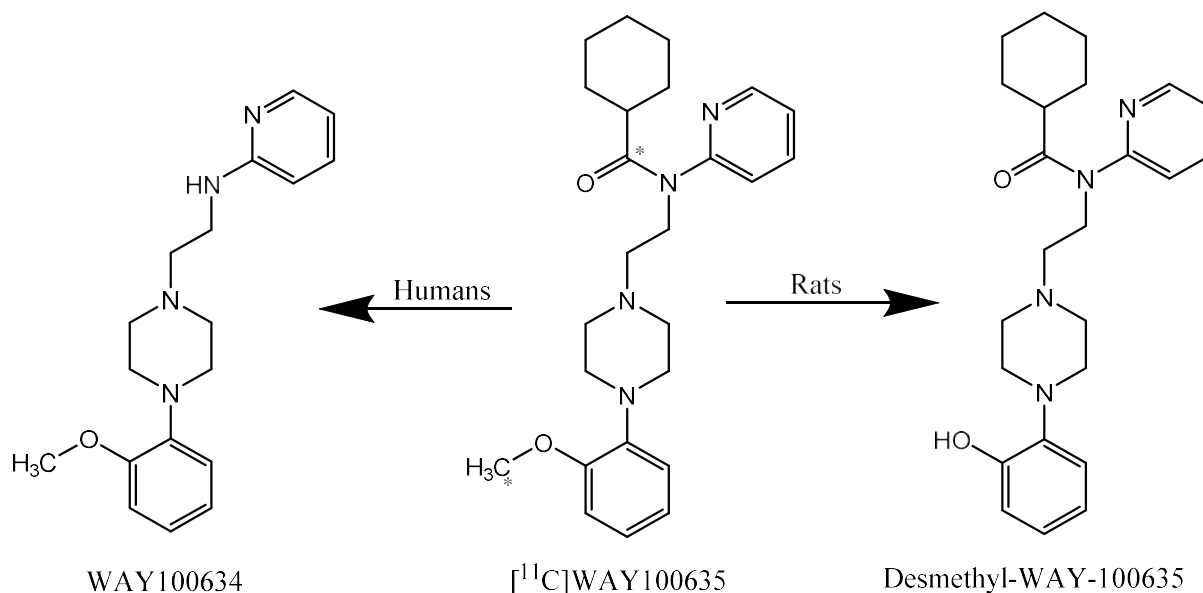
FIG. 36. The different metabolic pathways of WAY100635 in humans and rats (Courtesy of P. Elsinga, University Medical Center Groningen).

6.2. THE EFFECT OF DIFFERENT ANAESTHETICS

In contrast to human PET/SPECT scanning, anaesthesia is usually required for preclinical imaging as it is essential that the animal does not move during the scan. Surgery and invasive injections or blood sampling also need anaesthesia to minimize pain and guarantee the highest animal comfort. Anaesthetics are drugs that can be used for this purpose. They can be divided into two groups according to their function.

Local anaesthetics anesthetize a specific body region, but do not anesthetize the whole animal. Local anaesthetics can be administered via the skin, subcutaneous, intrathecal, or into the epidural space. They function as a reversible blockage of the nervous conduction. Local anaesthetics are usually not applied for imaging studies but find application in minor surgical operations.

General anaesthetics are used to anesthetise the whole animal. They are usually gases or volatile liquids (inhalant anaesthetics) which will be taken up by the animal while breathing. However, there are also general anaesthetics that have the form of non-volatile liquids or solids. These anaesthetics can usually be purchased preformulated and are injected intravenously, intramuscularly, or subcutaneously. They can be used as stand-alone treatments or combined with inhalation procedures of gases to generate full unconsciousness and have a better control over anaesthesia. General anaesthesia is used both for imaging studies and surgery. The depth of the anaesthesia can be varied depending on the specific need. Sometimes, support of breathing via intubation is needed when deep anaesthesia is used.

Commonly applied inhalant anaesthetics are halothane, isoflurane, sevoflurane or desflurane. Inhaled anaesthetics affect the CNS on multiple molecular targets. The modern lipid hypothesis is currently used to explain anaesthetic effects [80]. In short, anaesthetics change the membrane lateral pressure profile, which change the conformation of membrane ion channels and lead to the effect. For example, chloride channels (GABA-A receptors) and potassium channels are affected, and their enhanced activation by endogenous ligands in turn depresses other neurotransmission pathways, such as the serotonergic, glutamate or cholinergic pathway. Usually, inhalant anaesthesia is favourable due to rapid sedation and recovery; however, it requires specialized equipment for administration, and it might lead to a decrease of cerebral blood flow, and it is not well tolerated by all species. Of the drugs mentioned, halothane has the highest blood/gas partition coefficient whereas desflurane has the lowest. This means that desflurane has the fastest induction and recovery rate but requires more precise monitoring of the anesthetized animal. Isoflurane has been shown to irritate airways, which has not been seen with sevoflurane and desflurane. However, desflurane boils at room temperature which means special equipment is needed. The potency of inhaled anaesthetics is measured from the Minimum Alveolar Concentration (MAC) (Table 9). The lower the MAC, the higher the potency and the lower the amount needed to sedate an animal or human [81].

TABLE 9. MAC OF DIFFERENT ANAESTHETICS

Inhalation Anaesthetics	Mac (Human*)	Mac (Rats#)
Halothane	0.75%	0.88%
Isoflurane	1.17%	1.12%
Sevoflurane	2.29%	1.97%
Desflurane	6.6%	-
Nitrous oxide	104%	-

*Based on age of 40 years, #Based on adult Wistar rats

Besides inhalant anaesthetics, injectable anaesthetics are used for general anaesthesia; for example, propofol, barbiturates, or a combination of ketamine + (dexmedetomidine or diazepam or xylazine). Sometimes these injectables are also used in combination with inhalant anaesthetics to better control the anaesthesia [82]. Propofol activates GABA-A receptors to enhance their response to endogenous GABA. This leads to an inhibition of the glutamate receptor signalling and affects the nerve

depolarization in the voltage activated calcium current, which ultimately results in sedation and general anaesthesia. Propofol is used to induce and maintain anaesthesia. Barbiturates also bind to GABA-A receptors and anaesthesia is induced via the same pathway. However, barbiturates such as pentobarbital often result in side effects such as: hypotension, nausea, headache, drowsiness, bradycardia, respiratory depression, hepatotoxicity and skin rash. Therefore, barbiturates are only recommended for use in animals which will be sacrificed directly after the experiment. Ketamine is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist and blocks the hyperpolarization of activated cyclic nucleotide-gated potassium channels (HCN1). This blockade results in an anaesthetic effect. However, ketamine has also been shown to affect opioid receptors, such as mu, kappa, and delta receptors, at higher doses sigma receptors [68, 69, 81–83] and needs to be used with care if these receptors are the targets to be studied in the experiment. Ketamine is often given in combination with analgesics such as dexmedetomidine, diazepam or xylazine. These drugs are applied to reduce pain, avoid the neurotoxic effects of ketamine or induce muscle relaxation. Usually, 2 mg/kg of ketamine is injected intravenously into the test subject over 60 sec. The onset will be in 10–30 sec and produce 5–15 min of anaesthesia/dissociation. Longer studies (>15 min) require multiply intravenous injections of ketamine. This complicates its use, especially in animals where even lower doses need to be used. For this reason, ketamine is often applied in combination with other anaesthetics. In patients, often NSAIDs or gabapentin are used for pre-anaesthesia purposes, while opioids or ketamine are used for the induction and maintenance of anaesthesia, with a possible addition of local lidocaine during surgery. In Table 10, a limited overview of commonly applied anaesthesia protocols for animals is displayed.

TABLE 10. EXAMPLES OF DIFFERENT COMMONLY APPLIED ANAESTHESIA PROTOCOLS WITH DIFFERENT SPECIES

Animal Species	Anaesthesia Protocol
Mouse	1.4% isoflurane in 100% oxygen via the nose cone. Anaesthesia was injected with a flowrate of 200 mL/min in a combination of ketamine 100 mg/kg and 10 mg/kg xylazine.
Rat	Anaesthesia was induced at 3−3.5% isoflurane in 0.6% oxygen and maintained at 1.5−2.5% during scans.
Pig	On the scanning day, pigs were tranquilized by intramuscular injection of 0.5 mg/kg midazolam. Anaesthesia was induced by injection of a Zoletil veterinary mixture (1.25 mg/kg tiletamine, 1.25 mg/kg zolazepam, and 0.5 mg/kg midazolam). Following induction, anaesthesia was maintained by intravenous infusion of 15 mg/kg/h propofol.
Dog	Isoflurane was maintained at 2% in 100% oxygen (2 L/min) for 45 min. Anaesthesia injection intramuscular of ketamine and dexmedetomidine alone up to maximum doses (40 mg/kg and 0.25 mg/kg), and midazolam with increasing to maximum dose (1.5 mg/kg).
Non-human primate	Anaesthesia was induced by intramuscular injection of ketamine hydrochloride (~10 mg kg^{-1}). Anaesthesia maintained either by inhalation anaesthesia with sevoflurane 3.0 vol% in oxygen and medical air or by infusion of a mixture of ketamine hydrochloride (4 mg kg^{-1} h^{-1}) and xylazine hydrochloride (0.4 mg kg^{-1} h^{-1}) i.v. After induction of anaesthesia, animals were mechanically ventilated using an oxygen and air ventilator at an end tidal carbon dioxide fraction of 4–5 vol% and an inspired oxygen fraction of 0.3-0.4.

As with any drug, anaesthetics also lead to side effects, which makes it necessary to monitor the animal during anaesthesia. In a worst case scenario, the animal could die or wake up. Parameters to be controlled and monitored are temperature, heart and respiratory rate, pulse quality, mucous membrane colour, and the respiratory pattern [84]. If parameters deviate from the reference intervals, necessary adjustments need to be carried out such as increasing the concentration of the anaesthetics or temperature. Prolonged anaesthesia leads to hypothermia: consequently, measures are necessary to keep the animal warm. Heating beds, lamps or radiators are used for this purpose. Another commonly observed side effect is hyperglycaemia. High blood sugar levels can affect the function of the brain and the neuroprotective

properties that are found in the BBB. In particular, results from PET imaging using [^{18}F]FDG need to be carefully reviewed when using animals under anaesthesia. Sevoflurane and propofol are known to decrease the glucose metabolic rate as well as to reduce brain activity. Hypothermia and hyperglycaemia can also affect the blood circulation and mislead interpretations, especially in imaging experiments. Effects of anaesthesia can last for more than one week, which needs to be considered when performing longitudinal studies [85]. Anaesthesia usually causes a reduction in body weight in small animals, either due to stress or because animals eat less. Consequently, it is important that control groups receive the same amount and duration of anaesthesia.

Finally, anaesthetics can interact with other receptor systems, for example with the GABAergic receptor system. Drugs that are supposed to bind to these receptors could show a strongly reduced signal. For example, [^{11}C]L-deprenyl-D2, a radioligand selective for MAO-B in NHPs, showed a 77–80% (n=3) reduced specific binding anaesthesia with sevoflurane compared with ketamine/xylazine [70][86] (Fig. 37).
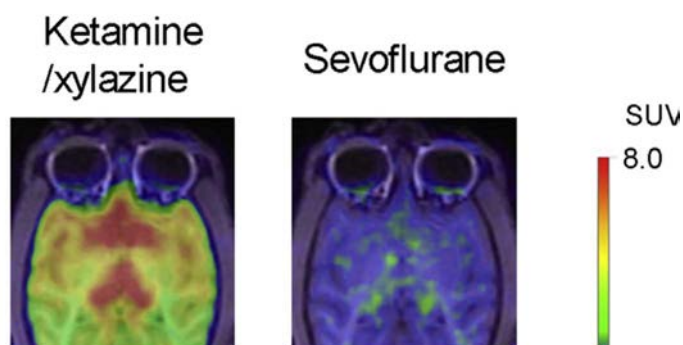
FIG. 37. PET images for [^{11}C]L-deprenyl-D$_2$ in NHPs anaesthetized with ketamine/xylazine (left) or sevoflurane (right). Average images from 27 to 93 min after radioligand injection. Image intensity is presented as a SUV (Reproduced from Ref. [86] with permission courtesy of [Br J Anaesth]).

It is preferable to avoid anaesthesia where possible. NHPs can, for example, be trained to stay still for the duration of the scan. However, such a set-up is very difficult to implement and almost impossible to set up for rodents. Lastly, rats can also be softly immobilized by wrapping them in a specially designed restraining suit for the duration of the experiment.

6.3. IMAGING TYPES

Imaging allows visualization of radiotracer kinetics non-invasively, which aims for most imaging studies to generate or extract qualitative data through one or more images. The type of imaging study chosen is based on the required information from the study.

6.3.1. Static imaging

Static imaging is the acquisition of a single image. It is a snapshot of the distribution of a tracer within a body part or within the whole body. Usually, static imaging is applied to molecular imaging probes that are already well described. Knowledge about the biodistribution of the respective tracer allows to select an optimal timepoint when to image the region of interest. Usually, this snapshot takes around 5–10 min. It represents the average concentration of activity of the target and gives semi-quantitative information of the uptake in the region of interest, for example as the SUV. The advantages of static imaging are its high throughput, relatively short scanning time of the animals and less demanding experimental set-up and data analysis. Parameters such as the accumulation rate or washout kinetics cannot be determined with static imaging. Therefore, dynamic imaging is usually used for newly developed receptor targeting tracers, especially if binding parameters such as the BP are determined by kinetic modelling.

6.3.2. Dynamic imaging

Dynamic imaging displays the biodistribution of a radiopharmaceutical over time. A dynamic imaging study consists of the acquisition of multiple static images that are plotted and displayed in sequential manner. Acquisition duration for each image (imaging frame) can be of the order of 10 sec to 20 min to capture the peak in brain uptake. Acquired data are continuously recorded by the scanner during acquisition and can be split into frames post acquisition. Therefore, the same dataset can be split into imaging frame sets in multiple different ways if necessary. Usually, dynamic imaging is simultaneously started with imaging agent administration. Consequently, it is possible to follow the tracer′s in vivo distribution and study its pharmacokinetics. In order to extract binding parameters out of the acquired data, mathematical modelling (kinetic modelling) is needed (see Sections 6.6 and 8). For this kind of modelling, the Time Activity Curve (TAC) of the tissue's concentration of radioactivity over time (i.e. which dynamic imaging provides) needs to be determined. For brain imaging, usually an arterial input function is also needed. It accounts for the transportation rate of the tracer from the blood (arterial input function) into the brain. To generate a full arterial input function, blood sampling is needed, which is usually limited in small animals such as like rodents. However, with an arteriovenous shunt and coincidence counter of the blood input function, significant blood loss can be averted. Metabolite analysis can be performed to assess the fraction of intact tracer. Also, the blood to plasma ratio can be assessed from discrete blood samples. Dynamic imaging allows the study of binding parameters such as the BP or other pharmacokinetic parameters of a tracer and even of a target (see Sections 6.7 and 8). Compared to static imaging, it is time consuming. Long scan times of 60–90 min is needed compared to 5–10 min for static imaging. This also results in longer reconstruction times [87]. Dynamic imaging is commonly used for new tracers or to study receptor binding [88].

6.3.3. Arterial input function

The tracer is delivered to the brain and other tissues with arterial blood, and tracer concentration can be assumed to be equal in all systemic arteries. Therefore, TAC for arterial blood (arterial input function) is used for kinetic modelling. To generate a full arterial input function, the concentration of the tracer in the blood needs to be assessed at different time points from the tracer injection until the end of dynamic imaging. The basic assumption of most kinetic modelling approaches is that only the intact tracer dissolved in plasma penetrates across the BBB, while radiometabolites do not. Furthermore, a fraction of unmetabolized tracer can be bound to plasma proteins, in which case it does not participate in the mass exchange between blood and brain. Hence, not only the total radioactivity concentration in the blood needs to be determined, but also the component corresponding to the intact tracer in plasma that needs to be quantified and possibly corrected for protein binding. This is most commonly done by withdrawing blood samples, often through implanted catheters, and analysing them with the aid of γ counters, radio-HPLC/TLC systems, etc. However, repeated blood sampling in small animals such as rodents is problematic because of their low overall vascular volume. Moreover, when the tracer is injected as a bolus, the initial part of the arterial input curve 'injection peak' is challenging to quantify properly without very frequent measurements, and quantification errors lead to biased estimates of kinetic modelling parameters. These challenges can be addressed by the following techniques:

(a) Continuous measurement of arterial blood activity by β or γ sensitive detectors without an actual sample withdrawal. In one implementation, arterial blood is made to flow past a coincidence counter through an arteriovenous shunt. In another implementation, β sensitive silicon diodes are implanted near the artery to measure radioactivity passing through it. In these cases, net blood loss resulting from the measurements is minimal, because the blood either does not leave the vessels or is promptly reinjected into the animal. In yet another implementation, the blood is drawn from an artery and is passed through a microfluidic system with a built-in β/γ detector. Although the withdrawn blood is not reinjected, the total withdrawn volume can be kept low due to the small total volume of the microfluidic system. These methods provide time–activity curves for whole arterial blood with the best (sub-second) temporal resolution;

(b) Estimation of arterial input function from the actual dynamic imaging data by including the heart of the animal into the field of view of the camera. The heart is constantly changing shape as it is pumping the blood around the body, but it is possible to extract the TAC from the left ventricle (oxygenated blood ready to be sent into the arteries) from the image by means of a mathematical technique called factor analysis. This is the least invasive method of all, but its temporal resolution is limited by the temporal resolution of the PET camera, which is on the order of several seconds;

(c) Compensation for the loss in circulating blood volume after conventional manual blood sampling by injecting an equal amount of saline into the animal through the same catheter after each sample withdrawal;

(d) Using population based arterial input functions: This approach assumes that the shape of the arterial input function is comparable in all experimental animals. This is a reasonable assumption, since the animals used for tracer evaluation usually belong to the same population, have similar ages and body weights, the distribution and metabolism of the tracer in them should follow similar trends. Therefore, only a limited number of samples corresponding to a limited number of time points is drawn from each individual animal, and then the data from all animals are pooled together to build the population based input function. In some implementations of the scheme, one experimental group is used to obtain PET imaging data in a minimally invasive manner, while another independent experimental group is used to obtain a population based arterial input function through invasive blood sampling and/or euthanasia and dissection.

It should be noted that although arterial input curves with high temporal resolution can be obtained through a detector flow by or by image analysis, these curves will only reflect total radioactivity concentration in the arterial blood. To correct them for tracer metabolism and/or for plasma protein binding, withdrawal and analysis of blood samples is essential. Therefore, conventional blood sampling cannot be completely avoided.

6.4. BLOOD BRAIN BARRIER

Radiopharmaceuticals that target proteins such as receptors, enzymes or other structures in the brain have to pass through the BBB. For many chemical entities such as polar small molecules, peptides, proteins such as monoclonal antibodies or larger molecules (polymers, nanoparticles, etc.), this barrier presents an insurmountable hurdle because of their polarity or size, or both. Recently, a set of new techniques has been developed to guide molecules until recently considered to be BBB impermeable into the brain. Focused ultrasound in connection with microbubbles [89], chemically induced BBB opening with drugs (e.g. mannitol), or receptor facilitated transport (e.g. using the transferrin receptor) are some techniques that have shown great promise. For example, focused ultrasound mediated delivery of ^{111}In labelled trastuzumab has been reported in 2021 for clinical application for the first time [89]. Future studies are required to show the full potential of these new techniques.

6.5. SPECIFIC BINDING

Usually, radiopharmaceuticals are designed to bind with high affinity and selectivity to a specific target. These properties of radiopharmaceuticals are normally assessed in in vitro studies, but in vitro data may not be representative of the in vivo situation. As a rule of thumb, the target affinity in vivo is sufficient if the B_{max} over K_d ratio (based on in vitro data) is ideally > 10. Affinity, however, should not be too high as the tracer uptake may reflect blood flow rather than target expression. B_{max} is defined as the total density of a receptor in tissue and K_d as the affinity of the tracer for this target. In molecular imaging, both parameters are connected to the outcome measure of BP that can easily be determined by PET. The BP is defined as BP = B_{max}/K_d or the ratio of the concentration of specifically bound and non-displaceable ligand in tissue. The affinity of the tracer for the receptor is assumed to be the same in all brain regions, but receptor densities (B_{max}) may be different. As such, a higher BP relative to the non-displaceable compartment (BP_{ND}) is expected in regions with a higher B_{max} value. To test the selectivity of the tracer, blocking or competition studies are usually performed. In a blocking/competition study the binding of the tracer will be measured in the presence of a second unlabelled ligand, providing

quantitative information of the specific binding of the tracer relative to baseline or control animals. The difference between a blocking and a competition study lies in the order in which the tracer and the unlabelled blocker/competitor are administered. In blocking studies, the blocker is given before the tracer, in competition studies the competitor is administered afterwards. In both cases, specificity of the tracer is shown when the block/competition lowers for example the BP to values of a reference region, which is absent of the target of interest. If there is no reference region, tracer uptake can be compared to a baseline scan or a scan of an untreated control group. Usually, the blocker is administered 5–45 min prior to tracer injection. The dose of the blocker/competitor is also case dependent. In general, competition studies have shown to be less sensitive for competition compared to blocking studies. Fig. 38 displays an example of a blocking study.
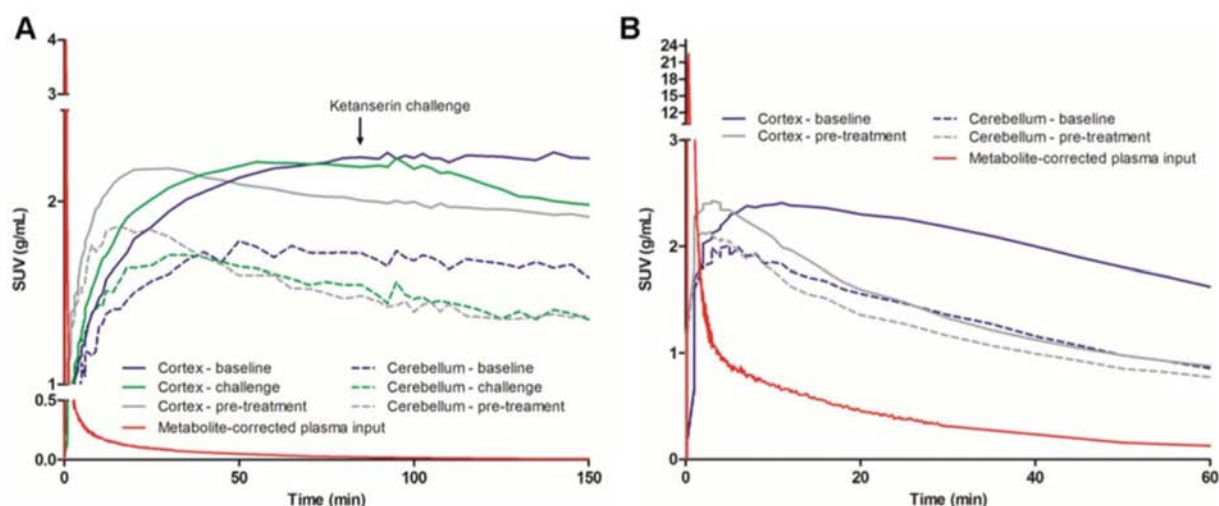
FIG. 38. TACs and metabolism corrected input curves for [^{18}F]MH.MZ (A) and [^{18}F]altanserin (B). Data are presented as SUVs. [^{18}F]MH.MZ: Baseline, challenge, pre-treatment. [^{18}F]Altanserin: Baseline, pre-treatment. PET images of the mean decay-corrected activity concentration after bolus injection of [^{18}F]MH.MZ (Reproduced from Ref [90]with permission courtesy of [Theranostics]).

6.6. EX VIVO STUDIES

Ex vivo studies are a powerful tool to determine the biodistribution of radiopharmaceuticals within the body. For radiopharmaceuticals that do not emit γ rays, they are the only way to determine such distribution. Ex vivo studies are also carried out to verify the measurements obtained by non-invasive imaging methods. It is important to remember that imaging data usually consist of counts stemming from the organ of interest, but also from blood within the tissue if not corrected for. For animals, such correction is not standard. Consequently, higher uptake values are often observed using imaging methods compared to ex vivo studies, especially if the tissue obtained from ex vivo studies was perfused (e.g. with saline) before measurement [83, 90]. Ex vivo studies within the CNS allow to dissect the brain into different brain regions. This enables assessment of smaller brain regions, without the confounding partial volume effects that are observed with imaging of small animals. For example, uptake in various brain regions such as the cortex or cerebellum can easily be determined. Ex vivo studies also allow to get tissue that can be used for ex vivo autoradiography or further analysis, for example, metabolism determinations or omics, which are discussed in the following sections.

A typical ex vivo study in rodents is carried out as follows:

(a) Radiopharmaceutical administration: Most often by intravenous injection, but intraperitoneal or subcutaneous routes are also sometimes used;
(b) Distribution phase: The activity is allowed a certain amount of time to distribute throughout the rodents' bodies, while the rodents are awake or anesthetized;

(c) Perfusion: If it is essential to remove blood from the tissue before radioactivity determination, or the tissue needs to be fixated for subsequent biomarker analysis, the animals can be subjected to whole body perfusion with or without a fixating agent once the distribution phase is over;

(d) Euthanasia: The animals can be sacrificed by decapitation, anaesthetic overdose, intracardiac injection of pentobarbital, heart extirpation, cervical dislocation, or a combination of the mentioned techniques.

(e) Tissue dissection: Tissue samples or whole organs are extracted for further analysis;

(f) Radioactivity determination: Radioactivity associated with organ or tissue sample, or homogenate is measured using suitable detector system based on the emission type and energies of the associated radionuclide;

    i. Radioactivity determination: For isotopes that generate γ photons or annihilation photons upon decay (typical PET and SPECT isotopes, e.g. ^{18}F or ^{99m}Tc) or decay to γ emitting daughters (some therapeutic isotopes, e.g. ^{225}Ac), direct γ counting of intact tissue samples can be used; activities of pure α and β emitters (e.g. ^{3}H, ^{212}Bi) can be measured with liquid scintillation counting of tissue homogenates mixed with scintillation cocktails or, in certain cases, with Cerenkov counting of intact samples;

    ii. Ex vivo autoradiography and staining: Tissue samples are frozen, sliced on the cryotome, thaw mounted on glass slides and exposed against phosphor imaging plates or radiation sensitive film to obtain autoradiographic images of radioactivity distribution in tissue sections. Same slides can later be processed and stained to reveal general histological features or specific molecular landmarks in the sample;

    iii. Tissue preparation for metabolism studies or omics: Tissue samples can also be homogenized and/or digested to extract radiometabolites from tissue samples or perform omics analyses extraction of radiometabolites generally following standard bioanalytical protocols.

6.6.1. Staining

Staining methods are applied to tissue sections from in vitro or ex vivo experiments with radiotracers to assess the correspondence between the distribution patterns of radioactivity and the intended target of the radiotracer. The target can be defined as a concrete population of biomolecules, concrete cell population or gross Region of Interest (ROI) in the tissue. The distribution of specific biomolecules or cells can be assessed by immunohistochemistry using antibodies specifically recognizing these molecules or cell markers. More generally, regions of interest can be defined by highlighting tissue architecture with the aid of histological staining methods, such as hematoxylin-eosin staining (nuclei/cytoplasm), Nissl or Golgi-Cox staining (neurons) or Luxol staining (myelin) [91]. Staining normally requires fixation of the tissue, which can be performed by exposing the slides to paraformaldehyde vapours or soaking them in fixating solutions. Staining of brain aggregates such as β amyloid, tau or α synuclein is important to characterize and study brain diseases such as AD or Parkinson`s disease. Unspecific binders such as Thioflavin S or Congo Red as well as specific antibody based binders for specific pathology even exists for various aggregation conformations and fragments. Besides, staining target verification can be performed by Western blot or PCR techniques.

6.6.2. Tracer evaluation by ex vivo mass spectrometry

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is a sensitive enough analytical method to detect trace amounts of organic analytes in tissues. Therefore, it is possible to evaluate candidate radiotracers in a non-radiolabelled form by studying their biodistribution with the aid of LC-MS/MS. The experimental scheme is very similar to that used in conventional ex vivo evaluations of radiotracers, but instead of counting radioactivity, the obtained tissue samples are homogenized, the homogenates are extracted and analysed by LC-MS/MS [92]. The major disadvantage of this method is the high amount of effort required for sample processing and the establishment of analytical methods. The major advantage, apart from the elimination of the need for handling radioactive material, is that multiple candidate compounds can be evaluated simultaneously in the same population of animals, as

long as tracer conditions are still satisfied. Even sophisticated experiments such as receptor occupancy determination can in principle be performed with the aid of LC-MS/MS [93]. If the ROI containing the target to which the candidate radiotracer should bind is too small to be excised for homogenization, the distribution of the candidate compound can instead be studied with high spatial resolution (10–100 μm) in tissue sections by means of imaging mass spectrometry techniques such as MALDI or DESI. However, the measurements obtainable with these techniques are far less quantitative than LC-MS/MS or radioactivity counting.

6.6.3. Metabolites

Like all other drugs, radiopharmaceuticals can undergo metabolism. Usually, radiopharmaceuticals are administered intravenously and consequently, they are distributed via the blood stream to other organs. In Phase I drug metabolism, radiopharmaceuticals are chemically modified by a variety of enzymes to introduce reactive and polar groups. The most common modifications are carried out by cytochromes P460 which oxidize radiopharmaceuticals and introduce for example hydroxyl groups or dealkylate amines, (thio)ethers or esters. Phase I metabolism also encompasses reactions such as reductions or hydrolysis. In phase II metabolism, introduced reactive and polar groups are conjugated with charged species such as glutathione, sulfate, glycine or glucuronic acid. Conjugation products have as such an increased molecular weight and are even more polar than Phase I metabolites. Consequently, they can often not diffuse across membranes and stay in the blood stream before they are actively excreted via the liver or the kidneys, for example. Metabolites that still carry the radioactive radionuclide are called radiometabolites. They can lead to challenges for imaging as well as for targeted radiotherapy studies. For example, in PET studies the camera cannot distinguish between the signal from the intact tracer or its respective radiometabolite(s). As radiometabolites often result in metabolites that accumulate non-specifically and result in a non-displaceable binding component, quantification of a specific signal that lies behind this component may be difficult [94]. Fig. 39 illustrates the respective challenge.
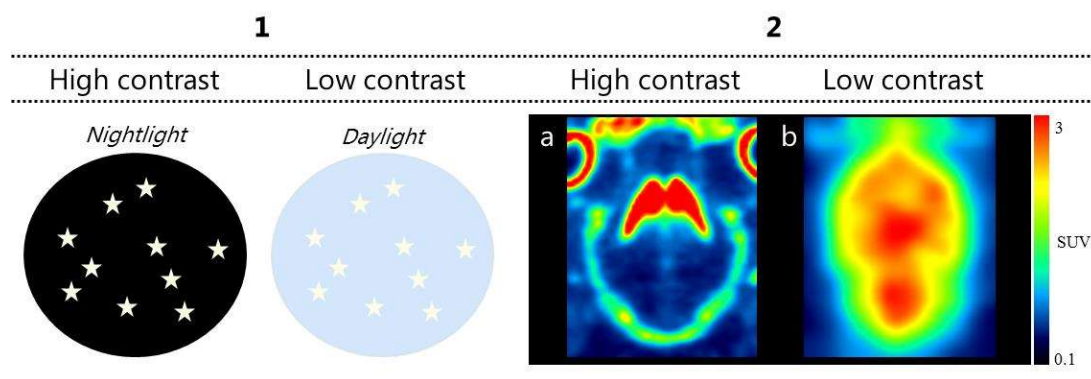
FIG. 39. Schematic presentation of the importance of contrast within PET images. 1) Visualization of high and low contrast by an analogy of the visibility of the stars during night and day. 2) Horizontal sections through summed PET images of rodent brain with high and low contrast. a) [^{18}F]Fallypride at baseline b) [^{11}C]Pimavanserin after pretreatment with elacridar (P-gp inhibitor) (Reproduced from Ref. [94]with permission courtesy of [Neuropharmacology]).

One way to mitigate this challenge partially is to use a reference region in which the non-displaceable binding component can be determined and afterwards subtracted from the signal received in the region of interest. However, if the non-displaceable binding component is too high, this mitigation loses its power. If formed radiometabolites show specific binding to any targets in the brain, specific binding of the parent radiopharmaceutical becomes even more difficult to image. In this case, the specific binding component of the radiometabolite need to be blocked. If the radiometabolite binds to the same target, differentiation between the signal from the metabolite and its parent is in many cases impossible. For brain tracers, it is beneficial that radiometabolites when formed within the brain (i.e. that cannot pass through the BBB) are rapidly removed from the brain and quickly excreted from the blood stream. Ideally, the radiotracer should rapidly enter the brain and selectively bind to the target without forming

any radiometabolites. Radiometabolites also influence the dosimetry of an injected radiopharmaceutical. Formed radiometabolites could accumulate in tissues and even prevent the use of the radiopharmaceutical because of radiation protection concerns caused by such accumulation of radiometabolites. This is especially relevant for targeted radionuclide therapy studies, but it also plays a role in imaging studies. Ideally, radiometabolites are excreted fast and do not accumulate in radiation sensitive organs to any greater extent.

For these reasons, it is important to determine the metabolism profile of a specific radiopharmaceutical and its biodistribution time course. Usually, the rate, amount, and concentration of radiometabolites are determined first. This is usually done in blood samples. The blood sample volume differs between species. In rodents, only small amounts can be taken for analysis. Therefore, high sensitivity analysing equipment is often required. For comparison, blood samples with a maximum volume of 0.1 mL can be drawn from mice: and in turn, 1.5 mL from rats, 300 mL from pigs and 500 mL from humans. Plotting the measured relative abundance of unmetabolized parent tracer over time makes it possible to determine the metabolism rate and determine the arterial input function, which is needed for quantification purposes. Existence of brain metabolites is more difficult to determine. Usually, animals are sacrificed at specific timepoints, their brains are perfused (e.g. with saline), removed and homogenized, and this homogenate is analysed for radiometabolites. The metabolism rate and concentration can be determined as described before. Another possibility is to carry out microdialysis. Attention needs to be paid to species differences. As described above, the formation rate and identity of radiometabolites may vary from species to species. Generally, larger species possess a slow metabolism. For example, [^{18}F]MH.MZ — a tracer for selective 5-HT$_{2A}$R imaging — displayed a similar radiometabolite profile in rodents or pigs, but did not show any metabolism in humans over a timeframe of 180 min [95]. Radiometabolites are usually analysed by TLC and reverse phase HPLC. The use of TLCs is more economic but less accurate and work less well for ^{11}C tracers. In contrast, the use of HPLCs is more accurate and even radiometabolites with minor chemical property changes can be resolved from the parent. However, the use of HPLCs is more expensive and takes longer. Other analysis methods are based on SPE, protein precipitation or liquid–liquid extraction. These methods are fast and simple to execute, but not very accurate. A deeper overview of various methods to analyse radiometabolites is provided in Ref. [13].

6.7. MICRODIALYSIS

Microdialysis is a method that makes it possible to analyse the composition of interstitial tissue fluid with the aid of microdialysis probes implanted at relevant locations. A microdialysis probe is composed of tubing and a semi-permeable membrane which together form a loop contour (Fig. 40). As the perfusing solution is pumped through the probe, it undergoes mass exchange with the interstitial liquid. Compounds dissolved in the interstitial liquid are transferred to the infusate through the semi-permeable membrane. The composition of the infusate pumped out of the probe can then be analysed, for example by HPLC, to track the concentrations of molecules of interest. The technical aspects of various microdialysis set-ups are reviewed in Ref. [96].

Important feature of the microdialysis set-up is that the molecular weight cut-off of the semi-permeable membrane that can be adjusted to exclude proteins. Thus, for small molecules, either the total concentration or only the free (non-protein-bound) concentration can be measured, depending on whether the molecular weight cut-off is, respectively, high or low.

In the context of CNS radiopharmaceutical evaluation, microdialysis is useful to monitor interstitial concentrations of the endogenous ligands for the targets to which the investigated CNS radiopharmaceutical is supposed to bind. For example, when a radiopharmaceutical binds to certain neurotransmitter receptors and is displaced by a competing cold drug, how much of the drug actually gets across the BBB can be measured at the post-administration time, and for how long it remains present in the brain. Similarly, microdialysis makes it possible to follow the release of endogenous neurotransmitters elicited by injected drugs or electrical stimulations. Sensitivity to endogenous neurotransmitter is a sought after property for some radiotracers targeting neurotransmitter receptors, and independent evaluation of neurotransmitter burst is important for a rigorous assessment of this

property. Following endogenous neurotransmitter release by microdialysis has an intrinsic problem, the neurotransmitter is released into the synaptic cleft, while the microdialysis probe measures interstitial concentration of the neurotransmitter. Only some of the released neurotransmitter diffuses into the interstitial space, so microdialysis measurements may greatly underestimate the actual synaptic levels of the neurotransmitter.
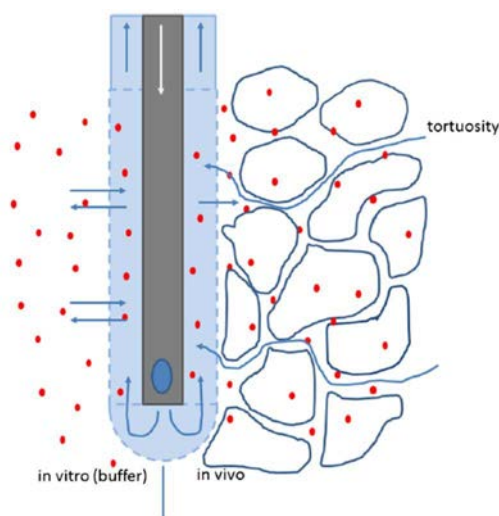
FIG. 39. Microdialysis - Principle of action (Reproduced from Ref. [96]with permission courtesy of [AAPS J]).

6.8. NON-HUMAN PRIMATE PET UTILITY IN RADIOTRACER DEVELOPMENT

The use of NHPs in the development of new CNS radiotracers is of great benefit in evaluating new candidate molecules for decisions on which molecules to move into human trials. The use of NHPs can also be of importance to establish radiation dose, which is a prerequisite for human PET.

6.8.1. In vitro characterization using NHP brain tissue

NHPs can be a great asset in early evaluation of binding characteristics of novel radiotracers using in vitro techniques such as autoradiography. A set of radiotracers can be readily evaluated using brain sections exploring specific binding, level of non-specific binding and binding affinity. The similarities between NHPs and humans has been readily seen in studies showing great translatability in binding profiles in NHPs compared to humans [97].

6.8.2. Advantages with NHP and similarities with humans

The similarities between NHPs and humans in terms of binding characteristics is not only true in vitro, as described above, but it is also often the case in vivo. This is illustrated in the development of the DAT radioligand [^{18}F]FE-PE$_2$I, first evaluated using autoradiography and preliminary in vivo studies in NHPs which demonstrated its prospects [98, 99]. The radioligand was developed at the Karolinska Institute involving all the key steps, from in vitro characterisation including selectivity and sensitivity, then in vivo experiments in NHPs (example images are shown in Fig. 41) and then all the steps up to and including first in human studies [100, 101]. The development process of [^{18}F]FE-PE$_2$I described in Refs [100,101] illustrates the steps followed from a good candidate and the utility in applied research in Parkinson's disease [102]. Development of other radioligands will be considered in the following sections as illustrations, including the development of a novel radioligand 5-HT1B receptor, namely [^{11}C]AZ10419369 serving as an example on utility of NHPs in development of novel radioligands.
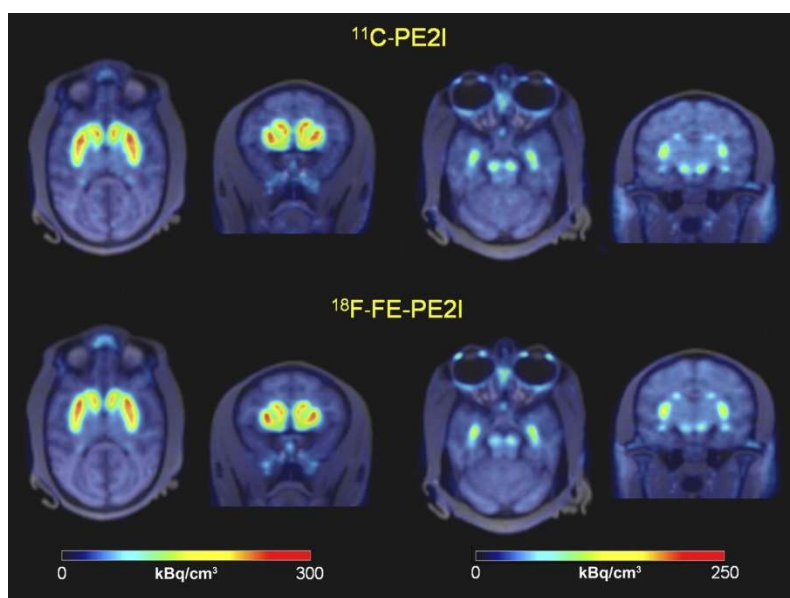
FIG. 40. Example figure of [^{18}F]FE-PE2I in an NHP compared to the carbon-11 labelled counterpart (Reproduced from Ref. [100]with permission courtesy of [J Nucl Med.])

6.8.3. Possible issues or considerations with NHP PET

Before moving on to the advantages and utility of using NHPs as a foundation for evaluating potential novel radioligands to study biological or pathological processes/entities in humans, we will address some evident disadvantages. It is of course an overall aim of all research to limit the use of animals and especially higher species such as NHPs. As discussed below there are many ethical and methodological issues that need to be considered. With improvements in the utility of other species in radioligand development and even in silico methods, the number of NHPs utilized in projects can be reduced and with improved methodology, this will also reduce the numbers further. Nevertheless, the research questions that can be addressed utilizing NHPs that are described below are such, that it may be difficult to foresee a near future without the need of NHPs in radioligand and drug development.

6.8.3.1. Ethical considerations

First of all, there are obvious ethical aspects of utilizing NHPs that need to be considered. Several ethical considerations have been discussed in Ref. [13] that also apply when NHPs are used in the development of novel radioligands. NHPs are only to be used when the research question cannot be addressed reliably, when using preclinical methods or lesser species. It should also be clear that other species such as rats or mice can serve as good models for providing valid information for further development. It needs to be fully established that the resemblances to human brain anatomy and physiology is of high importance to provide results pivotal for further research that would be of eventual benefit to humans, either due to better understanding of diseases, new diagnostic tools, or new therapeutics.

All NHP research is expected to undergo ethical review by a national or regional vetting agency, depending on the country's ethical organisation and regulation. It is important that the housing and maintenance of animals meet high quality in animal welfare. This can be ensured by fulfilling the criteria to be accredited by AAALAC International. Furthermore, the facility could also be approved for research following good laboratory practice definitions, further ensuring good quality and standards. The housing situation needs to be in larger areas furnished to ensure that the NHPs can express natural behavioural needs and social interactions in their groups.

Furthermore, the experimental procedures need to be planned in such a way to limit the stress on the animal as much as possible. This includes, for example, training NHPs for sedation, keeping the anaesthesia as short as possible, offering pain relief and taking other measures to ensure a good recovery

58

of the NHPs. It is also important not to perform experiments that are unnecessary if there is enough data to progress the project to the next step. It usually is enough to collect images of two NHPs to know if a radioligand shows promising characteristics for progress into human subjects.

6.8.3.2. Cost related to use of other species and possible differences

Besides the ethical aspects, there are other limiting factors that are important to consider. One thing is the very high cost involved with NHPs for breeding, procuring, and housing for an extended time. This could be a limiting factor in the inclusion of NHPs in academic research, but also in projects where there is high scientific need. This could influence the use of cheaper animal models which could hamper the validity of the results, or even provide erroneous results, either by stopping a perfectly good molecule or moving forward with a molecule that later shows that is does not work in humans. One example of this phenomenon is the development of the 5-HT1B radioligand [11C]AZ10419369 that was initially evaluated in NHPs [103] showing good properties and then later validated in human subjects showing excellent reliability to measure 5-HT1B receptors in vivo [104, 105]. In a back translational study the ability for [11C]AZ10419369 to study 5-HT1B receptors in rodents (mice, rats, guinea pigs) was examined [106]. The results showed that the initial studies revealed basically no brain uptake of [11C]AZ10419369, but after blocking of ABC transporters with cyclosporin A, there was a marked increase in [11C]AZ10419369 uptake. If [11C]AZ10419369 have only been evaluated in rodent models for the utility of in vivo imaging, it could have been the case that a very good radioligand would have been stopped pre-emptively based on non-translatable data.

6.8.3.3. Lack of target for disease biomarkers

Another limitation with NHP PET could be that it is not possible to study targets that are not expressed or present in the healthy NHP brain. This limits the possibility to study specific binding, although it can still provide pivotal information needed to move forward to human subjects. As an example, the development of a radioligand targeting mutated huntingtin for research in Huntington's disease was recently published [107]. Although the aggregated radioligand targeting mutated huntingtin is not present in the NHP brain, it was still possible to collect important information on brain uptake, clearance, metabolism, the brain uptake to plasma ratio, the grey matter to white matter ratio, etc. This combined information was important for guidance of which several lead compounds showed the most promising characteristics for further development, dosimetry evaluation and subsequent evaluation in human subjects including patients with Huntington's disease.

6.8.3.4. Anaesthesia

Lastly, one needs to consider any potential influences that the anaesthesia might have on the kinetics of the radioligand, such as sensitivity to blood flow alterations; effect on the affinity of the radioligand potential caused by alterations of the target, or release of endogenous neurotransmitters. To serve as an example, a study evaluating the effect of different anaesthesia on the radioligand binding of [11C]AZD9272 or [11C]L-deprenyl-D2 to monoamine oxidase-B (MAO-B) showed marked differences between sevoflurane compared with ketamine/xylazine [108]. The researchers found a marked reduction in signal from both radioligands during anaesthesia with sevoflurane compared to ketamine/xylazine. This is an example where anaesthesia has a marked effect on the outcome measurement which in many cases is not true, which should be considered when designing a PET study.

6.8.4. Validation process using NHP PET

Although using NHPs as the species of choice for validating novel radioligands includes both ethical considerations and accessibility challenges, it is still the most suitable model to evaluate and predict the behaviour of molecules and translate into further human studies. The validation process consists of several important steps for which NHPs can be used and are discussed below.

6.8.4.1. Selectivity, sensitivity, and go/no-go decision making

One of the key questions that can be addressed using NHP PET is the selectivity of a novel radioligand in an in vivo setting adding to the already established selectivity in vitro. This is usually done either as a pre-treatment study or a displacement study, the former is easier to carry out. For a pre-treatment study there is first a PET scan under baseline conditions and subsequently, either on the same day or a separate day, a PET scan before an agonist or antagonist molecule selected for the target is administered. The timing of the pre-treatment is determined on the kinetics of the competitor, and it is usually designed so the Cmax of the competitor is in middle of the PET examination. This could be within minutes prior to the second PET or sometimes a few or several hours prior to. The use of ^{11}C radioligands is usually preferred for these kinds of studies making it possible to do both baseline and pre-treatment PET on the same day. This reduces the strain on the animal due to fewer sedations and the risk of variability that could be inferred by doing the PET within a minimum of 4–6 weeks in between, depending on if the arterial cannulation is or is not performed. Longer rest is needed if arterial cannulation is performed since the invasive procedure requires a longer recovery time. The use of the pre-treatment paradigm to establish radioligand selectivity is nicely shown in the work done validating the 5-HT1B radioligand [^{11}C]AZ10419369 [102] and its ^{18}F labelled counterpart [^{18}F]AZ10419096 [109]. In both instances, a baseline PET was performed followed by a pre-treatment PET experiment using a 5-HT1B antagonist AR-A000002 administered 30 min prior to the second PET. Clear reduction of [^{11}C]AZ10419369 and [^{18}F]AZ10419096 binding, respectively, could be seen both visually and quantitively confirming the selectivity of each radioligand and providing information on the level of non-displaceable binding. Alternatively, selectivity can be evaluated with displacement instead of pre-treatment with the difference that the blocking compound is administered during the PET experiment rather than before. This is shown in the paper by Dahl et al. where they perform both pre-treatment and displacement in their evaluation of a novel Histamine Type-3 receptor radioligand called [Carbonyl-^{11}C]AZ13198083 [110]. For displacement, the blocking compound was given 60 min after the radioligand administration where a rapid reduction of [Carbonyl-^{11}C]AZ13198083 was seen demonstrating rapid reversibility of the radioligand adding on the information established with pre-treatment experiments.

To follow the description of pre-treatment studies, there will be a description of the use of NHPs to study the occupancy of novel drugs as an important tool to design in further phase one studies in humans. These studies are often followed by occupancy PET studies in humans, but serve as a good foundation to guide such studies due to the high translatability discussed above. As an example, the study by Varnäs et al. evaluating the novel psychotropic drug AZD3676 targeting both 5-HT1A versus 5-HT1B receptors was used [111]. To evaluate 5-HT1A the radioligand [^{11}C]WAY-100635 was used and for 5-HT1B [^{11}C]AZ10419369 was employed. Three NHPs were used at either baseline or after different doses of AZD3676 were applied. The results showed a dose dependent relationship between plasma exposure and target occupancy for both targets. It was also shown that the level of occupancy was equal for both targets, which was said to be surprising based on the available background data, highlighting the need to study these relationships in vivo in an adequate model.

Important go/no-go decisions for further studies include evidence of sufficient brain penetration, evidence of selectivity, the feasibility of being studied in NHPs, no data suggesting the presence of radiolabelled metabolites entering the brain, and reversible kinetics favourable for reliable kinetic modelling. Another possibly important piece of information guiding the selection of specific candidates over others could be the level of non-specific binding, which should be as low as possible.

6.8.4.2. Reliability

The use of NHPs to research the reliability of outcome measures in test/retest studies is unusual, mainly due to ethical reasons since the information gained is often not needed as a foundation for go/no-go decisions. It is often the case that test/retest studies need to be performed anyway in humans, making it not worth the strain on the animals, the inferred costs, etc. Nevertheless, it could be in some instances it is deemed necessary; for example, researchers from Yale PET centre recently published a good reliability of a novel SV2A radioligand [^{18}F]SDM-16 [58], where they reported investigations of

selectivity using both pre-treatment and displacement paradigms as further examples on top of those provided above. They also included valuable information on radiation exposure established in whole body dosimetry PET studies.

6.8.4.3. WB dosimetry

To move a radioligand into human clinical investigations, it is crucial to establish the radiation exposure of a novel radioligand and evaluate if there is any accumulation of radioligand or labelled metabolites in critical organs. This information is an important part of the investigator's brochure and part of the Investigational Medicinal Product Dossier (IMPD) together with other supporting data for the production of the radioligand, clinical safety including acute toxicology and preclinical data supporting further studies in humans. As mentioned above, Ref. [58] includes the description of the whole body experiments, which is typically done in two NHPs where the organs are delineated covering all major organs. The residence times per organ are then extracted and the absorbed dose is obtained using a program such as the commonly used OLINDA/EXM® software. The estimated effective dose usually determined in µSv/MBq then dictates the radiation dose inferred by a given dose in MBq. It is usually defined as an equivalent dose in mSv, which then needs to comply with any local or national regulation on the level of exposure that a given healthy person or patient is permitted to receive. This can vary greatly between countries if there are healthy younger or elderly patients included in the study.

6.8.5. Endogenous neurotransmitter release

The use of established radioligands or the use of novel ones to evaluate the sensitivity to study endogenous neurotransmitter release can sometimes be of interest. In a quite recent review by Finnema, et al., the lack of radioligand sensitive to neurotransmitter release was discussed, and the challenges to find good radioligands were highlighted [112]. As an example of how PET in NHPs can be used to study endogenous neurotransmitter release, the evaluation of the 5-HT_{1B} radioligand [^{11}C]AZ10419369 is used again [113]. In the project, the researchers utilized both the simpler pre-treatment paradigm and a more sophisticated equilibrium approach. In the pre-treatment experiments, they first did [^{11}C]AZ10419369 PET at baseline, but also after a dose of fenfluramine that induces serotonin release. It was then evident that there was a clear reduction of [^{11}C]AZ10419369 binding after pre-treatment caused by competition of the 5-HT_{1B} receptors by endogenous serotonin. In equilibrium studies, the radioligand is first administered as a bolus with sub-sequent constant infusion. The advantage is that one can study direct observation of changes after the administration of a blocking compound, or, as in this case, fenfluramine that induces endogenous serotonin release having an impact on the binding kinetics of [^{11}C]AZ10419369 towards the 5-HT_{1B} receptors. The authors were then able to show that the fenfluramine administration had a dose dependent impact on the BP of [^{11}C]AZ10419369, confirming the radioligand is sensitive to changes in endogenous serotonin levels in vivo.

6.8.6. Methodological considerations

There are some methodological limitations which may or may not be present at different PET centres depending on the software analysis in use. For example, ROI delineation may be more complicated and less sophisticated than for human PET studies where less developed tools for automatic segmentation are available and therefore, a lack of available atlases for automatic ROI delineation. Manual ROI delineation on MRIs and manual or semi-automatic co-registration of MRI to PET can be applied here and these steps are labour intensive, time consuming and rely heavily on the expertise of the researcher doing the analyses. This also applies for whole body studies where a not always combined PET/MRI or PET/CT system is available, then, when drawing ROIs on PET images, the anatomical expertise and experience of the researcher is even more important with an inherent risk of bias and interrater variability.

6.9. QUANTIFICATION OF IMAGING DATA

Provided that the imaging cameras are properly calibrated for the radionuclide used, reconstructed images show the distribution of the radioactivity concentrations (decay corrected). If a dynamic scan was performed, i.e. data acquired at different time intervals since the start of the acquisition were reconstructed separately, then a series of images corresponding to individual acquisition periods (frames) is available. To get the average concentration of radioactivity in ROI (often called volume of interest if it is defined in a 3-D space), one needs to average the values from all voxels comprising the region of interest. This already enables the comparison of radiotracer uptake between regions of interest in the same subject or between different frames of the same scan. The radioactivity concentration in the ROI acquired in different frames plotted against middle timepoints of these frames is called a TAC. However, absolute radioactivity concentrations cannot be compared between different subjects (animals) who have different body weights and received different activities. Therefore, the radioactivity concentration needs to be normalized to the subject's body weight and injected radioactivity. The two most popular normalization methods are:

(a) Expressing radioactivity concentrations as % injected dose per mL tissue (%ID/mL, %ID/g for ex vivo studies where tissue samples are weighed);
(b) Expressing radioactivity concentrations as SUVs, which correspond to the ratio of actual radioactivity concentration in a given ROI or voxel to the average radioactivity concentration in the whole body. The average radioactivity concentration is easily calculated by dividing the injected radioactivity by the subject's body weight, and then used to convert radioactivity concentrations in individual ROIs/voxels into SUVs.

Local radioactivity concentrations expressed as %ID/mL or SUVs can be aggregated from different test subjects to assess the radiotracer uptake variability and compare the average magnitude of this uptake in each ROI for different experimental groups.

However, the most relevant parameters for CNS radiotracers are their affinity towards their targets (Kd) and the density of those targets in the regions of interest (B_{max}). These parameters cannot be obtained directly from radiotracer uptake measurements because they only directly translate into concrete radiotracer concentrations when the target binding of the radiotracer, as well as its influx and efflux from the brain, are at equilibrium. It is possible to achieve a constant radiotracer concentration in plasma by injecting a bolus of the radiotracer and then applying a constant infusion of the same radiotracer. In that case, the radiotracer in plasma will be equilibrated with the radiotracer in the brain, and the equilibrium tissue/plasma ratios can be measured directly. However, this set-up is technical. Simple bolus injection of the tracer is much more widely used. Moreover, some tracers bind to their target in an irreversible manner, so that equilibrium can never be reached. Hence, there is a need for quantifying the binding parameters of the tracers from non-equilibrium time activity curves by means of kinetic modelling. Kinetic modelling approaches are discussed in detail in Section 8.

6.10. GO/NO-GO CRITERIA TO MOVE FURTHER TO CLINICAL TRANSLATION

To warrant further translation of a candidate CNS radiotracer, the following needs to be established:

(a) The radiotracer should have sufficient target specificity, i.e. its estimated B_{max}/K_d ratio (based on in vitro data) for the intended target in the intended ROI should be at least 10 fold higher than for all other targets to which the radiotracer can bind;
(b) The radiotracer should penetrate across the BBB in sufficient amounts to generate strong target specific signals. The rule of thumb threshold value is a peak brain concentration corresponding to an SUV of 1.5 or more. Efflux pumps in rodents are known to have higher activity than in large animals or humans, therefore an efflux pump blockade may be applied during the initial evaluation in rodents. If such, evaluation shows promising results in terms of other parameters except BBB penetration, then BBB penetration should be assessed later in larger species;

(c) Sufficient specific binding should be observed within a feasible scanning time (< 90 min). The desired specific to non-specific binding ratio is 2 or more, corresponding to $BP_{ND} > 1$. For robust kinetic modelling, the washout rate of a target bound tracer needs to be properly quantified, hence the specific binding should preferably be reversible within the timeframe of the scan;

(d) No lipophilic metabolites should be detected in the brain tissue since they confound the interpretation of imaging data. Ideally, no radiometabolites should be formed until completion of the PET scan, but if the tracer is metabolized (which happens in most cases), then all radiometabolites should be much more hydrophilic than the parent tracer.

Another two important parameters for the clinical translation of CNS radiotracers are the toxicity of the tracer molecule, and the tissue and organ doses resulting from tracer administration. Excessive toxicity or high radiation dose incurred by a non-target organ (organs excreting the tracer such as liver or kidneys are the most vulnerable) can prevent the candidate tracer from being translated to humans. However, it only becomes worthwhile to conduct a whole body dosimetry assessment for a radiotracer after it has shown sufficiently good brain penetration, target selectivity, specific binding and metabolic profile. With regards to toxicity risks, they are greatly reduced for compounds with the intended use as diagnostic radiotracers, because they will only be administered to humans in microdose amounts.

6.11. CONCLUSION

Preclinical in vivo testing is carried out to predict how a radiopharmaceutical behaves in humans in order to come up with the best design for the first in-human evaluation. In recent years, it became more and more obvious that species differences have to be strongly considered while analysing the translatability from animal to humans. Differences in protein concentration, metabolism profile or efflux transporter dependency are only some examples that vary from species to species. Different outcomes have been seen in rodents, pigs, NHP and humans as well as in naïve and diseased models. Discrepancies in the predictions made for human data from non-human primate data and vice versa are especially astonishing. Consequently, rejecting a radiopharmaceutical to be translated from animals into humans needs to be carefully considered. A case by case scenario applies. Future studies need to focus on predictability and reveal in which cases results from animals can be translated into the human situation, in which cases they cannot and how to improve this predictability.

7. TRANSLATION TO THE CLINIC

Before new PET tracers can be used in humans, non-clinical safety studies need to be conducted. Recommendations on the required non-clinical safety studies can be found in an international guideline, entitled ICH M3 (R2) [114] 'PET tracers for imaging of targets in the brain are typically non-carrier added radiolabelled small organic molecules that are usually administered once, or at most only a few times. Consequently, the injected mass of the PET tracer is sufficiently small that the microdose approaches in the Section 7.1 of the ICH M3 (R2) guideline apply. According to the ICH guideline M3(R2) the recommended non-clinical study for microdose applications in humans should include assessment of non-clinical pharmacology, pharmacokinetics, toxicity and dosimetry. The results of these non-clinical safety studies should be included in the IMPD, investigational new drug application, or a similar document that should accompany the clinical trial application of the new PET tracer.

7.1. NON-CLINICAL PHARMACOLOGY AND PHARMACOKINETICS

Non-clinical pharmacology studies need to include a proper characterization of the target binding properties of the tracer, as well as its off-target binding and non-specific binding. Typically, in vivo tracer binding properties are assessed in competition studies, in which a selective competitor for an on-target or off-target binding side is administered prior to the tracer (blocking studies), or during the acquisition of the PET scan (displacement studies). Such studies have been described in more detail in

Section 6. When PET tracers are administered in microdose quantities, pharmacological effects are not expected. In case of extremely potent radioligands, the verification dose to be administered to patients may be required to be less than 1/100th of the pharmacologically active dose (scaled on mg/kg basis). Furthermore, adequate estimates of the pharmacokinetics of the tracer are required. Plasma clearance and target binding kinetics can be estimated from dynamic PET scans and serial arterial blood samples obtained in kinetic modelling studies, as will be described in Section 8. Since PET only measures radioactivity, irrespective of its source, tracer metabolism also needs to be investigated. For CNS PET tracers, it is particularly important that radioactive metabolites do not cross the BBB. Therefore, not only plasma samples, but also brain tissue needs to be investigated for the presence of metabolites, as is described in Section 6. Small amounts of metabolites in the brain may be treated as being part of the non-specific binding compartment, provided that the metabolites do not bind to the target receptor. Non-clinical pharmacology and pharmacokinetics studies need to be performed in a pharmacodynamic relevant animal model, i.e. a model that properly reflects the target expression levels that are observed in humans, as well as the changes in target expression due to the disease, disease progression and treatment.

7.2. SPECIES DIFFERENCES

The metabolic and pharmacokinetic data obtained in preclinical studies need to be carefully extrapolated to humans. For CNS radioligands, species differences may result in significant differences in the imaging quality and measurement accuracy. However, almost no significant effects on safety are expected because the injected amounts of radiotracer are at the sub-nanomolar level. The effect of species differentiates on image quality and measurement accuracy come from various factors, including species differences in drug metabolism, expression levels of drug efflux transporter, pharmacokinetics, etc. which will affect the tracer clearance and distribution. An example of a clinically applied PET tracer that shows large species differences in metabolic stability, is the estrogen receptor ligand [18F]FES. In humans, [18F]FES is bound to the sex hormone binding globulin in plasma and as a result it is protected from metabolic degradation [115]. In rodents, however, the tracer is rapidly degraded, because they don't have any sex hormone binding globulin. Consequently, specific binding of [18F]FES in rodents is substantially lower than in humans. Also, differences in the expression of endogenous compounds that compete with the PET tracer for the same binding site could be a reason for species differences. For [18F]FLT, for example, this phenomenon is the cause for species differences. Rats have much higher plasma concentrations of thymidine than humans. This endogenous thymidine strongly reduces tracer accumulation, because [18F]FLT and thymidine are carried across the cell membrane by the same transporter and are both phosphorylated by the same thymidine kinase enzyme [116].

Another possible factor resulting in species differences is related to the differences in the target molecule itself in the CNS. Differences in target molecule expression levels and binding affinity can have a large impact on the usefulness and predictive value of preclinical results in the tracer development process.

A typical example of species differences in receptor expression level on metabotropic glutamate receptor type 1 (mGluR1) kinetic analysis of [11C]ITMM is presented here [117-119]:

(a) The mGluR1 expression levels are high in rodents, middle in humans, and low in NHPs (Table 11);
(b) In NHPs and humans, the mGluR1 expression can be quantified with [11C]ITMM in target regions with the highest mGluR1 expression (cerebellum), but it proved difficult to quantify the mGluR1 expression in brain regions with a moderate receptor expression (thalamus and striatum);
(c) In rodents, [11C]ITMM PET can be used to quantify the mGluR1 expression in regions with a moderate receptor expression, such as thalamus and striatum.

Species differences in receptor density and affinity of the radioligand in large part account for the differences in the behaviour of [11C]ITMM in rodents, NHPs and humans (Table 11).

TABLE 11. SPECIES DIFFERENCE OF mGluR1 DENSITY AND THE [^{11}C]ITMM OUTCOME MEASURES, BP (BP_{ND}) AND VOLUME OF DISTRIBUTION (V_T)

Region	Rat		Monkey		Human	
	B_{max} (nM)*	BP_{ND} (111)	B_{max} (nM)*	V_T (112)	B_{max} (nM)*	V_T (113)
Cortex	47.1	1.9	10	1.3	26	0.7
Hippocampus	68.8			0.9		
Striatum	74.1	2.8		0.9		0.5
Cerebellum	403.2	4.7	53	2.5	82	2.6

Region	Rat		Monkey		Human	
	B_{max} (nM)*	BP_{ND} (111)	B_{max} (nM)*	V_T (112)	B_{max} (nM)*	V_T (113)
Cerebellum/Cortex	9.1	2.5	5.3	1.9	3.2	3.7
B_{max}/K_D						
Cortex		3.7		0.8		2.0
Striatum		5.9				
Cerebellum		34.1		4.2		6.5

B_{max} data was obtained from [120]

7.3. TOXICITY STUDIES

This section describes preclinical toxicity studies to be performed in the CNS radioligands, especially for short half-life positron labelled small molecule organic compounds, before the ligand is used clinically for the first time at the intended medical institution. The toxicity studies need to be performed under established reliability criteria such as good laboratory practice. Available non-clinical or clinical safety study data produced by other institutions are to be used if confirmed to be reliable. Further discussion on requirements for toxicological studies in radiopharmaceuticals is provided in a position paper of the Radiopharmacy Committee of the European Association of Nuclear Medicine (EANM) [121].

7.3.1. Test substances

Toxicity studies of CNS PET tracers are conducted with the active ingredient (unlabelled; may be synthesized with an alternative process) or the labelled final dosage form (which meets the quality criteria and has undergone radioactive decay) as test substances. Studies are conducted at a dose that is at least 100 fold the clinical dose as calculated based on body weight. The ICH M3 guideline [114] recommends a maximum dose of 1000 fold the dose to be administered to humans on a mg/kg basis for intravenously injected tracers.

7.3.2. General guidance and consideration of preclinical toxicity studies

The ICH harmonized tripartite guidance describes approaches to single and repeated dose studies that conform to the requirements of the following notifications by the ICH Consensus Guideline on microdose studies and non-microdose exploratory clinical studies [114].

Although these notifications require preclinical pharmacology studies for setting the clinical dosage through the estimation of the effective dose based on preclinical data, preclinical pharmacology studies to estimate the effective dose are not typically performed for PET tracers, because extremely low dose PET ligands are not expected to produce the pharmacologic effects associated with a therapeutic dose of the ligands. Guidance issued by the U.S. Food and Drug Administration uses weight based (rather than pharmacologic effect based) criteria for imaging agents [122]. Safety pharmacology studies,

however, may be required for CNS PET tracers. This could have agonistic activity even in tracer amounts, such as opioid receptor ligands and nicotinic acetylcholine receptor ligands.

The ICH M3 guideline [114] describes two approaches for non-clinical general toxicity studies to support microdose trials that can be applied to CNS PET tracers:

    (a) Approach 1: If the total dose to be administered to any subject does not exceed 100 μg, either as a single dose or divided doses, an extended single dose toxicity study can be performed;

    (b) Approach 2: If the total cumulative dose does not exceed 500 μg (given max 5 administrations of not more than 100 μg with an interval of at least 6 half-lives), a 7 day repeated dose toxicity study needs to be performed.

Since the administered dose per injection of an CNS PET tracer is usually less than 10 μg, in almost all cases approach 1 can be applied.

7.3.3. Extended single dose toxicity studies

Guidance for the toxicity studies required for human studies, in which the total administered tracer dose ≤100 μg, is presented here:

    (a) Administer a dose that is 100 fold to 1000 fold the clinical dose as calculated based on body weight. On the following day, perform haematology tests, blood chemistry tests and necropsy, and histopathological examination. Evaluate delayed toxicity and recovery two weeks post dose. In typically designed studies in rodents, ten animals of each sex are assigned to each group evaluated in the tests on the day after its administration, and five animals of each sex are assigned to the group(s) selected for evaluation on Day 14. In typically designed studies in non-rodents, three animals of each sex are assigned to each group evaluated in the tests on the day after its administration, and two animals of each sex are assigned to the group(s) evaluated on Day 14;

    (b) Perform the studies in both sexes of one species (normally a rodent species);

    (c) Administer the test substances intravenously. Perform a toxicokinetic study if using another route of administration;

    (d) No genotoxicity study needs to be performed if the individual amounts of compounds and impurities contained in the final dosage form do not exceed 100 μg. An acceptable daily intake of 120 μg of genotoxic impurities assumes continuous exposure over 1 month [123]. This figure was calculated based on an excess cancer risk of no more than 1 in a million. Any information from studies or structure activity relationship assessment suggesting genotoxicity obtained in the process of a nonclinical study needs to be included in the toxicity study results;

    (e) Investigations of local irritation of the test substance are not recommended, because of the trace doses involved. However, if the final drug formulation uses a new vehicle for intravenous administration, local irritation of the vehicle needs to be evaluated.

7.3.4. Seven day repeated dose toxicity studies

Guidance for the toxicity studies required for repeated dose studies with ≤5 administrations of ≤100 μg per administration and a cumulative dose ≤500 μg is presented here:

    (a) Perform the studies as 7 days repeated dose toxicity studies that match the clinical dosing regimen and at a dose that is at least 100 fold, but not more than 1000 fold, the clinical dose as calculated based on body weight;

    (b) On the following day, perform haematology tests, blood chemistry tests, necropsy, and a histopathological examination. Evaluate delayed toxicity and recovery 2 weeks post dose;

(c) Genotoxicity does not need to be evaluated if the individual amounts of compounds and impurities contained in a single dose do not exceed 100 μg and the total amount in a single dose does not exceed 500 μg. Any information from studies or structure activity relationship assessment suggesting genotoxicity obtained in the process of a nonclinical study needs to be included in the toxicity study results;

(d) The other requirements are identical to those for single dose studies.

7.3.5. Threshold of toxicological concern

The position paper by the Radiopharmacy Committee of the EANM proposes that toxicity tests may not be required for PET tracers administered at a dose <1.5 μg, as judged on a case by case basis [121]. This proposal was based on the threshold of toxicological concern concept, as described in [124]. According to the threshold of toxicological concept in this guideline, an intake of 1.5 μg of a mutagenic impurity is associated with a negligible risk (<1 per 100 000) and therefore can be used as an acceptable limit for most pharmaceuticals. It should be noted, however, that the IHC M7 guideline [124] applies to mutagenic impurities in pharmaceuticals and does not refer to the active ingredient. The proposal by the Radiopharmacy Committee of the EANM [124] has not been implemented in guidelines for non-clinical safety studies yet and application of the threshold of toxicological concept needs to be discussed beforehand with the local competent authorities.

7.4. DOSIMETRY, ALLOMETRIC SCALING

Estimation of human radiation dose is mandatory for the development of new CNS radioligands before entering a human clinical study. Internal radiation exposure in humans is estimated based on animal study data using an established methodology, such as that of the Committee on Medical Internal Radiation Dose of the Society of Nuclear Medicine and Molecular Imaging of the United States. Tissue dissection using rodents was the most common method used for estimating radiation dose with extrapolation of animal data to human data normalized to interspecies variations. These estimated human radiation dose data are used to determine the maximum allowable injection dose in human clinical studies. Clinical exposure data produced by other medical institutions are used if confirmed to be reliable.

7.4.1. General consideration of acceptable dose for clinical trial volunteers

Most of the human effective dose (1–10 mSv for each administration) of the current CNS PET tracers fall into Category IIb risk (Table 12) [125]. For Category risk Iib, the social benefit will be related to an increase in knowledge of disease cure or prevention. It is undesirable for the same volunteer to repeatedly take part in investigations involving radiation exposure; therefore, radiation and ethical committees need to ascertain that this is not occurring. Investigators need to address this aspect as part of their submission for approval of a proposed study.

Although the population based ICRP risk classification, as presented in Table 12, is widely accepted, it does not take the characteristics of individual subjects into account, such as concurrent treatment (e.g. radiotherapy), life expectancy and gender. If PET tracers will only be used in a specific study population, age and gender, adjusted risk categories could be applied.

TABLE 12. RISK CATEGORIES AND CORRESPONDING LEVELS OF SOCIAL BENEFIT [125]

Level Of Risk	Risk Category (Total Risk)	Corresponding Effective Dose Range (Adults) MSV	Level Of Social Benefit
Trivial	Category I (Less than 10^{-6})	<0.1	Minor
Moderate to intermediate	Category IIa (10^{-6}–10^{-5})	0.1–1	Intermediate to moderate
	Category IIb (10^{-5}–10^{-4})	1–10	
Moderate	Category III (More than 10^{-4})	>10*	Substantial

To be kept below deterministic thresholds except for therapeutic experiments

7.4.2.   Estimating radiation exposure from rodent biodistribution

The classical dissection method is easily employed in many facilities at a relatively low cost. Below are the tips for biodistribution study especially for dosimetry evaluation:

(a) Dissection time points are selected in such a way that the clearance of radioactivity from the organs is sufficiently covered, especially in the case of slowly excreted tracers labelled with ^{18}F or longer lived radionuclides.
(b) Leaked urine at the time of euthanasia and bladder contents is recovered and added to the bladder data for the calculation.
(c) The residence times of relevant organs is calculated by integration of the area under the decay uncorrected mean time–activity curves, extrapolated to infinity. Residence times for a 70 kg adult human male phantom are estimated from the measured rat residence times based on the difference in tissue to body weight ratio (g/kg) [126].
(d) The radiation absorbed dose and effective dose for human adults are estimated using OLINDA/EXM software [127].

As an alternative for dosimetry estimation based on ex vivo biodistribution studies, dosimetry calculations could be derived from serial whole-whole body PET scans of the animal [128]. Such an imaging approach could strongly reduce the number of laboratory animals required and avoid spillage of body fluids like urine. A challenge of imaging based dosimetry in small animals could be the delineation of major organs in the images. Since PET may not provide sufficient anatomical information, simultaneous acquisition of a CT or MRI scan for organ delineation may be required. To avoid partial volume effects, small regions of interest need to be drawn that represent the content of the whole organ.

Although the rodent derived effective doses are roughly comparable to the human derived doses in most cases [129], sometimes considerable differences are found for critical organ dose estimates and pharmacokinetics in certain cases. Therefore, human dosimetry studies for radiation risk assessment may still be necessary for the initial clinical application of new CNS radioligands. In the case of ultra short half-lived PET radionuclides such as ^{11}C ($t_{1/2}$ = 20 min), dosimetry studies can be abandoned [130]. Because the radioactivity decay is much faster than the elimination of radioactive compounds from the body, it results in the effective dose < 5 mSv/MBq.

7.5. INVESTIGATIONAL MEDICINAL PRODUCT DOSSIER

Besides the successful completion of the non-clinical studies, the production of the PET tracer needs to be validated according GMP guidelines before the tracer can be applied in humans. In Europe, an IMPD has to be prepared as part of the clinical trial application process. In other parts of the world, documents with a similar content are required (e.g., the investigational new drug application in the USA). The IMPD should provide not only a summary of the results of non-clinical pharmacology and toxicology studies (and clinical data, if available), but also a large amount of data related to the production tracer, including a description of the properties of the radiopharmaceutical, the manufacturing process, the formulation, potential impurities, the procedures for quality control, the process validation results and

stability. The EANM has published a practical guideline for the preparation of an IMPD, dedicated to radiopharmaceuticals [131]. Typical examples of IMPDs of PET tracers have been attached to the EANM guideline that can be adjusted to the situation a specific institution. Since national regulations may vary, the competent authorities should always be contacted to verify the local requirements.

8. PRECLINICAL KINETIC MODELLING AND QUANTIFICATION OF BRAIN PET TRACERS

Although PET imaging is generally considered as a quantitative in vivo brain imaging technique, it is not straightforward to relate the PET signal to the underlying pathophysiological parameters of interest, such as, amongst others, cerebral blood flow, metabolism, receptor expression level or enzymatic activity. The aim of kinetic modelling is to better understand and define the relationship between PET measurements and underlying brain tissue parameters and, especially for new PET tracers, to demonstrate to which extent the PET signal indeed depends only on the actual brain tissue parameters of interest or whether other parameters are also of importance. Specifically on a preclinical level, kinetic modelling data provide valuable information for the potential clinical translation of new PET ligands, as it can confirm target engagement, elucidate binding characteristics and establish whether binding can be considered as reversible or irreversible, thus helping to define clinical PET imaging protocols for both accurate and precise quantification. In addition, kinetic modelling provides reference data to evaluate possible simplifications of imaging procedures, even at the preclinical level, and to define simple and accurate patient protocols to broaden clinical applicability of a PET tracer.

In this section, first the quantitative endpoints of kinetic modelling are discussed together with different approaches to estimate these endpoints. Next, an overview of different input functions is given to account for changes in tracer availability, which is followed by a presentation of simplified quantification approaches. Finally, although most approaches are generic and valid for both clinical and preclinical PET imaging, some comments that are specific for small animal PET imaging are given.

For the remainder of this section, it is assumed that PET imaging is performed with the tracer principle being valid, i.e. that the amount of injected tracer is a trace amount and does not cause pharmacological effects or changes in brain function. In addition, the pathophysiological brain parameters of interest need to be imaged in a steady state, i.e. they are not changing themselves during a PET scan. From a methodological point of view, it is assumed that appropriate algorithms have been used to provide reconstructed PET data that are proportional to the radiotracer concentration in brain tissue and accurately represent the underlying radiotracer distribution in the brain.

8.1. QUANTITATIVE ENDPOINTS

Although PET radiotracers are selected for their high affinity and selective binding to a specific target of interest, they can also show non-specific, off-target binding to molecules that are structurally or functionally similar to the main target of interest. Therefore, the time varying PET signal or activity concentration per unit brain tissue is not only determined by the in situ specific binding of the PET tracer to the target of interest, but also by non-specific binding and the amount of free tracer in blood and brain tissue [132]. As such, the measured PET signal $C_{PET}(t)$ can be represented as the superposition of different signals as shown in Eq. 2:

$$C_{PET}(t) = (1 - V_B)(C_F(t) + C_{NS}(t) + C_S(t)) + V_B C_B(t) \qquad (2)$$

Where:
t represents the time after tracer injection;
$C_F(t)$ is the tissue concentration of free or unbound tracer;
$C_{NS}(t)$ and $C_S(t)$ are the tissue concentrations of non-specific and specific bound tracer, respectively.

Often, the tissue concentration of non-displaceable tracer binding $C_{ND}(t)$ is considered as the sum of the free tracer concentration $C_F(t)$ and the concentration of non-specifically bound tracer $C_{NS}(t)$ (Fig.

42). In addition, $C_B(t)$ represents the tracer concentration in arterial blood while V_B represents the fractional blood volume within the tissue of interest ($0 \leq VB \leq 1$).
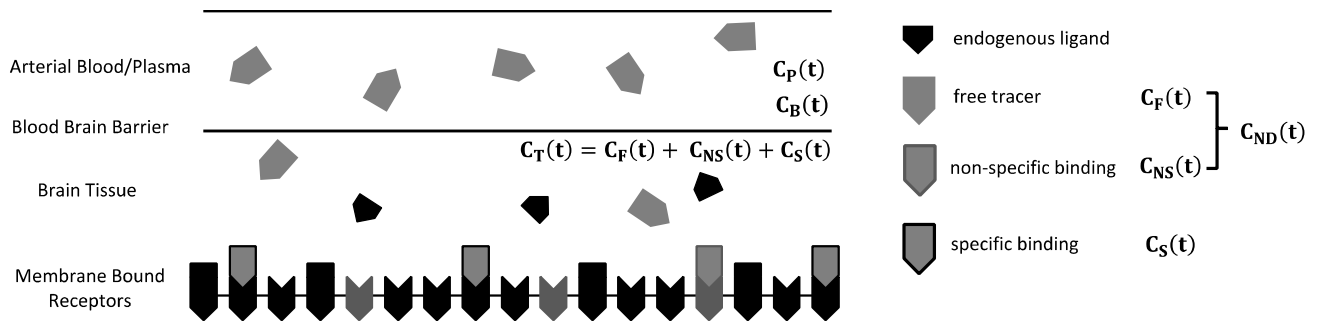
FIG. 41. Schematic representation of the different states of a PET receptor ligand in brain tissue (Courtesy of M. Koole, Catholic University of Leuven).

For PET tracers with reversible binding kinetics such that the tracer is also cleared from brain tissue, a tissue distribution volume V_T can be defined as the ratio, at equilibrium, of the total tissue tracer concentration C_T to the parent radioligand concentration in arterial plasma C_P, separated from possible radiometabolites: see Eqs (3) and (4):

$$V_T = \frac{C_T}{C_P} = \frac{C_F + C_{NS} + C_S}{C_P} = V_F + V_{NS} + V_S \tag{3}$$

$$= \frac{C_{ND} + C_S}{C_P} = V_{ND} + V_S \tag{4}$$

which can be divided into different tissue distribution volumes (V_F, V_{NS}, V_S and V_{ND}) corresponding to the different states of the tracer in tissue (C_F, C_{NS}, C_S and C_{ND}, respectively). This is based on the assumption that radioactive metabolites are not entering brain tissue and only a free, intact tracer in arterial plasma is functionally active and interacting with the target of interest.

In addition, the BP (relative to the non-displaceable compartment) BP_{ND} can be defined as in Eq. (5):

$$BP_{ND} = \frac{V_S}{V_{ND}} = \frac{V_T - V_{ND}}{V_{ND}} \sim \frac{C_S}{C_F} = \frac{B_{avail}}{K_D} = \frac{k_{on} B_{avail}}{k_{off}} \tag{5}$$

which is directly related to the concentration of available targets B_{avail} and the affinity of the tracer $1/K_D$ for the target. Provided there is sufficiently high specific activity, such that it can be assumed that the tracer principle is valid, BP_{ND} will be proportional to the number of available receptors, i.e. those receptors that are not occupied by endogenous ligands or by administered drugs.

If the binding affinity of the PET tracer and/or density of binding sites are high, tracer kinetics can become very slow with a corresponding high BP_{ND} or V_T. For these tracers, BP_{ND} or V_T can only be estimated reliably with long scanning times in order to measure and quantify the slow tracer clearance rate from brain tissue. However, if the tracer clearance rate from the tissue becomes too slow or tracer binding is irreversible because of trapping or metabolism of the tracer, the trapping or net influx rate constant Ki, representing inward transport and irreversible binding of the radiotracer, needs to be considered as a quantitative endpoint even when tracer binding is reversible, but too slow to be captured with a reasonable scanning time.

8.2. TRACER KINETIC MODELLING

In steady state PET studies where a PET tracer is constantly infused to maintain tracer concentration in arterial blood at a constant level, the distribution volume is easily derived from constant concentration

ratios in equilibrium. However, in general PET scanning is not performed under equilibrium conditions as the time varying brain tissue response is measured after bolus injection of the tracer, so that equilibrium conditions are not reached during the PET scan. In order to still derive an accurate estimation of the appropriate quantitative endpoints, a kinetic model is applied to relate the time varying tracer concentration in tissue, measured with a dynamic PET scan, with the input function to brain tissue represented by the time dependent tracer concentration in arterial plasma following bolus injection. The most common kinetic modelling approach is based on compartmental models where the tracer concentration in each compartment is assumed to be homogeneously distributed and where different compartments are considered to describe not only the time varying concentration in physically different compartments, such as plasma and brain tissue, but also the different time varying (binding) states of the tracer in brain tissue [133]. Hence, a three tissue compartment model would be needed to describe the kinetics of a free, specifically bound, and a non-specifically bound tracer in tissue as presented in Fig. 43. However, in general, the information contained within a dynamic PET data is too limited to allow for a robust distinction between the kinetics of these three kinetic compartments. Therefore, the complexity of a three tissue compartment model is often reduced to a 2TCM by assuming that the exchange of tracer between free and non-specifically bound tracer compartments is much faster than the exchange between free and specifically bound tracer compartments. As such, a 2TCM consists of a merged (non-displaceable) compartment representing both free and non-specifically bound tracer concentration in tissue and a second compartment representing the specific tracer concentration. In addition, a third compartment (or better source) represents intact tracer concentration in arterial plasma (see Fig. 43).
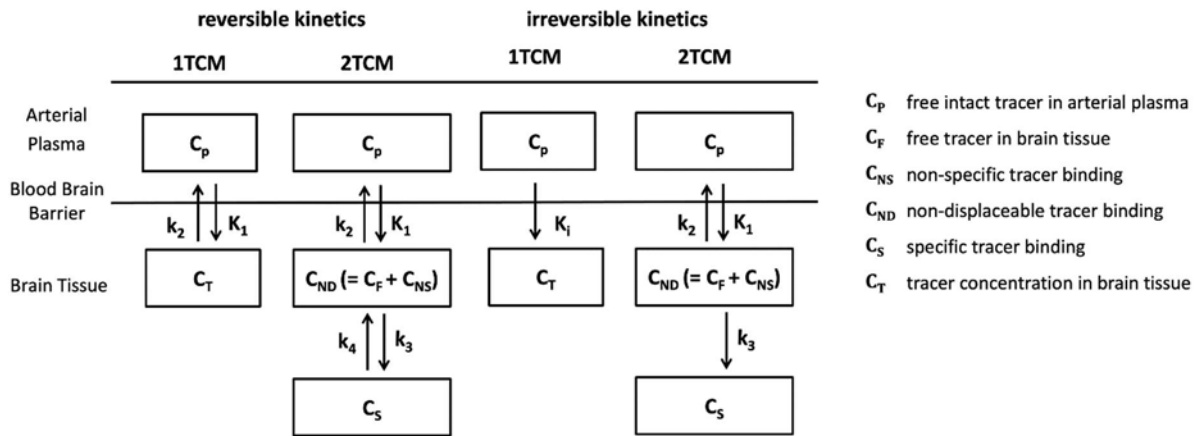
FIG. 42. Schematic overview of the different compartments for reversible one and two tissue compartment models (1TCM-2TCM) and for irreversible tracer kinetics together with the corresponding exchange or transport rate constants (Courtesy of M. Koole, Catholic University of Leuven).

This reduced, two tissue compartment model can be described by two differential equations where tracer concentration in each compartment is considered to be zero at tracer injection (and start of the dynamic PET scan), and where tracer concentration in the arterial plasma is driving the model: see Eqs (6) and (7):

$$\frac{dC_{ND}(t)}{dt} = K_1 C_P(t) - (k_2 + k_3)C_{ND}(t) + k_4 C_S(t) \tag{6}$$

$$\frac{dC_S(t)}{dt} = k_3 C_{ND}(t) - k_4 C_S(t) \tag{7}$$

K_1, k_2, k_3 and k_4 are the exchange or transport rate constants, which describe the exchange of tracers between different compartments, and which are representative for the steady state of the underlying

system for a given PET radioligand. K_1 includes a perfusion dependent component. It is indicated by a capital letter to indicate a change of medium (i.e. from plasma to tissue) and has mL∗cm^{-3}∗min^{-1} as unit, whereas k_2, k_3, and k_4 indicate the fraction of tracer transferred per unit time and have min^{-1} as unit. These exchange rate constants are also assumed to be time invariant over at least the duration of the PET scan. By setting the derivatives $\frac{dC_{F+NS}(t)}{dt}$ and $\frac{dC_S(t)}{dt}$ to zero which corresponds to equilibrium conditions, the exchange rate constants can be used to derive quantitative endpoints such as tissue distribution volume or BP, as per Eqs (8) and (9):

$$BP_{ND} = \frac{k_3}{k_4} \tag{8}$$

$$V_T = \frac{K_1}{k_2}\left(1 + \frac{k_3}{k_4}\right) \tag{9}$$

If the compartments of non-displaceable and specific tracer binding are kinetically undistinguishable because of a fast exchange of tracer between both compartments, the compartment model can be further simplified to a single tissue compartment (1TCM), represented by only one differential equation, as per Eq. (10):

$$\frac{dC_T(t)}{dt} = K_1 C_P(t) - k_2 C_T(t) \tag{10}$$

With the washout rate constant from tissue k_2 being reduced by the specific tracer binding and with the tissue distribution volume $V_T = \frac{K_1}{k_2}$ being the only quantitative endpoint.

If k_4 becomes too small, an irreversible 2TCM is described by Eqs (11) and (12):

$$\frac{dC_{ND}(t)}{dt} = K_1 C_P(t) - k_2 C_{ND}(t) - k_3 C_{ND}(t) \tag{11}$$

$$\frac{dC_S(t)}{dt} = k_3 C_{ND}(t) \tag{12}$$

When a steady state is reached in the compartment with non-displaceable tracer binding such that $\frac{dC_{ND}(t)}{dt} = 0$ and $C_{ND}(t) = \frac{K_1}{k_2+k_3} C_P(t)$, this results in the following differential equation for the specifically bound tracer concentration Eq. (13):

$$\frac{dC_S(t)}{dt} = \frac{K_1 k_3}{k_2+k_3} C_P(t) = K_i C_P(t) \tag{13}$$

with the influx or trapping rate constant K_i having the same unit mL·cm^{-3}·min^{-1} as K_1.

Kinetic parameters can be estimated by fitting an analytical solution of these differential equations to the measured dynamic PET data. On the other hand, nonlinear fitting procedures are needed which are time consuming and sensitive to noise, which make them less suitable for estimating kinetic parameters on a voxel basis. However, the coupled differential equations can be reformulated in a linear form by transforming both arterial plasma data and measured dynamic PET data. Using this approach, transfer rate constants are not estimated separately, but the slope of a straight line after a certain time t* corresponds to either V_T (for reversible tracers) using the Logan plot [134] or K_i (for irreversible tracers) using the Patlak plot [135]. As such, the presence of a straight line gives an immediate visual confirmation of reversible or irreversible tracer kinetics, while the slope allows immediate visual assessment of tissue distribution volume of uptake rate. In addition, these linearised models allow for optimal estimates to be computed directly in one iteration using linear regression. Consequently, these linear least squares fitting methods are easy to implement, computationally very efficient and therefore

very convenient for generating voxel wise parametric images. Moreover, graphical methods rely on the area under the curve of the measured data. Since, for a bolus injection, the peak contribution to the total area under the curve of the input function is limited, these approaches are less sensitive to inaccuracies in peak estimation. On the other hand, a linearised approach does not give information on any of the rate constants k_1 to k_4 and, therefore, it is not possible to distinguish a change in transport (perfusion) from a change in binding. In addition, the optimal time t* may depend on the actual kinetics within each voxel.

From a translational point of view, it should be noted that once a valid kinetic modelling approach has been established for preclinical imaging of a PET tracer, care is still needed with a straightforward clinical translation because of potential species differences, especially since it can be expected that both tracer metabolism and tracer kinetics are faster in animals.

The choice for a specific model is governed by various factors including the properties of the tracer. If the tracer is inert and simply diffuses into and out of brain tissue, 1TCM would be an appropriate model while 2TCM considers the retention of the tracer in brain tissue because of specific binding, while still assuming reversible tracer kinetics. Measures such as the akaike information criterium [136–138], model selection criterion which is a reciprocal modification of akaike information criterium or bayesian information criterion can be used to select the most appropriate model configuration by considering both model complexity and a smaller residual sum-of-squares error due to a better fit. While akaike information criterium is a commonly used method for model comparison, conclusions are generally in line with other model selection methods.

Reliable estimates of tissue distribution volumes using either 1TCM or 2TCM, require both an uptake and a washout phase with the clearance rate of the tracer matching the half-life of the PET radionuclide. This tissue clearance rate is in part determined by the affinity of the tracer for the target and by the target density. Ligands with higher affinity targeting a rather dense target population tend to stick longer to the target molecules, often delaying washout beyond the usable measurement time of a PET radionuclide. If the affinity is such that the radioligand shows only very modest washout from brain tissue during the PET measurement, the washout rate cannot be determined reliably, thereby hampering the calculation of macroparameters such as V_T or BP$_{ND}$. In terms of transport rate constants, also known as microparameters, this means that a reliable estimation of k_4 cannot be obtained. In this case and in cases where the tracer is metabolized or trapped in tissue, tracer binding needs to be considered irreversible and k_4 set to zero. Instead of V_T or BP$_{ND}$, the influx rate constant K_i, also named metabolic rate (for metabolic tracers), the trapping rate, or accumulation rate constant, should be considered as the quantitative endpoint. This assumption is, for instance, valid for measuring energy metabolism using the glucose analogue 2-fluoro-2-deoxy-d-glucose labelled with ^{18}F ([^{18}F]FDG). This glucose analogue is trapped in brain tissue after being metabolized in the mitochondria to FDG-6-PO4 by the hexokinase enzymatic action. For a PET measurement up to 1 h post injection, dephosphorylation (k_4) of the FDG-6-PO4 is not observed and the assumption that $k_4 = 0$ is valid.

8.3. INPUT DATA FOR PET KINETIC MODELLING

To apply kinetic modelling, the so called TAC or radiotracer concentration over time in brain tissue or even individual voxels need to be accompanied by the time dependent activity concentration of intact tracer in arterial plasma.

8.3.1. Time activity curve

A TAC for a brain ROI can be extracted from the dynamic PET scan, which measures the radiotracer distribution in the brain at successive time points after tracer injection. However, the extraction of an appropriate TAC that represents tracer kinetics in the underlying brain region can be challenging because of the limited PET resolution and hence associated partial volume effects with surrounding tissues, especially in small laboratory animals. In addition, manual delineation of specific brain regions can be time consuming and observer dependent. Therefore, in human applications, methodologies have been

developed that allow for automatic volume of interest generation using an atlas based approach [139] (Fig. 44), where dynamic PET data are spatially normalized to a standard space, such as Montreal Neurological Institute (space, using a non-linear transformation). This spatial normalization allows anatomically predefined brain regions in standard space to be projected onto the dynamic PET data to extract the corresponding regional TACs. If a high resolution anatomical MR is available, the MR dataset can be used to achieve a more accurate spatial normalization that doesn't depend on quality or the distribution pattern of the dynamic PET data. Moreover, high resolution anatomical MR data can be used to correct for partial volume effects between different tissue classes such as grey matter, white matter and cerebrospinal fluid or between different brain regions [140]. This especially applies when comparing healthy volunteers with elderly subjects or with patients where regional cerebral atrophy is suspected. However, the optimal use of partial volume correction techniques depends on the brain pathology under investigation, the characteristics of the PET tracer that was used, and the brain regions of interest, and therefore need to be considered carefully for each application separately [141, 142].
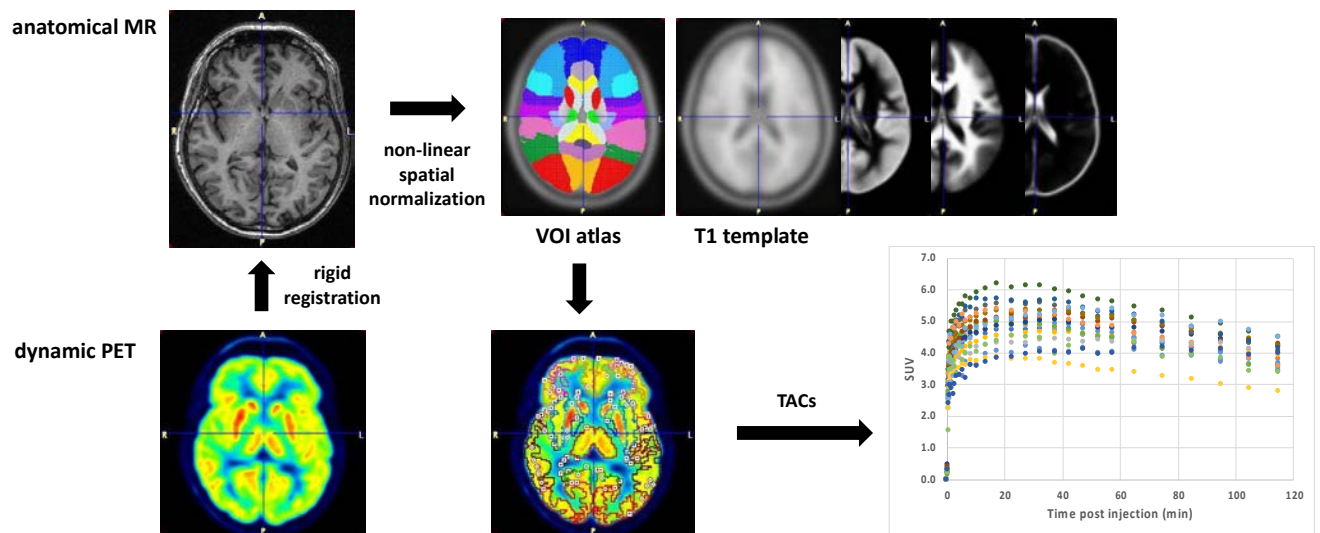
FIG. 43. Processing pipeline for an atlas-based approach to automatically define brain regions and extract the corresponding time activity curves from the dynamic human PET data (Courtesy of M. Koole, Catholic University of Leuven).

For small animal brain PET imaging, similar atlas based approaches can also be considered, projecting anatomically predefined brain regions in standard space, such as the Paxinos space, onto the dynamic PET data after the spatial normalization to extract the corresponding regional TACs.

8.3.2. Input functions

In addition to a tissue TAC, an input function is needed, which represents the radiotracer concentration delivered to the brain. Even if the tracer resides in both plasma and blood cells, only the ligand in arterial plasma is available for transport for most brain PET radioligands. Therefore, arterial blood sampling is performed during the PET scan, where blood samples are collected through an arterial line, plasma is separated from the cellular blood fraction, and radioactivity in the plasma is corrected for any radioactive metabolites resulting from metabolism of the tracer by enzymes in either plasma or liver. In this way an input function of the free intact radiotracer in plasma is obtained at successive time points after tracer injection. However, this approach has major drawbacks in that drawing arterial blood sampling is an invasive procedure, and manual sampling and analysis of blood samples requires a substantial amount of work, although this can be substantially reduced using automatic, online sampling systems (see Fig. 45). In addition, errors due to incomplete or irregular sampling, sampling of small blood volumes yielding limited count statistics, and irregular or unreliable metabolite analyses may affect overall quantification. Moreover, cross-calibration between the blood sampling and PET system needs to be secured. Especially for preclinical PET studies, these measurements require arterial cannulation by a precise and tedious surgical intervention with a substantial mortality risk. Therefore, this approach limits

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repeated scanning of animals to a maximum of two time points within a short time interval, thereby reducing the potential of PET imaging to monitor interventions over longer time windows. In addition, the total blood volume that can be extracted during sampling in rodents is limited [143].

Since arterial sampling procedures are logistically challenging, less cost effective and not suitable for high throughput PET imaging, efforts have been made to determine the input function from the dynamic PET data by isolating the signal from the carotid arteries in the case of human PET studies. For animal models, one can consider extracting the PET signal from structures with a large blood pool that are still in the field of view of the PET scanner such as the heart cavity. This so-called Image Derived Input Function (IDIF) has the main disadvantage that the signal may be confounded by partial volume effects, especially in the case of rodent studies, because of the limited PET resolution. Consequently, this non-invasive alternative to arterial sampling is methodologically very challenging and has only been implemented successfully for a limited number of tracers [144]. One of the issues is that even a visually acceptable IDIF will typically show inaccuracies in peak estimation, which will have a different impact on the quantitative parameters depending on tracer kinetics. Another important limitation is that the parent compound cannot be distinguished from its radioactive metabolites and that the plasma radioactivity concentration cannot be separated from the whole blood activity concentration. Therefore, limited numbers of either arterial or venous blood samples is still needed to estimate the differences between plasma and whole blood activity concentrations, and to calculate the percentage of intact parent tracer in arterial plasma. As such, one could also consider an average input function or metabolite curve with late venous blood samples to perform a patient specific scaling of the population based input function [145, 146] or metabolite curve [147]. Another promising development is the IDIF parameterization and estimation of patient specific IDIF parameters simultaneously with the parameters of the kinetic model [148]. However, practice has proven that a limited number of blood samples is still needed to make the parameter estimation more reliable. So, although the number of arterial blood samples can, in theory, be reduced, an IDIF approach has only been used to its full extent for a limited number of PET tracers [149, 150] and only rarely resulted in imaging procedures with a substantially reduced invasiveness for the patient. In addition, an IDIF approach needs to be thoroughly validated for each tracer for each tracer and kinetic model separately prior to routine preclinical use, as population based input functions or metabolite data, although appropriately scaled using venous or arterial blood samples, may not apply for other patient populations or animal models because of the effects of different disease states.
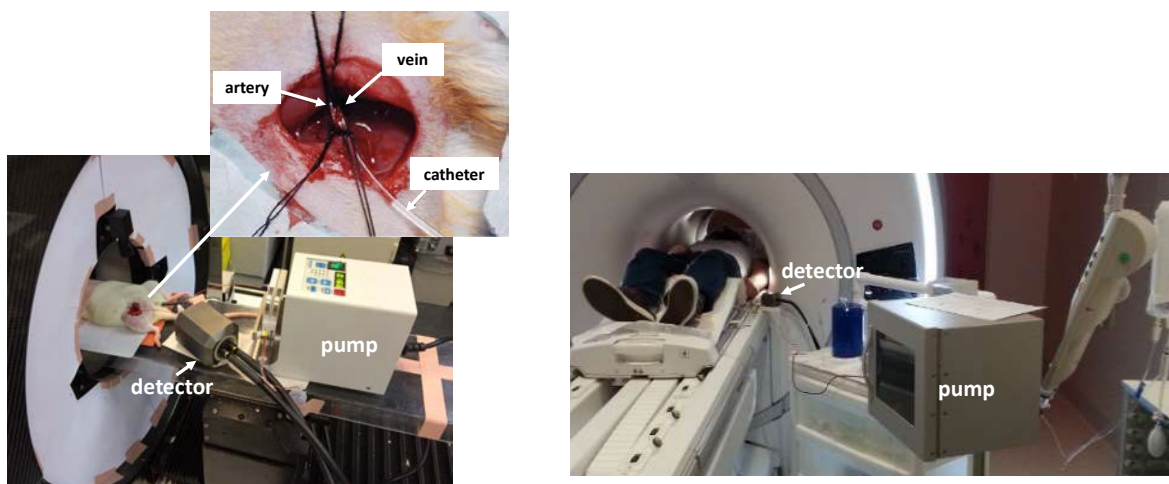

FIG. 44. Typical setup of an online, automatic detection system to measure the activity concentration in arterial blood both in a rat model (left) and in a clinical setting (right) (Courtesy of M. Koole, Catholic University of Leuven, Belgium).

8.3.3. Reference tissue models

To avoid arterial blood sampling, other input functions can be considered, which are still image based and therefore do not require measurements other than a dynamic PET scan. If the activity concentration can be determined in a brain tissue that does not express the target of interest, this tissue can be considered as a reference tissue and the activity concentration in this reference tissue can be considered as representative for the non-displaceable tracer uptake in the target brain regions. Consequently, BP_{ND} can be estimated for any target region using the tissue distribution volumes of reference (V_R) and target tissue (V_T) as follows (Eq. 14):

$$BP_{ND} = \frac{C_S}{C_{ND}} = \frac{C_T - C_R}{C_R} = \frac{V_T - V_R}{V_R} \tag{14}$$

where C_T and C_R are the tracer concentrations in the target and reference region, respectively, and assuming V_R equals V_{ND}. It's worthwhile noticing that in this context $\frac{V_T(t)}{V_R(t)}$ is often termed the distribution volume ratio (DVR) such that $BP_{ND} = DVR - 1$. It should also be pointed out that in the absence of a reference region, BP_{ND} can be estimated numerically, but this estimate is often not reliable. The quantitative parameter that is used most frequently in the absence of a reference tissue, is the distribution volume V_T which can be estimated more reliably.

In addition, the corresponding TAC of this reference tissue can be used as input function for a reference tissue model to estimate BP_{ND} for a target region using only the dynamic PET data after a bolus injection of the tracer. This way neither arterial blood samples nor labour intensive radiometabolite analyses are needed and dependency on other devices such as a well counter or dose calibrator is completely avoided. In terms of reference tissue model, the Simplified Reference Tissue Model (SRTM) is the most used model [151] in which tracer kinetics in both target and reference regions are described adequately by a 1TCM (in accordance with Eq. 10).

$$\frac{dC_T(t)}{dt} = K_1 C_P(t) - k_{2a} C_T(t) \tag{15}$$

$$\frac{dC_R(t)}{dt} = K_{1R} C_P(t) - k_{2R} C_R(t) \tag{16}$$

where $C_P(t)$, $C_T(t)$ and $C_R(t)$ represent the tracer concentrations in plasma, target tissue and reference tissue, respectively, at time t. The subscript R refers to the kinetic parameters of the reference tissue while the subscript `' refers to an apparent kinetic parameter.

Solving Eq. (16) for $C_P(t)$ gives the following expression for the tracer concentration in plasma (Eq. 17):

$$C_P(t) = \frac{1}{K_{1R}} \left(\frac{dC_R(t)}{dt} + k_{2R} C_R(t) \right) \tag{17}$$

Substitution of Eq. (17) into Eq. (15) results in a non-linear operational equation of $C_T(t)$ as function of $C_R(t)$, R_1 ($=K_1/K_{1R}=k_2/k_{2R}$), k_2 ($=k_{2a}\cdot(1+BP_{ND})$) and BP_{ND}, which can be fitted using $C_R(t)$ as (reference tissue) input function. Receptor Parametric Mapping (RPM) a parametric implementation of SRTM, allows for fits at the voxel level by using a basis function approach [152]. The number of fit parameters can even be reduced to two (R_1 and BP_{ND}) by imposing the same (reference tissue) k_{2R} ($=k_2/R_1$) in a second run of RPM [153].

The operational equation for $C_T(t)$ can also be linearised by integrating both sides (after substitution of Eq. (17) into Eq. (15)), as shown in Eq. (18):

$$C_T(T) = R_1 C_R(T) + k_2 \int_0^T C_R(t)dt - k_{2a} \int_0^T C_T(t)dt \tag{18}$$

Eq. (18) is similar to the multilinear reference tissue model [154] and computationally efficient to generate voxel wise BP_{ND} parametric maps. On the other hand, BP_{ND} is the only valid quantitative endpoint. Therefore, model validation using full kinetic modelling is essential, especially if non-displaceable tracer uptake could be affected by brain penetrating radiometabolites or disease related permeability changes of the BBB [155]. Selection of the appropriate reference tissue also needs to be supported by histology data. In addition, preclinical PET imaging after pre-dosing with a blocking agent, whose selectively binds with high affinity to the same target and is non-toxic at higher doses, can identify a specific brain region as reference tissue and can verify whether non-displaceable tissue distribution volume is identical for reference and target regions.

8.4. SIMPLIFIED QUANTITATIVE ENDPOINTS USING STATIC PET SCANNING

Although dynamic PET scanning has the potential to provide much more information on tracer kinetics, static PET scanning at a single, late time point after tracer injection is often preferred, mainly because more patients can be scanned and more PET scans being performed with the same tracer batch. On the other hand, rescaling the activity concentration measured at this single late time point by an appropriate factor is the only option to account for differences in tracer activity, body weight, tracer concentration in plasma, tracer metabolism rate, time differences between injection and scanning.

8.4.1. Simplified quantitative endpoint for irreversible tracer kinetics

The SUV is frequently considered to account for differences in injected activity and weight between animals and/or persons. The SUV is defined as the radioactivity concentration in tissue divided by the injected amount of radioactivity normalized to either body weight, lean body mass or body surface area. For irreversible tracer kinetics, one can assume that:

$$C_T(t) \approx K_i \int C_P(t) \tag{19}$$

Where K_i is the net influx constant. Dividing both sides of Eq. (19) by injected activity per body weight, results in (Eq. 20):

$$\frac{C_T(t)}{A_{inj}/BW} = SUV \approx K_i \frac{\int C_P(t)}{A_{inj}/BW} \tag{20}$$

Now, assuming that the plasma integral is proportional to the injected activity per body weight with the same proportionality constant across subjects or animals, the following approximation is valid:

$$SUV \sim K_i$$

However, to make sure this assumption is valid, it needs to be ensured that the systemic distribution volume for a PET tracer with irreversible binding characteristics is consistent among animals or subjects. Therefore, imaging procedures such as the time interval between tracer administration and PET scanning as well as procedures for animal or patient preparation such as fasting and patient or animal condition during the tracer uptake phase, need to be standardized to reduce variability and increase repeatability of SUV based quantification. In addition, the physiological state of the animal needs to be monitored closely during preclinical PET scanning to make sure that body temperature is maintained, and anaesthetics are optimally used to prevent large changes in tracer metabolism rate and tracer distribution throughout the body between animals or between consecutive scans of the same animal. Finally, it needs to be confirmed that the anaesthetics themselves do not affect the biological process under study.

8.4.2. Simplified quantitative endpoint for reversible tracer kinetics

If during a certain time interval of the dynamic PET scan, the ratio between the tracer concentration in brain tissue and plasma becomes constant, the apparent tissue distribution volume can be estimated

based on this ratio which generally overestimates the true underlying tissue distribution volume [156]. Although the tissue distribution volume based on this pseudo equilibrium between tissue and plasma is biased, some of the errors cancel out when a ratio of apparent distribution volumes is calculated such that this ratio can be a valid approximation of the DVR between two tissues. This means that in the case of a reference tissue, the ratio of the target to standardized uptake values reference (SUVR) can be considered as an approximation of DVR or BP_{ND} when the ratio of tracer concentration between target and reference tissue becomes constant. Although the term SUVR is commonly used, this ratio can also be calculated directly from the measured radioactivity concentrations without conversion to SUV. In addition, SUVR is generally more robust than SUV, since it is only image based and does not require cross-calibration with the dose calibrator. This SUVR approach is often used for brain receptor studies where the cerebellum can be considered as a reference region or for unilateral brain diseases where the corresponding contralateral brain region can be used as healthy reference tissue. However, any SUVR approach based on a single, static PET scan at a late time point after tracer injection, always needs to be validated based on the transient or pseudo equilibrium observed between target and reference region during dynamic PET scanning. This validation needs to be repeated for each new animal model and intervention.

8.5. ADDITIONAL COMMENTS FOR PRECLINICAL PET IMAGING

In a preclinical context, dynamic PET data can provide valuable information on tracer characteristics for clinical tracer validation even without an input function or reference tissue. For instance, a steadily increasing TAC could be suggestive of irreversible tracer binding or radio metabolites entering the brain, thereby confounding the PET signal. In addition, a dynamic PET scan can be repeated for the same animal but under different conditions. As such, TACs after pre-treatment with a drug with high affinity and selectivity for the same target as the PET tracer, should decrease tracer uptake compared to baseline conditions as fewer targets are available for tracer binding. As a result, tracer kinetics under blocking conditions should also be much faster with faster clearance from tissue. If pre-treatment doses are in the range where complete blocking can be expected, the reduction of the PET signal can be indicative for the fraction of specific tracer binding. In addition, displacement, or chase experiments where an additional cold compound is administered during the dynamic PET scan to displace tracer binding, can be used to confirm in vivo binding kinetics. Therefore, although these experiments are more observational in nature rather than quantitative, they can provide valuable information on tracer kinetics and specific binding without being invasive or logistically and technically challenging.

In terms of tracer injection in small animals, only smaller amounts of tracer can be injected into animals due to their much smaller volume. In addition, the specific activity of high affinity radioligands needs to be high enough to prevent violation of the tracer principle, which severely limits the use of PET ligands in mice [30, 157]. In addition, tail vain injection can be challenging such that it is worthwhile to check whether injection was successful, or paraveneous, because this will significantly hamper quantification.

9.   CONCLUSIONS

The primary objective of this publication is to provide a base guideline for the development of CNS tracers for clinical application. Therefore, the different stages have been extensively described.

The first stage is to understand which biological target that plays a vital role in the neurological disease of interest needs to be investigated. In Section 2 an extensive list is presented on brain targets for which CNS tracers have been developed for human studies. Being on this list does not necessarily mean that the tracer has reached a mature stage of evaluation. The majority of tracers are labelled with ^{11}C but a shift is observed to an increasing number of ^{18}F variants facilitating increased availability.

There is a great need to develop radiotracers for CNS targets for which currently no tracer is available at all. These can either be historical targets for which it has been challenging to develop an appropriate imaging agent, or new emerging potential targets. Emerging targets for which tracers are studied in a preclinical setting are mostly involved in neuroinflammation.

To date, most CNS imaging agents have been small molecules. However, recent work demonstrating antibodies and larger molecules can enter the CNS and be useful for imaging, suggest this is an emerging area of development.

The development of a new radiopharmaceutical is described as a multidisciplinary process that involves contributions from different fields of research including nuclear chemistry, synthetic chemistry, pharmaceutics, molecular biology and drug pharmacology. General selection criteria to design or choose the optimal radiotracer have been described, since the success rate to develop a successful tracer is still quite low, a novel approach has been described in Section 3 for the improvement of the success rate for a new CNS tracer. Regarding the choice of the radionuclide, ^{11}C and ^{18}F are the main players as they are the ideal radionuclides to be incorporated in small molecules that can readily penetrate the brain. There may come a chance for radiometals when it is possible to allow antibodies to enter the brain by using transport systems like transferrin. For sites without a cyclotron, there are alternatives such as amyloid imaging agents labelled with ^{68}Ga that have been recently reported. There are also emerging applications of radiolabelled antibodies for CNS imaging, but these are still in their infancy. Then, longer lived radionuclides including ^{89}Zr may be possible to use.

To a considerable extent the success of a PET radioligand does not depend only on the biological characteristics but also on an efficient and practical radiolabelling method which will enable widespread use. Due to the short half-life, time is obviously a critical parameter for the radiochemistry development. In principle, the entire synthesis including the purification, isolation, and formulation of the radioligands should not take more than two half-lives. Taking longer than two half-lives will result in a lowering of the molar activity (A_m) which is often critical for the PET measurements. With the introduction of total body PET systems with increased sensitivity, it will be possible to apply radiotracers with lower A_m and apply low yielding production methods. Furthermore, prolonged scanning protocols with ^{11}C labelled radiotracers will be possible.

GMP production of CNS tracers is required in most countries to allow clinical studies. Worldwide, there is a huge variation in regulations with respect to GMP tracer production. Use of synthesis modules are recommended to protect workers against radiation. In addition, synthesis modules offer the advantage of using audit trail and achieving more constant production conditions.

The first step in translating newly developed tracers is in vitro testing to evaluate some properties of CNS tracers. These tests on metabolism, lipophilicity, affinity for BBB may serve as go/no go decision points to continue translation or not. In addition, a better understanding of the metabolic pathway can lead to establish the optimal labelling position within the molecule. It is very important how in vitro assays are performed and at which concentration level. For example, tracers for molecular imaging are produced with high molar activity. This means, when a tracer is dosed at 200 MBq, less than 10 ug mass will be administered. Therefore, for in vitro assays to screen the behaviour, the tracer procedure needs to be performed at the same low tracer level concentrations. Also, the use of 3-D cell systems will reflect a more precise in vivo situation. Furthermore, new developments in screening assays using organ on chip systems will reflect a better in vivo situation and support in making the right decisions for the selection of the appropriate compounds to go in vivo.

When it is decided to continue preclinical evaluation in vivo, several additional tracer characteristics can be studied. Although already tested in vitro, metabolism and BBB penetration can be tested in vivo. Furthermore, other unique features such as pharmacokinetics and non-displaceable non-specific binding can be assessed. By intelligent designing of the experiments, interaction of different receptor systems can be investigated, and intervention studies can be done by behavioural tests and by administering a challenge dose. Different animal species and disease models can be chosen. However, it is important

that target expression is ideally in the same range as would be expected in human disease. Because of species differences, it is recommended to test the CNS tracer in two animal models before going to clinic. An important confounding factor in preclinical imaging is the effect of anaesthesia. It is preferable to avoid anaesthesia where possible as it can largely affect biodistribution of the tracer. NHPs can, for example, be trained to stay still for the duration of the scan. However, such a set-up is very difficult to implement and almost impossible to set up for rodents. Lastly, rats can also be softly immobilized by wrapping them in a specially designed restraining suit for the duration of the experiment.

The use of NHPs in the development of new CNS radiotracers is of great benefit in evaluating new candidate molecules for decisions on which molecules to move into human trials. The use of NHPs can also be of importance to establish the radiation dose which is a prerequisite to do human PET studies. Several challenges have been described in this publication related to ethical considerations, the high costs of NHP PET studies, the lack of target for disease biomarkers, and the use of anaesthesia. Important go/no-go decisions for further studies include evidence of sufficient brain penetration and evidence of selectivity of the radiotracer. It is feasible to get important information on the presence of radiolabelled metabolites, their BBB penetration and reversible kinetics favourable for reliable kinetic modelling through NHP studies. Another possibly important piece of information guiding the selection of specific candidates over others could be the level of non-specific binding, which should be as low as possible.

Preclinical in vivo testing is carried out to predict how a radiopharmaceutical behaves in humans in order to come up with the best design for the first in human evaluation. In recent years, it became more and more obvious that species differences need to be strongly considered for translation from animal to humans. Differences in protein concentration, metabolism profile or efflux transporter dependency are only some examples that vary from species to species. Different outcomes have been seen in rodents, pigs, NHPs and humans as well as in naïve and diseased models. Discrepancies in the predictions made for human data from non-human primate data and vice versa are especially astonishing. Consequently, one needs to carefully consider rejecting a radiopharmaceutical to be translated from animals into humans. Future studies need to focus on predictability and reveal in which cases results from animals can be translated into the human situation, and in which cases not and how to improve this predictability.

Before new PET tracers can be used in humans, non-clinical safety studies need to be conducted. PET tracers for imaging of targets in the brain are typically non carrier added radiolabelled small organic molecules that are usually administered once, or at most, only a few times. Consequently, the injected mass of the PET tracer is sufficiently small that the microdose approaches of the ICH M3 guideline [114] apply. According to this guideline, the recommended non-clinical studies for microdose applications in humans should include assessment of non-clinical pharmacology, pharmacokinetics, toxicity and dosimetry. The results of these non-clinical safety studies should be included in the IMPD, investigational new drug application, or similar document that should accompany the clinical trial application of the new PET tracer.

Although PET imaging is generally considered as a quantitative in vivo brain imaging technique, it is not straightforward to relate the PET signal to the underlying pathophysiological parameters of interest, such as, amongst others, cerebral blood flow, metabolism, and the receptor expression level or enzymatic activity. The aim of kinetic modelling is to better understand and define the relationship between PET measurements and underlying brain tissue parameters and, especially for new PET tracers, to demonstrate to which extent the PET signal indeed depends only on the actual brain tissue parameters of interest or whether other parameters are also of importance. Specifically on a preclinical level, kinetic modelling data provide valuable information for the potential clinical translation of new PET ligands, as it can confirm target engagement, elucidate binding characteristics, and establish whether binding can be considered as reversible or irreversible, thus helping to define clinical PET imaging protocols for both accurate and precise quantification. In addition, kinetic modelling provides reference data to evaluate possible simplifications of imaging procedures, even at the preclinical level, and to define simple and accurate patient protocols to broaden clinical applicability of a PET tracer.

From a translational point of view, it should be noted that once a valid kinetic modelling approach has been established for preclinical imaging of a PET tracer, care is still needed with a straightforward clinical translation because of potential species differences, especially since it can be expected that both tracer metabolism and tracer kinetics are faster in animals.

This publication describes all the above features in detail. There are many challenges ahead of us. However, it can be concluded that the application of CNS radiotracers in nuclear medicine is rapidly developing both in size and in scientific level because of the multidisciplinary research community.

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ABBREVIATIONS

AD	Alzheimer's disease
BBB	Blood brain barrier
BCRP	Breast cancer resistance protein
BP	Binding potential
cGMP	Current Good Manufacturing Practices
CNS	Central nervous system
CO	Carbonyl
DAT	Dopamine transporter
EANM	European Association of Nuclear Medicine
FDG	2-[^{18}F]fluoro-2-deoxy-D- glucose
FTM	Fluid thioglycollate medium
GABA	g-aminobutyric acid
GE	Genetically engineered
GMP	Good Manufacturing Practice
HBSS	Hanks' Balanced Salt Solution
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HI	Hydroiodic Acid
IDIF	Image Derived Input Function
IMPD	Investigational Medicinal Product Dossier
KO	Knockout
LC-MS	Liquid Chromatography Mass Spectrometry
MAC	Minimum Alveolar Concentration
MDR	Multi drug resistance
MPO	Multiparameter optimization
NADPH	Nicotinamide adenine dinucleotide phosphate
NHP	Non-Human Primates
P-gp	p glycoprotein
PBS	Phosphate buffered saline
PD	Parkinson's disease
PET	Positron Emission Tomography
ROI	Region of Interest
QA	Quality assurance
QC	Quality control
SERT	Serotonin transporter
SPECT	Single photon emission tomography

SPE	Solid Phase Extraction
SUV	Standardized uptake value
SUVR	Standardized uptake values reference
TAC	Time activity curve
TLC	Thin Layer Chromatography
TSB	Tryptose soy broth
TSPO	Translocator protein

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