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Metabolite studies *in-vitro* and *in-vivo*:
From preclinical evaluation to clinical trials

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Mag.rer.nat. Lukas Nics

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Univ.-Prof. Mag. Dr. Karl-Heinz Wagner

Current PhD-thesis was performed within the Radiochemistry and Biomarker Development Unit, Department of Nuclear Medicine at the Medical University of Vienna.

The working group for Radiopharmaceutical Sciences was founded in 2004 by Priv.-Doz. Mag. Dr. Markus Mitterhauser and assoc. Prof. Priv.-Doz. Mag. Dr. Wolfgang Wadsak who conjointly lead the team as equal partners since then. In 2011, the Radiochemistry and Biomarker Development Unit was founded integrating already existing working groups in radiochemistry and radiopharmacy.

The interdisciplinary nature of this group is evident: it comprises of approximately 20 scientists deriving from many different disciplines, such as chemistry, pharmacy, nutritional sciences, physics, medicine and engineering, all devoted to the final goal: the development and preclinical evaluation of novel PET tracers, specifically targeting receptors, transporters and enzymes.

The Radiochemistry and Biomarker Development Unit has currently research cooperations with the Department of Psychiatry and Psychotherapy, Clinical Pharmacology, Neurology and Cardiology as well as the Centre for Brain Research and the Centre of Excellence for High-field MRI (all at the Medical University of Vienna). Furthermore, close collaboration with the Departments of Inorganic Chemistry, Pharmaceutical Technology and Biopharmaceutics as well as Drug and Natural Product Synthesis (all from the University of Vienna) exist. International cooperations comprise the radiochemical and/or radiopharmaceutical facilities at: ETH Zurich (Switzerland), University Clinic Tuebingen (Germany), University Clinic Muenster (Germany), UCLA (Los Angeles, USA) and the CAMH Toronto (Canada).

***„You can't always get what you want -
But if you try, sometimes you just might find
You get what you need.“***

M. Jagger, K. Richards, 1968

Danksagung

Ganz besonders danken möchte ich meiner lieben Frau Eva, die mir während meiner Dissertationszeit nicht nur mit Rat, Tat und viel Geduld zur Seite stand, sondern mir auch den Rückhalt gab (in welcher Hinsicht auch immer) der oftmals nötig war. >>„danke liebes Würmchen“<<

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>>„Markus, danke dafür, dass du den Spagat zwischen Freundschaft und Chef so gut hinkriegst und auch, dass du immer wieder Zeit findest mit mir über alles was uns bewegt zu reden (egal zu welcher Jahreszeit aber immer unter freiem Himmel) – denn draussen ist es herrlich.“<<

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Preface

Formally, I performed my PhD studies in Nutritional Sciences at the Department of Nutritional Sciences, Faculty of Life Sciences at the University of Vienna. My entire practical scientific work took place at the Department of Nuclear Medicine, Radiochemistry and Biomarker development unit at the Medical University of Vienna of the General Hospital of Vienna.

All scientific research questions regarding this manuscript are mainly subjected and answered to radiopharmaceutical aspects. Nevertheless, the scientific field of radiopharmacy is related both to nuclear medicine and nutritional sciences. The field of physiology is found in both scientific category groups in a specific case. The behaviour of radiopharmaceuticals (i.e. drugs) is explained by pharmacokinetics and pharmacodynamics, where metabolism and biotransformation represent sub-areas of pharmacokinetics. Nevertheless, it is the same behaviours to food and food related questions in the field of nutritional sciences.

The current thesis mainly deals with enzymatic research questions which are regarding biochemical topics. Enzymatic processes, which take place in humans and animals (*in-vivo*) and the required preclinical evaluation (*in-vitro*) are included in the scientific portfolio of nutritional sciences as well as, accordingly, in radiopharmacy. That implies that all biochemical techniques and optimized methods that were used during my practical work can be applied to scientific research questions regarding nutritional sciences if required.

However, I want to point out that no information concerning feasible applicability and relevance to the fields of nutritional science will be discussed in this manuscript.

Abstract

The present thesis deals with several aspects of the metabolism of tracers for Positron-Emission-Tomography (PET). The exact determination of the current ratio of intact and metabolized tracer is a prerequisite for a further quantification and safe application of these molecules in living organisms.

As a model substrate, a major part of this thesis dealt with [¹⁸F]FE@SUPPY (5-(2-[¹⁸F]fluoroethyl) 2,4-diethyl-3-(ethylsulfanylcabonyl)-6-phenylpyridine-5-carboxylate). Furthermore, the ligands [¹¹C]Me@APPI (1-(3-([¹¹C]methylamino)-1-phenylpropyl)-3-phenyl-1H-benzo[d]imidazol-2(3H)-one), [¹⁸F]FE@SNAP (2-[¹⁸F]fluoroethyl 3-((3-(4-(3-acetamido phenyl) piperidin-1-yl)propyl)carbamoyl)-4-(3,4-difluorophenyl)-6-(methoxymethyl)-2-oxo-1, 2,3,4-tetrahydropyrimidine-5-carboxylate) and [carbonyl-¹¹C]WAY100635 (N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)-cyclohexane[¹¹C]carboxamide) served as substrates to evaluate their metabolic behaviour using *in-vitro* as well as *in-vivo* methods.

This thesis is divided in 4 sub-sections:

- 1) Single enzyme experiments using carboxylesterase;
- 2) Multi-enzyme experiments using hepatic liver microsomes containing Cytochrom P-450 as well as metabolic experiments using pooled plasma;
- 3) Experiments to assess the penetration of radiotracers of the blood-brain-barrier; and
- 4) *in-vivo* metabolite quantification in a clinical study

ad 1) All of the evaluated research PET-tracers carry ester or amide moieties. The cleavage of these functions via carboxylesterase belongs to the first line of metabolism. Thus, the Michaelis-Menten kinetics of the enzymatic decomposition *in-vitro* gives a first estimate for the *in-vivo* stability of the tracer (bearing in mind, that esterases are responsible for the major part of metabolism of esters and amides *in-vivo*). The different metabolites have been analyzed by dedicated high-pressure-liquid chromatography (HPLC) methods.

ad 2) As a further step towards a wider applicability and a higher degree of predictability of our “single-enzyme data”, the next logical step was the application of “almost the entire”

enzymatic apparatus for metabolic degradation. Hepatic microsomes and plasma from human and rat origins were prepared under standard conditions and our PET-tracers were subjected to enzymatic treatment. The metabolites were analyzed by HPLC methods.

ad 3) Additionally to the metabolic behaviour of PET-tracers *in-vitro*, information and prediction of the penetration of the blood brain barrier (BBB) is of high interest. C18-HPLC methods were used to obtain the partition coefficient (log P), a measure for lipophilicity of the compound of choice, giving an indication if a tracer is able to pass the BBB. Immobilized artificial membrane (IAM) chromatography (specific HPLC columns are commercially available) is a new method to provide information on compound-membrane-interactions and represents the solute distribution between the aqueous phase and the membrane. This is a systemic approach for molecules which main mechanism of transport across cell membranes is passive diffusion.

ad 4) Having information on the *in-vitro* behaviour of the molecules is followed by the evaluation of the *in-vivo* behaviour. As part of a clinical study, it was of particular importance to develop a fast and reliable HPLC method to determine the extent and velocity of the metabolic process of a radiotracer which was administered to humans. After defined time points, the metabolites were extracted from blood and quantified by radio-HPLC.

The main focus of this thesis is the implementation of various enzymatic methods (*in-vitro* and *in-vivo*) using human tissues (enzymes and blood) to enhance the knowledge on the metabolic behaviour of investigated PET-tracers. Thus, predictions to a certain extent should be enabled. The introduced and optimized methods will become standard procedures in the development of new PET-radiotracers to assess their metabolic status.

Zusammenfassung

Die vorliegende Dissertation hat sich mit verschiedenen Aspekten des Metabolismus von Positronen-Emissions-Tomographie (PET)-Tracern beschäftigt. Die genaue Bestimmung der jeweils aktuellen Verhältnisse von intaktem und metabolisiertem Tracer ist eine Voraussetzung für eine sichere Anwendung und in weiterer Folge der Quantifizierung dieser Moleküle im lebenden Organismus.

Für einen großen Teil der Dissertation diente [¹⁸F]FE@SUPPY (5-(2-[¹⁸F] fluoroethyl)2,4-diethyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate) als Modellsubstanz. Aber auch [¹¹C]Me@APPI (1-(3-([¹¹C]methyl amino)-1-phenylpropyl)-3-phenyl-1H-benzo[d]imidazol-2(3H)-one), [¹⁸F]FE@SNAP (2-[¹⁸F]fluoroethyl3-((3-(4-(3-acetamidophenyl)piperidin-1-yl) propyl)carbamoyl)-4-(3,4-difluorophenyl)-6-(methoxymethyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate) und [carbonyl-¹¹C]WAY100635 (N-(2-(4-(2-methoxyphenyl) piperazin-1-yl)ethyl)-N-(pyridin-2-yl)-cyclohexane[¹¹C]carboxamide) dienten als Substrate für die Evaluierung der metabolischen Eigenschaften sowohl *in-vitro* als auch *in-vivo*.

Die Dissertation gliedert sich folgendermaßen:

- 1) Einzelenzym-Experimente mit Carboxylesterasen;
- 2) Multienzym-Experimente mit Lebermikrosomen, welche den Komplex Cytochrom P-450 enthalten sowie mit gepooltem Plasma;
- 3) Experimente, um das Passieren von Radiotracern über die Blut-Hirn-Schranke vorherzusagen; und
- 4) *in-vivo* Metabolismus Experimente im Zuge einer klinischen PET-Studie

ad 1) Alle evaluierten Forschungstracer haben innerhalb des Moleküls entweder Ester- oder Amidbindungen. Die Spaltung dieser Bindungen über das Enzym Carboxylesterase gehört zu den frühen metabolischen Einflüssen. Deshalb liefert uns auch eine Kinetik (Michaelis-Menten-Kinetik) dieser *in-vitro* stattfindenden Spaltung erste Informationen über eine

Stabilität der Tracer – mit dem Hintergrund, dass Esterasen für einen Großteil der Ester- und Amidspaltung verantwortlich sind.

ad 2) Als weiterer Schritt für eine breitere Anwendbarkeit und für einen höheren Grad an Vorhersagbarkeit im Vergleich zu „Einzelenzym-Daten“ eignet sich die Anwendung des „kompletten Enzym-Apparates“ für die Erstellung einer Kinetik. Sowohl Lebermikrosomen als auch Plasma (beides aus Mensch und Ratte) wurden unter physiologischen Bedingungen mit unseren PET-Tracern inkubiert um ein metabolisches Profil zu erhalten. Die Analysen wurden mit der Hochdruckflüssigkeits-Chromatographie (HPLC) durchgeführt.

ad 3) Zusätzlich zum metabolischen *in-vitro* Verhalten der PET-Tracer ist es von entscheidender Wichtigkeit, Informationen über ein mögliches Passieren der Blut-Hirn-Schranke (BHS) zu gewinnen. C18-HPLC Methoden werden verwendet, um den Verteilungskoeffizienten ($\log P$) zu erhalten. Dieser $\log P$ -Wert ist ein physikalischer Parameter des Tracers und dient der Abschätzung, ob das Molekül die BHS passieren könnte. Da der $\log P$ eine sehr unspezifische physikalische Größe ist, wurde eine HPLC-Chromatographie mit immobilisierten künstlichen Membranen (IAM) eingesetzt, um genauere Informationen über Substrat-Membran-Interaktionen des Tracers zu bekommen. Diese Methode erlaubt auch, dessen Stoffverteilung zwischen wässriger Phase und Membran zu berechnen. Dieser systematische Ansatz ist nur für Moleküle zulässig, deren Hauptmechanismus für das Passieren der BHS die passive Diffusion durch Zellmembranen darstellt.

ad 4) Wenn das *in-vitro* Verhalten der verwendeten Moleküle bekannt ist, folgt der finale Schritt: die Evaluierung der Situation *in-vivo*. Im Zusammenhang mit einer klinischen Studie war es von entscheidender Wichtigkeit, eine schnelle und zuverlässige HPLC Methode zu entwickeln, um die Abbaurate des Tracers in Menschen messen zu können. Zu bestimmten Zeitpunkten wurde den Probanden Blut entnommen und aus diesem die Metaboliten bestimmt. Die Quantifizierung wurde mittels Radio-HPLC durchgeführt.

Die Haupt-Fragestellung dieser Dissertation ist die Implementierung einer Reihe verschiedener enzymatischer Methoden (*in-vitro* und *in-vivo*) unter der Verwendung von Humanmaterial (sowohl Enzyme als auch Blut), um das Wissen über das metabolische

Verhalten unserer PET-Forschungstracer zu steigern. Dadurch soll tieferes Verständnis sowie eine gewisse Vorhersagbarkeit erreicht werden, wodurch die Weiterentwicklung der Tracer leichter lenkbar wird. Die entwickelten und optimierten Methoden werden nunmehr bei der Evaluierung neuer Liganden standardmäßig Anwendung finden.

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Background / Introduction

1. Drug development

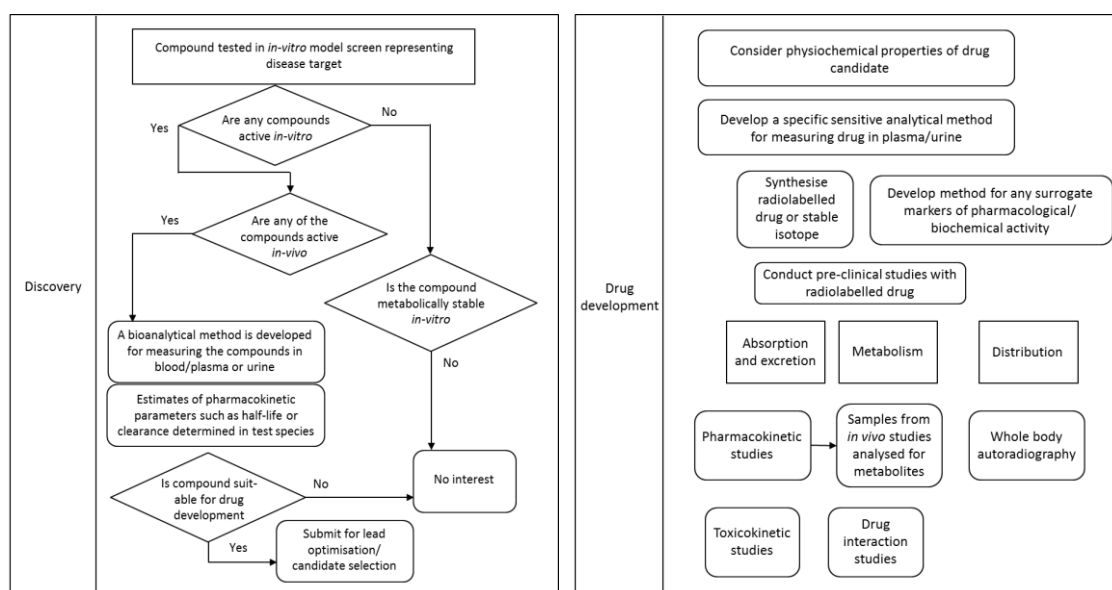
1.1 GENERAL

Drug research includes many different disciplines united by a common goal – the development of new therapeutic and/or diagnostic agents. For the purpose of getting new substances for clinical use there are two fields to distinguish– discovery and development. The former implies the establishment of a hypothesis of the target region (enzyme, receptor, etc.) which alters depending on various diseases. Drug discovery stands also for establishing appropriate models for screening of the new drugs using *in-vitro* and *in-vivo* applications to receive information about its biological activity [Lin, 1997]. The second stands for the stage of development which lasts between five and ten years [Evans, 2004] and serves the evaluation of toxicity and specificity of new drugs. While serendipities play an important role to consider the synthesis of new drugs, *in-silico* models as well as a basic knowledge in metabolism are of crucially relevance [Lappin, 2006]. Only a low percentage of all interesting chemical components is predestined for clinical application. Most of the components turn out to be inefficient, not potent enough or have potential toxic characteristics. Scientists of multidisciplinary fields should engage in a process to identify diseases, to develop chemical agents with biological, chemical and pharmacological properties to finally get drugs of high quality. An ideal drug candidate needs to have specific drug-like properties, including chemical and enzymatic stability, solubility, low clearance by the liver or kidney, permeation across biological membranes, potency and safety [Liederer, 2005].

If a target of interest (e.g. receptor or transporter) is identified, medicinal chemists start with a series of empiric and semi-empiric structure-activity tests to modify, if necessary, the structure of the compound for an optimal *in-vivo* activity [Lin, 1997]. In early discovery, *in-silico* models [Zhu, 2011, Gupta, 2010, Vaz, 2010], *in-vitro* studies using material from human and animal origin such as hepatocellular and subcellular fractions and/or

recombinant enzymes [Baranczewski, 2006, Dalvie, 2009, Anderson, 2009, Obach, 2011] as well as enzyme families are used to predict the disposition of new molecular entities in humans [Penner, 2012]. But also *in-vivo* studies using a range of experimental animal models [Jolivet, 2005, Tang, 2006, De Buck, 2007] are carried out in order to get prognoses of these molecules of choice.

Special conditions for *in-vitro* screenings are used for testing the receptor-affinity of drugs. During this series of screening processes the number of potential drug candidates gets lower and lower, until a small number (perhaps only one) is chosen for further development [Lappin, 2006]. Nevertheless, it is not possible to assume, that good *in-vitro* results of a drug can be translated one-to-one to their *in-vivo* behaviour – especially if the substance shows insufficient bioavailability and expected duration of action is not predictable. An increasing awareness should be given to pharmacokinetic and metabolism, since both play a key role the drug screening processes. It is of high importance to evaluate the metabolic behaviour but also the pharmacokinetics as soon as possible to obtain the maximum information concerning the *in-vivo* behaviour of the selected molecule [Lin, 1997]. Drugs, which have an optimum binding affinity to the target receptor- or transporter-protein are not chosen automatically, but rather those which have the optimum – or optimized – characteristics. The correct choice of a component is always a compromise between water solubility, toxicity and other parameters. At this early stage *in-silico* and *in-vitro* methods are compiled, to develop the new compound using typically cell-based tests, enzyme kinetics but also a few animal data, which are of interest at a later point in the research process [Lappin, 2006]. Cross-species comparison of metabolic profiles and their behaviour, which are provided in *in-vitro* studies, may lead to the right selection of animal species [Penner, 2012]. It should be guaranteed, that those animal species are exposed to all major human metabolites [Dalvie, 2009, Anderson, 2009].



[Eddershaw, 2004]

Figure 1: Bioanalysis, pharmacokinetic and drug metabolism steps in drug discovery and drug development

Unfortunately, due to the short time frames during development but also due to the often small amounts of test substances, animal studies are often reduced to a minimum of selected species. It is important to select the suitable animal species and a convenient experimental design to get reliable results on absorption, metabolism and elimination of pharmaceutical substances. Otherwise it might happen that a promising drug is discarded due to a weak experimental design or an inappropriate animal model [Lin, 1997]. Moreover, it is of importance to compare human and animal data in a very early state in order to avoid a wrong choice of models.

Many drugs (e.g. psychotropic drugs) are transformed to one or more biological active metabolites [Baldessarini 1990]. If this is the case, the metabolites should, from a pharmacokinetic point of view, differ from the lead compound in their biological behaviour such as distribution and metabolism. From the pharmacological point of view the metabolite and the parent compound could act by the same, a similar or an antagonistic mechanism. To gather as much knowledge as possible on kinetics of active metabolites is essential for the therapeutical outcome, but also important to explore possible toxicological effects of specific pharmaceuticals [Lin, 1997]. Unfortunately, metabolite studies are often

carried out relatively late in the drug developing process. It would be optimal, if the main information on the whole metabolic behaviour of the selected drug is already known before clinical process is initiated. Preliminary knowledge of the *in-vitro* behaviour on tissues of human origin, the identification of enzymes which are responsible for metabolic processes, but also the information of possible polymorphisms, which can lead to potential toxicities, are needed to optimize clinical trials.

However, the role of scientists who deal with metabolic processes of new pharmaceuticals is more than “only” *in-vitro* and *in-vivo* screening. It is further needed to gain information about absorption, distribution, metabolism and excretion (ADME) of these drugs [Lin, 1997].

It is not only progress done when using *in-vitro* methods regarding metabolite studies [Wrighton and Stevens, 1992, Guillouzo, 1993, Berry, 1992, Chapman, 1993], but also our strongly learning outcome about drug-metabolizing enzymes such as Cytochrom P-450 [Henderson and Wolf, 1992, Gonzalez and Nebert, 1990] or Carboxylesterase [Wang, 1994, Hoskawa, 1990], that provide information about the stage in the process before clinical application. State of the art techniques such as high-pressure liquid chromatography (HPLC) connected to mass spectrometers (MS) and high-field nuclear magnetic resonance (HNMR) represent improved possibilities to receive information about the metabolism during an early discovery process [Fenselau, 1992, Evans 2004].

If a drug reaches the developing stage, costs are rising significantly, which makes it of huge importance to start a debate on the effectiveness of a pharmaceutical compound for selection [Lappin, 2006]. By that date at the latest, detailed information should be communicated to the authorities regarding the metabolic behaviour and the pharmacokinetic profile of the new drug.

If a drug is selected for further development it is common to administer the drug to human volunteers. Further testing of the drug is required to receive necessary information about long-term toxicity, carcinogenicity and reproduction toxicity before it can be finally approved for medical use [Lappin, 2006].

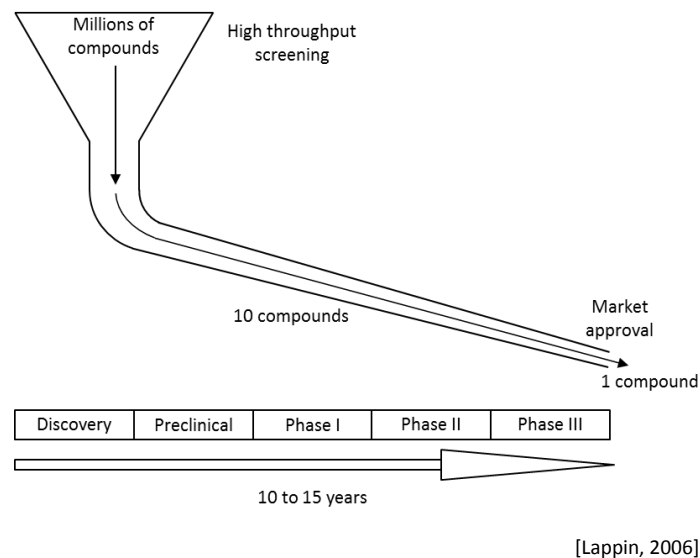


Figure 2: The drug development funnel

1.2 THE REGISTRATION PROCESS OF DRUGS

1.2.1 Phase I

In this phase an intensive series of clinical trials begins. All studies with human volunteers are performed considering the Helsinki agreement. A Clinical Trials Application (CTA in Europe) has to be administered to the regulatory authorities of the ministry of health. This application consists of all toxicological data and potential risks to human volunteers. Phase I trials take place, when the drug and the treatment showed promising in-laboratory tests. These trials usually consist of small numbers of healthy male volunteers. The number of volunteers is up to 100 in total. The goals are to explore whether the treatment is safe, the best way to administer the drug (oral, intravenous, etc.) are chosen and which amount is the optimal dose that causes the fewest side effects (e.g. effects of food on drug administration). In this phase it is of importance to know how the drug concentration changes over time (pharmacokinetics) and which symptomatic and physiological effects may be caused over time (pharmacodynamics) [Lappin, 2006].

1.2.2 Phase II

Once a treatment is found to be safe, it will be applied in a small number of patients (up to 300 in total) which are suffering from the target disease. The goals are to explore whether the treatment is successful and whether there are only few side effects, which may occur to the patients. These could be polymorphic effects and reactions with food and other drugs. Patients often have physical tests (e.g. blood tests) to carry out the pharmacokinetic of the drug as in phase I trials [Lappin, 2006].

1.2.3 Phase III

Phase III trials take place, if it has been shown that a treatment with evaluated drug helps some patients (in a phase II trial). It is usually performed in different countries (multi-center trial) with a huge number of patients (up to many thousands). It is a typical approach to compare the drug with a placebo as control or a marketed drug in a randomized double-blind study. Side effects evaluated by pharmacokinetic and pharmacodynamics studies and the mechanisms of treatment are examined in detail [Lappin, 2006].

1.2.4 Phase IV

After the drug has been accepted for use, postmarketing surveillance takes place in order to verify potential adverse effects. Due to the possible exposure of the drug to thousands or millions of patients after approval, statistical effects may arise which could not have been detected to this extent during earlier clinical trials. Depending on the outcome of phase IV adverse reports a further use or a withdrawal of the drug from the market can happen [Lappin, 2006].

1.3 PRECLINICAL EVALUATION

The preclinical phase is based on the selection of a target of choice combined to its evaluation and the following successful search for chemical compounds (tracer) which have suitable affinities. Tracers providing the best affinity profile are further used to generate

lead structures which have enhanced activity profiles such as increased potency, selectivity, bioavailability and reduced toxicity as well as undesirable side effects. The preclinical phase finishes with the examination process regarding pharmacodynamic and pharmacokinetic parameters *in-vitro* and *in-vivo*.

If a drug is selected for preclinical evaluation, it should be emphasized that the behaviour of the tracer is well investigated. A tracer should display high affinity to the target region which could be a receptor or transporter. Furthermore, it is of utmost importance that the tracer is highly selective to the molecular target and has a low tendency to non-specific binding. A tracer should, from a metabolic point of view – if degraded – not result in a breakdown to radio-metabolites which possibly interfere at the target tissue.

The preclinical evaluation supports the authorization process of novel medicinal products by providing background data from animal models. The process comprises the evaluation of ADME characteristics, but also dose finding, and efficacy of candidate compounds (in dedicated animal disease models and controls). Nowadays it is state of the art to use imaging techniques such as PET [Lin, 1997] and magnetic resonance (MRI) for the establishment of kinetic and distribution data sets.

2. Metabolism / Biotransformation

Metabolism, also referred to as biotransformation is the biochemical modification or alteration of molecules such as nutrients, amino acids, drugs but also a variety of toxins within the body. The overriding aim of every organism when exposed to toxic substances is to get rid of it as quickly as possible [Evans, 2004]. Sometimes the organism is not able to excrete those endogenous or exogenous chemicals via bile or urine. Endogenously synthesized molecules like steroid hormones or bile pigments, but also many xenobiotica, are highly lipophilic compounds which usually cannot be removed out from the body very effectively. Therefore, a very efficient metabolism is needed to render those non-polar substances into a more polar counterpart to make them “excretable”. Otherwise, they would accumulate within the body and potentially cause harmful effects. In the course of evolution the enzymatic system of mammals developed a strategy to eliminate chemicals

with possible dangerous characteristics from the body. Intermediates or products of those enzymatic modifications are called metabolites. Compared to the lead compounds, the formed metabolites have a different chemical characteristic and behaviour. An important chemical characteristic of formed substances is their increased water solubility and the possibility to be excreted.

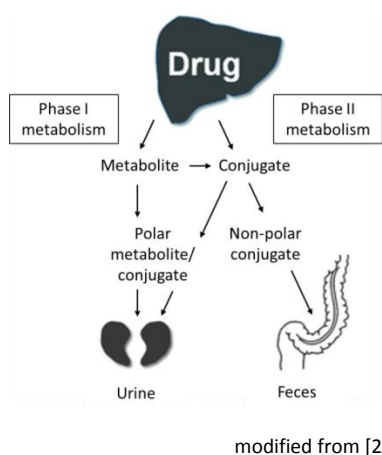
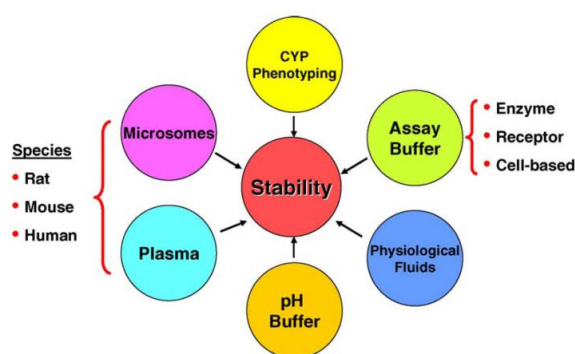


Figure 3: Phase I and Phase II of drug metabolism in the liver

Drug metabolism can be divided into two main paths: Phase I and Phase II. It is important to know that phase I and phase II enzymes play a vital role in both the de-toxification and elimination of different xenobiotica and drugs. It is an effective combination of these two enzymatic phases to finally eliminate all xenobiotic compounds from the body. In most cases molecules undergo phase I reaction first and then, if necessary, a phase II metabolism. In some cases phase II products undergo further phase I metabolism (e.g. sulphated steroids) [Manchee, 2004].



[Di, 2005]

Figure 4: Stability profiles in drug discovery

2.1 PHASE I METABOLISM

Phase I metabolism occurs in most tissues but the primary and first pass site occurs during hepatic circulation. Additional metabolism takes place mainly in the gastro-intestinal tract but also in epithelial, renal, skin and lung tissues. Nearly all phase I enzymes are located within the cells in the endoplasmatic reticulum. Utmost interest of the drug metabolism community is given to the hepatic cytochrome P-450 system [Eddershaw, 2004], which is the most important phase I oxidation system.

The metabolism of phase I enzymes includes a wide range of activities and is therefore splitted into the following categories: oxidation, hydration, reduction and hydrolysis. These categories describe so called functionalized reactions since they lead to the introduction or uncovering of key functional groups like -OH, -COOH, -NH₂, -SH and many more. These properties support the removal of xenobiotics directly from the body or via conjugation with the polar co-factors of phase II metabolising systems [Eddershaw, 2004]. It should be pointed out, that phase I metabolism may also give rise to reactive, potentially toxic metabolites which may bind covalently to tissue macromolecules [Manchee, 2004].

Enzyme	Reaction
Cytochrom P450s (CYPs)	oxidation, reduction
Monoamine oxidases (MAO)	oxidation
Flavin-containing monooxygenase (FMO)	oxidation
Alcohol dehydrogenase	oxidation
Aldehyde dehydrogenase	oxidation (reduction)
Xanthine oxidases	oxidation
Epoxide hydrolase	hydrolysis
Arboxylesterase and peptidase	hydrolysis
Carbonyl reductase	reduction

[Eddershaw, 2004]

Table 1: Major classes of phase I enzymes involved in drug metabolism

2.2 PHASE II METABOLISM

The general result of phase II metabolic reactions is the generation of much more hydrophilic and therefore more easily excretable compounds. This step is called conjugation. Resulting metabolites are largely devoid of pharmacological and/or toxicological activity [Manchee, 2004]. Phase II enzymes, which are responsible for phase II

metabolism are becoming more and more important in drug discovery and drug development. The major rate-limiting process to metabolic clearance is provided by phase I oxidations, but nevertheless there is a huge number of xenobiotics and drugs which are metabolically cleared by phase II enzymes [Miners, 1991]. A variety of phase II conjugating enzymes attack at functional groups of diverse molecules such as -OH, -COOH, -NH₂, -SH. Compared to the relatively high selectivity of a number of cytochrom P-450 subtypes (e.g. cholesterol biosynthesis), it is known that phase II enzymes are involved in the metabolism of xenobiotics and endogenous compounds [Manchee, 2004]. During phase II metabolism, different enzymatic reactions take place such as: glucuronidation, glycosidation, sulphation, methylation, acetylation and glutathione conjugation.

Enzyme	Reaction	Functional group
UDP-glucuronosyltransferase	Glucuronidation	-OH, -COOH, -NH, -NOH, -NH ₂ , -SH, ring N
UDP-glycosyltransferase	Glycosidation	-OH, -COOH, -SH
Sulphotransferase	Sulphation	OH, -NH, -NOH, NH ₂
Methyltransferase	Methylation	OH, NH ₂
N-Acetyltransferase	Acetylation	OH, NH ₂ , -SO ₂ NH ₂
Amino acid-N-Acyltransferase	Amino acid conjugation	-COOH
Glutathione-S-transferase	Glutathione conjugation	Epoxide, organic halide
Acyltransferase	Fatty acid conjugation	-OH

modified from [Gibson, 1994]

Table 2: Details of phase II enzymes

2.3 ENZYMES

2.3.1 General

Enzymes are large biological three-dimensionally structured proteins which are responsible for a vast amount of conversion reactions to sustain human life [Smith,

2006, Garret, 2009]. They are responsible for a variety of functions inside living organisms (e.g. work together in a specific order, creating metabolic pathways). During enzymatic reactions, substrates such as drugs, xenobiotics, endogenous compounds, etc. are converted into different product molecules. Enzymes are highly selective catalysts, and possess the property to accelerate the specificity and the rate of metabolic reactions. Most of the enzymes require organic or inorganic cofactors to assist in catalysis. They have a high

selectivity to molecules which belong to anabolic processes in contrast to broader substrate selectivity to catabolic associated molecules. Enzymes work by lowering the activation energy for a reaction, which raises the rate of the reaction extremely (up to millions of times faster compared to uncatalyzed reactions). Due to this advantage products are formed much faster and the equilibrium state of enzymes are reached more quickly. Enzymes are known to catalyze approximately four-thousand biochemical reactions [Bairoch, 2000].

Enzymes are generally classified into six main family classes and many more subfamily classes. The top level classification shows:

abbreviation	enzyme	catalyzed reaction
EC 1	Oxidoreductase	oxidation/reduction
EC 2	Transferase	transfer a functional group
EC 3	Hydrolase	hydrolysis of various bonds
EC 4	Lyase	cleave various bonds except hydrolysis/oxidation
EC 5	Isomerase	isomerisation changes with single molecule
EC 6	Ligase	covalent joining of two molecules

modified from [1]

Table 3: Main enzyme classes regarding to its function

Enzyme activities can be affected by other chemical compounds. Molecules which decrease enzyme activities are called inhibitors, which affect many drugs and venoms. Enzymes which increase activities are activators. Physicochemical parameters such as temperature, pressure, concentration and pH can alter the activity of enzymes.

2.3.2 Structure

Enzymes are globular proteins consisting of few (62 amino acid residues; e.g. 4-oxalocrotonate tautomerase) to up to over 2.500 residues (e.g. fatty acid synthase) [Chen, 1992, Smith, 1994]. Enzymes consist of long linear chains of amino acids (like all proteins) and fold to three dimensional structures which make them unique and specific. Most of the enzymes are much larger than the substrate they act on, but only 2-4 amino acids (active site) are directly involved in the catalytical process [3]. Because of the specific complementary geometric shapes of enzyme and substrate it is possible to fit them exactly into one another [Fischer, 1894]. This is often described as “key and lock” model.

2.3.3 Inhibition

The reaction rate of each enzyme can be negatively influenced by enzyme inhibitors. In many living organisms, inhibitors act as part of a feedback mechanism. If an inhibitor competes for the same enzyme as the substrate it is called competitive inhibitor [Price, 1979]. In that case it is not possible for substrate and inhibitor to bind to the enzyme at the same time. Binding of an inhibitor makes the enzyme unable to act. These types of inhibitor molecules frequently carry similar structures as the real substrate. If the substrate concentration rises, the inhibitor can be released from the enzyme.

An inhibitor can bind both, to the active binding site or to another position of the enzyme. Binding to another position effects a change in the shape of the binding-site; this is called allosteric effect. A higher substrate concentration does not effect a displacement of the inhibitor.

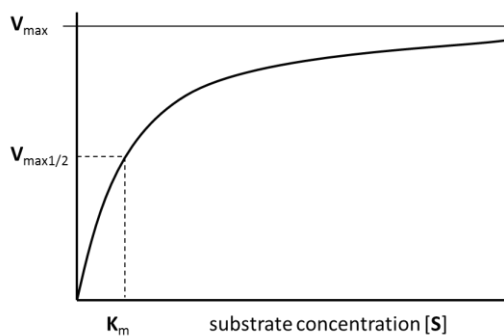
An uncompetitive inhibitor is only able to bind to the enzyme-substrate (ES) complex to result an enzyme-inhibitor-substrate (EIS) complex. This complex is enzymatically inactive.

A non-competitive inhibition takes place if the substrate and the inhibitor bind to the enzyme at the same time. The inhibitor does not bind to the active site. EI and EIS are enzymatically inactive.

2.4 KINETICS

Enzyme kinetics describe the reaction rate of a chemical process which is catalyzed by enzymes. Studying kinetics can disclose catalytic mechanisms of investigated enzymes. It also shows its role in metabolism or if and how an inhibitor alters its turnover rate. A special characteristic of a catalyzed reaction in contrast to an uncatalyzed reaction is a typical saturation kinetic.

At a defined enzyme concentration and a relatively low substrate concentration which slightly increases, the reaction rate rises linearly. The enzymes are only partly filled with substrate. An increasing substrate concentration leads to an increasing reaction rate. At high substrate concentration the enzyme is no longer able to catalyze the metabolism of every substrate molecule. The reaction rate reaches the theoretical maximum because of a complete occupation of the enzymes binding sites. At this time point the reaction rate is determined by the intrinsic turnover-rate of the enzyme – the maximum velocity ($V_{\max}$) is reached. The substrate concentration $[S]$ which is calculated at a turnover-rate at half-maximum of $V_{\max}$ is denoted by the Michaelis constant.



$$v = \frac{V_{\max}[S]}{K_m + [S]}$$

Figure 5: Michaelis-Menten kinetics and corresponding equation

2.4.1 Michaelis-Menten-kinetics

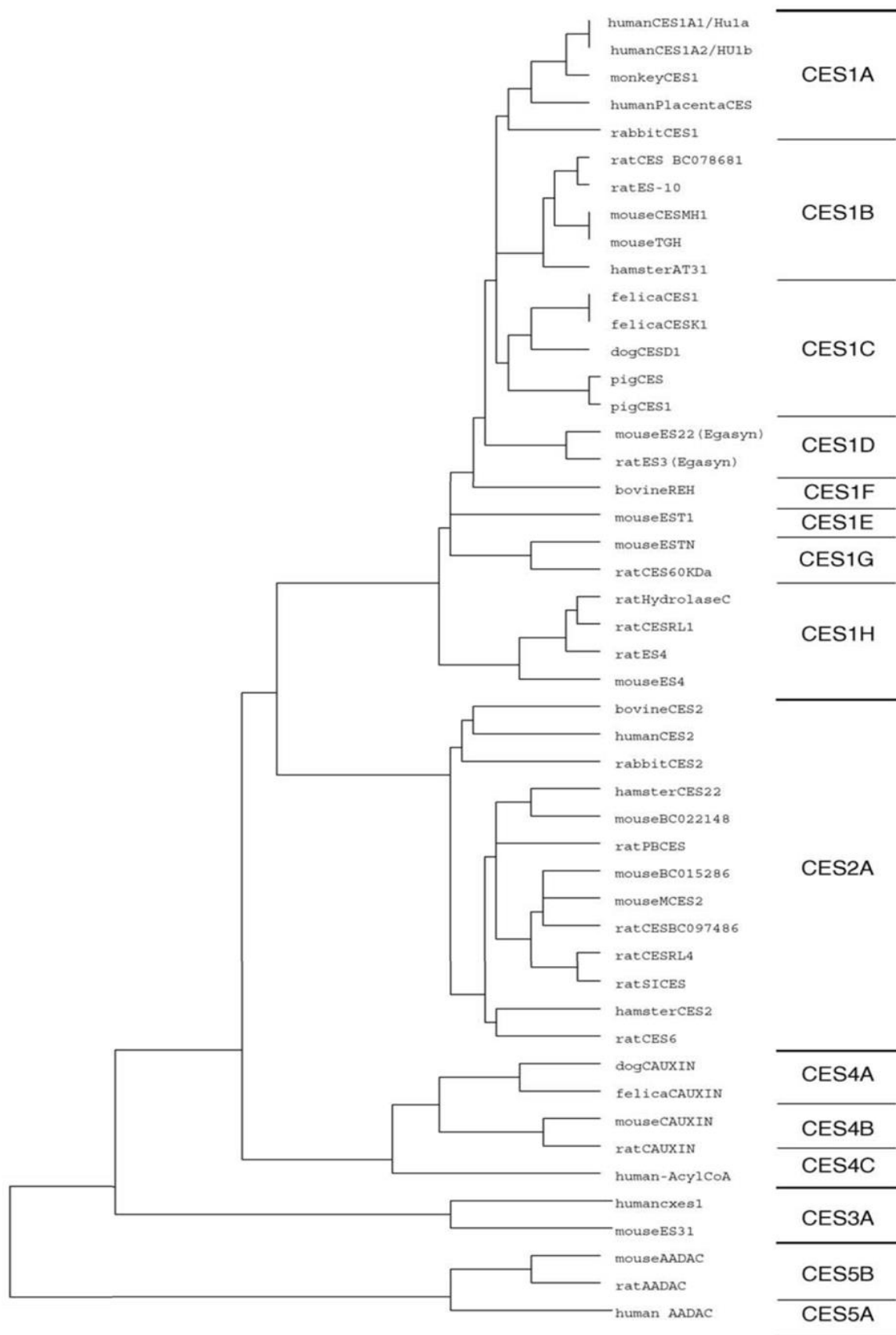
The best and most simple model of enzyme kinetics in biochemistry was described by Leonor Michaelis and Maud Menten – and referenced as the Michaelis-Menten kinetics. It is an equation which describes the turnover rate of enzymatic reactions referring to the substrate concentration and the reaction rate [Michaelis, 2011].

2.5 CARBOXYLESTERASE

2.5.1 General

Mammalian CES (EC 3.1.1.1) plays a highly important role in the hydrolytic biotransformation of a huge number of structurally diverse ester containing drugs and prodrugs [Sato, 1998, Yoshigae, 1998, Imai, 2003]. CES are members of the α/β -hydrolase fold family and show ubiquitous tissue expression profiles [Imai, 2006]. CES comprise a multigene family which is located mainly in the endoplasmatic reticulum (ER) in hepatocytes but also in the cytosol of many tissues and organs [Sato 1987, Heyman, 1980, Heyman, 1982]. Those serine esterases are involved in both drug metabolism and activation [Imai, 2006].

Mammalian CES isoenzymes are classified into five groups (CES1-CES5) and subgroups according to their high homology and similarity of characteristics [Sato, 2006]. CES 1 and CES 2 are the most different isoenzymes within the CES group and are differentiated on the basis of substrate specificity, tissue distribution, immunological properties and gene regulation [Imai, 2006]. Human CES basically consists of two main isoenzymes namely human carboxylesterase 1 (hCE-1, CES1A1, HU1) and carboxylesterase 2 (hCE-2, hiCE, HU3). The CES1 family includes the major forms of CES isoenzymes (more than 60% of homology of human CES). Thus, this family could be splitted in eight subfamilies (CES1A to CES1H) [Sato, 2006] (Figure 3). A third, brain specific CES was found in 1999 by Mori et.al., named hBr3 (hCE3) but reported insufficiently [Imai, 2006]. A new isoenzyme of human CES, CES 3 was identified in 2004 [Sanghani, 2004] and is expressed in a very low level in the liver and gastrointestinal tract [Quinney, 2005].



[Satoh, 2006]

Figure 6: Phylogenetic tree of the carboxylesterase superfamily

2.5.2 Structure

CES contain an N-terminal hydrophobic signal peptide that marks them for transporting through the ER. The His-X-Glu-Leu (HXEL) sequence which is located at the C-terminus of the protein is able to bind with KDEL receptor for retention in the luminal site of the ER [Potter, 1998]. Serine, Histidine and Glutamine which are the amino acid residues of the catalytic triad and four cysteine amino acids which are likely involved in specific disulfide bonds are similarly positioned in each CES [Imai, 2006]. CES are glycoproteins and their carbohydrate modifications are necessary for enzyme activity [Kroetz, 1993]. The first crystal structure of mammalian CES was reported in 2002 (rabbit CES1) and showed that CES share the serine hydrolase fold, which was observed in other esterases [Bencharit, 2002].

2.5.3 Tissue distribution

If CES play an important role in the detoxification process of xenobiotics, the typical expression happens in the related epithelia of most organs. High levels of CES activity are found in the blood of mammals, because some of the isoenzymes are destined for export into plasma [Sato, 2006]. However, in the blood of humans no such activity can be detected [Yan, 1995, Guemai, 2001, Li, 2005]. Expression profiles of gene encoding CES are mainly regulated by nutritional status, hormonal factors and xenobiotics. Although several intensive studies regarding the consequences of regulation of CES by chemicals and drugs have been conducted, there is still a lack of information about their regulations [Sato, 1998, Hosokawa, 1988, Furihata, 2003, 2004].

The highest levels of hydrolytic CES activity of many mammalian species are located in the liver [Sato, 1998] and in lower levels in many other tissues [Morgan, 1994]. Regions, where moderate to considerable mammalian CES activity is prevailing are in small intestine [Gaustad, 1992], skin [McCracken, 1993a], heart [Dean, 1995], muscle [Nambu, 1987], blood [Welch, 1995], lung [McCracken, 1993a, Dean, 1995], testis [Yan, 1995], respiratory/nasal tissue [Nikula, 1995,] adipose tissue [Tsujita, 1992], leucocytes [Laohapand, 1985] and the central nervous system (CNS) [Dean, 1995, Yamada, 1995]. Human CES was found in liver [Yamada, 1994, Sato, 2002] small intestine [Sato, 2002] plasma [Welch, 1995, McCracken,

1993b] brain [Yamada, 1994, Ehrich, 1995], lung [Satoh, 2002] stomach [Lund-Pero, 1994], colon [Lund-Pero, 1994], macrophages [Munger, 1991], monocytes [Zschunke, 1992, Saboori, 1991] and in the CNS [Hojring, 1977].

The highest expressions of CES 1 are in the liver and in lower amounts in macrophages, lung epithelia [Munger, 1991], heart, testis and other tissues. In gastrointestinal tract, CES 1 is very poorly expressed. CES 2 is mainly found in small intestine, but also in colon, kidney, liver, heart, brain and testis [Satoh, 2002], but essentially absent in all other organs [Schwer, 1997, Xu, 2002].

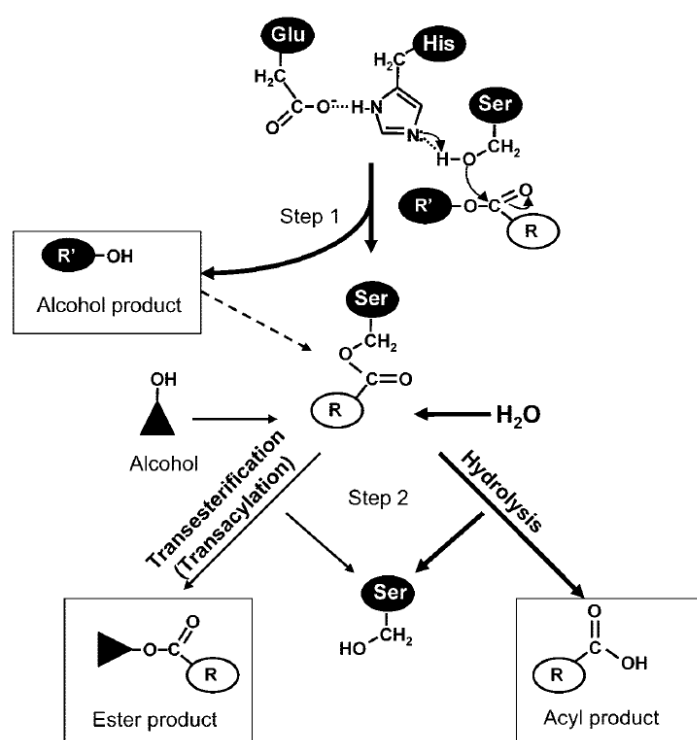
The activity of human CES was detected mainly in the microsomal fraction of the liver but also in high capacity in the lysosomes. Lysosomes contribute significantly to the esterolytic capacity of the liver [Satoh, 1998]. CES which is found in human plasma is primarily synthesized in the liver and then secreted into the periphery via Golgi apparatus [Robbi, 1992, Murakami, 1993, Yan, 1995].

The presence of CES in capillary endothelial cells serves the purpose to protect the CNS from toxic esters and is maybe therefore a necessary part of the BBB system [Yamada, 1994]. The distribution of the CES in human and rat brains seems to be different [Yamada, 1995].

2.5.4 Pharmacological and chemical aspects

The hepatic CES is able to catalyze the hydrolysis of many different xenobiotics such as drugs, chemicals (toxicants) which come from the environment, carcinogens and pesticides but also endogenously formed substrates. Food additives, such as preservatives, stabilizers, flavour enhancers, coloring agents, emulsifiers, antifoaming agents and humectants are also metabolized by this enzymatic group if they are corresponding molecules. The CES enzyme family is able to catalyze ester containing substrates to the respective alcohol- and free acidic moiety. From a chemical point of view, this fate happens to ester-, amide- and thioester-bond compounds. CES are also involved in the detoxification process of diverse drugs, carcinogens and environmental toxicants [Satoh, 1998]. CES use a catalytic triad (Ser-His-Glu) for catalysis, which is located at the base of a deep catalytic gorge [Bencharit, 2002, 2003a,b]. They cleave various ester bonds in a two-step process that includes the formation

and degradation of an acyl-enzyme intermediate. The first step contains the binding of the acyl hydroxyl group of the substrate molecule to the hydroxyl group of serine to form an acyl-enzyme complex while the alcohol moiety is released. Then, after the attack of histidine-activated water on this acyl-enzyme complex, the acid moiety is released too. It is of importance to have a distinct microenvironment which surrounds these molecules to facilitate the substrate binding as for the release of the alcohol and/or acyl compounds [Imai, 2006].



[Imai, 2006]

Figure 7: The two-step catalytic mechanism of mammalian carboxylesterase

The hydrolysis of various compounds by CES not only implies the inactivation of drugs to increase their water solubility for excretion. It also means that prodrugs can be activated to their biologically active form [Sato, 1987, Heymann, 1980, 1982, Leinweber, 1987].

2.6 MICROSOMES

In cell biology, microsomes are vesicle-like artifacts (approximately 100 nm in diameter) re-formed from pieces of the endoplasmic reticulum (ER) when eukaryotic cells are broken-up in the laboratory. Microsomes are not ordinarily present in living cells [Voet, 2011]. To separate the microsomal fraction from cells (mainly from hepatocytes), they have to be homogenized and then separated from other cellular debris by differential centrifugation.

Microsomes contain the enzymatic superfamily cytochrome P-450.

2.7 CYTOCHROME P-450

2.7.1 General

The cytochrome P-450 (CYP450) intracellular proteins are a family of haem proteins resulting from expression of a gene super-family that currently contains around 1000 members in species ranging from bacteria through to plants and animals [Hasler, 1999]. CYP450 are necessary for the detoxification of chemicals and the metabolism of drugs [Lynch, 2007]. CYP450 are the major enzymes involved in drug metabolism and bio-activation, accounting for about 75% of the total number of different metabolic reactions [Guengerich, 2008]. In contrast to most enzymes, CYP450 are involved in xenobiotic metabolism and have evolved a broad substrate specificity which enables them to metabolise a very wide range of compounds to which an organism may be exposed [Evans, 2004].

These cell-membrane bound mammalian proteins encode enzymes which are involved in: the metabolism of pharmaceuticals, xenobiotics and pollutants, arachidonic acid metabolism and eicosanoid biosynthesis, sterol and bile acid synthesis, steroid synthesis and catabolism, vitamin D₃ synthesis and catabolism, retinoic acid hydroxylation, biogenic amine and neuroamine metabolism and orphan CYP450 of unknown function [Nebert, 2002]. The name “cytochrome P450” derives from the fact that these proteins have a haem group and

an unusual spectrum. CYP450 shows an absorbance band maximum in the range of 450 nm [Nelson, 2009] after the reducing agent sodium dithionite was added to diluted microsomes which were previously gassed with carbon-monoxide [Hasler, 1999].

Sterols	Xenobiotics	Fatty Acids	Eicosanoids	Vitamins	Unknown
1B1	1A1	2J2	4F2	2R1	2A7
7A1	1A2	4A11	4F3	24A1	2S1
7B1	2A6	4B1	4F8	26A1	2U1
8B1	2A13	4F12	5A1	26B1	2W1
11A1	2B6		8A1	26C1	3A43
11B1	2C8			27B1	4A22
11B2	2C9				4F11
17A1	2C18				4F22
19A1	2C19				4V2
21A2	2D6				4X1
27A1	2E1				4Z1
39A1	2F1				20A1
46A1	3A4				27C1
51A1	3A5				
	3A7				

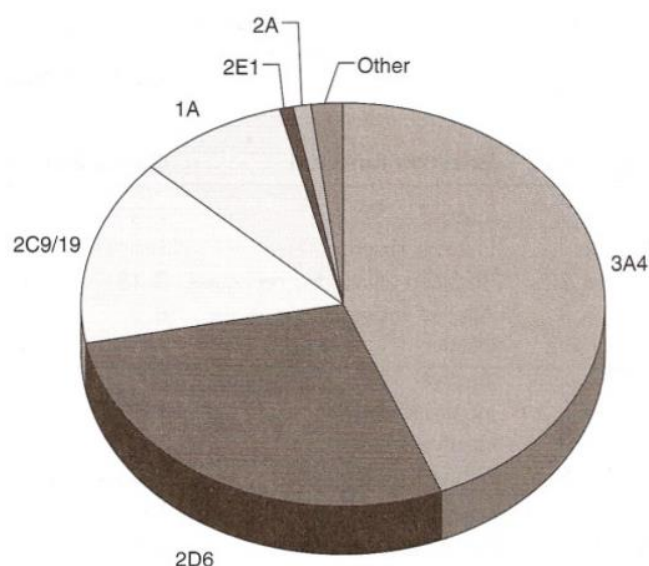
[Guengerich, 2005a, 2005b]

Table 4: Classification of human CYP450 based on major substrate classes

In humans, CYP450 are known for their central role in phase I drug metabolism where they are of critical importance to two of the most significant problems in clinical pharmacology: drug interactions and interindividual variability in drug metabolism [Danielson, 2002].

2.7.2 Nomenclature

The nomenclature is based on the amino-acid sequence of each isoform rather than a particular reaction or substrate of individual CYP450 [Eddershaw, 2004, Nelson, 2009]. If an isoform has a sequence homology of more than 40%, it belongs to the same CYP gene family such as CYP1, CYP2, CYP3, etc. If the homology of an isoform is greater than approximately 55-60%, then they belong to the same subfamily such as CYP1A, CYP2B, etc. [Eddershaw, 2004, Nelson, 2009]. Every individual isoform is given a number such as CYP2A1, CYP2A2, etc. [Eddershaw, 2004]. Humans have 57 CYP450 genes [Nielsen, 2005], which are subdivided to 18 families and 44 subfamilies. There are more than 9000 known CYP450 sequences [Nelson, 2009].



[Eddershaw, 2004]

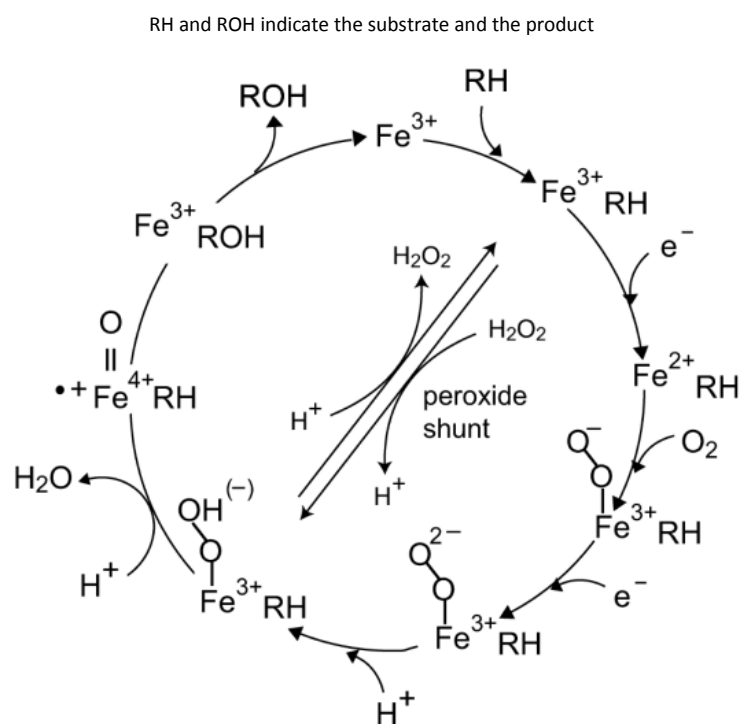
Figure 8: Percentage of commonly prescribed drugs metabolized by each CYP isoform

2.7.3 Tissue distribution

CYP450 enzymes are distributed ubiquitously in the human body but predominantly expressed in the liver. They can also be found in the small intestine (reducing drug bioavailability), lungs, placenta and kidneys [Slaughter, 1995]. CYP450 are predominantly membrane-associated proteins [Berka, 2011] and bound to membranes within the ER or in the inner membrane of mitochondria.

2.7.4 Biochemical Aspects

CYP450 act together with the flavoprotein NADPH-cytochrome P450 reductase which is involved in the transfer of electrons from NADPH to CYP450 [Eddershaw, 2004]. CYP450 “activate” molecular oxygen for the oxidative metabolism of a great variety of lipophilic organic chemicals.



[Sakaki, 2012]

Figure 9: Reaction cycle of cytochrom P-450

2.7.5 Pharmacogenetic aspects

Drug metabolism via CYP450 enzymes exhibits genetic variability (also called polymorphism) that influences a patient's response to a particular drug [Weinshilboum, 2003]. Humans which inherit two "wild type" alleles are so called "extensive" metabolizers. Polymorphism occurs when a variant allele replaces one or two wild type alleles. Variant alleles usually encode CYP450 enzyme that has reduced or no activity [Wilkinson, 2005]. A subject which has one wild type and one variant allele has reduced enzyme activity whereas somebody with two copies of variant alleles is called "poor metabolizer". It may also happen that humans inherit multiple copies of wild-type alleles, which results in an excess enzyme activity. That phenotype is termed "ultrarapid" metabolizer [Phillips, 2001].

2.7.6 Drug Interaction

Many drug interactions are the result of an alteration of CYP450 metabolism [Michalets, 1998]. Drugs can be metabolized by one single or even by multiple CYP450 enzymes [Daly, 2003]. Drugs that cause CYP450 metabolic drug interactions are referred to as either

“inducers” or “inhibitors” [Lynch, 2007]. An inducer is able to increase the CYP450 enzyme activity because of increased enzyme synthesis. An inhibitor blocks the metabolic activity of one or more CYP450 enzymes. It has been described that drugs can do both: be metabolized by and inhibit the same enzyme, or be metabolized by one and inhibit another enzyme [Ray, 2004].

3. Receptors

3.1 GENERAL

Receptors are molecules, which are ubiquitarily distributed in the organism and mostly found on the surface of various cell-types. Receptors receive chemical or physical signals from different origins coming from outside or inside of the cell. A binding process of substrates to the binding center of these receptors, which is called the “active centre” or “active site”, generates signals which direct the cell to some action (e.g. to die, to divide, to allow certain molecules to enter or exit). These substrates or ligands are generally small molecules like peptides, hormones, neurotransmitters, toxins but also pharmaceutical drugs.

Receptors are protein molecules existing in different dimensions such as the 40 kilodalton (kD) – Immunoglobuline G receptor [Tosi, 1988] or the 215 kD – mannose 6-phosphate receptor [Brown, 1986]. Receptor proteins could be cell surface proteins which are embedded in the plasma membrane or nuclear receptors which are integrated in cytoplasm or nucleus.

Each receptor recognizes and often binds just one distinct ligand which has a specific shape. The drug must interact with complementary surfaces on these receptors. This specific

binding of the ligand to the receptor, which follows the so called „key-lock principle“, activates or inhibits a specific biochemical pathway. The binding of a drug to a receptor is determined by stabilizing effects such as: hydrogen bonds, ionic bonds, van der Waals forces and even covalent bonds. The drug-receptor binding process describes a reversible reaction which is moving towards the equilibrium and then a constant interplay between complex formation and complex dissociation.

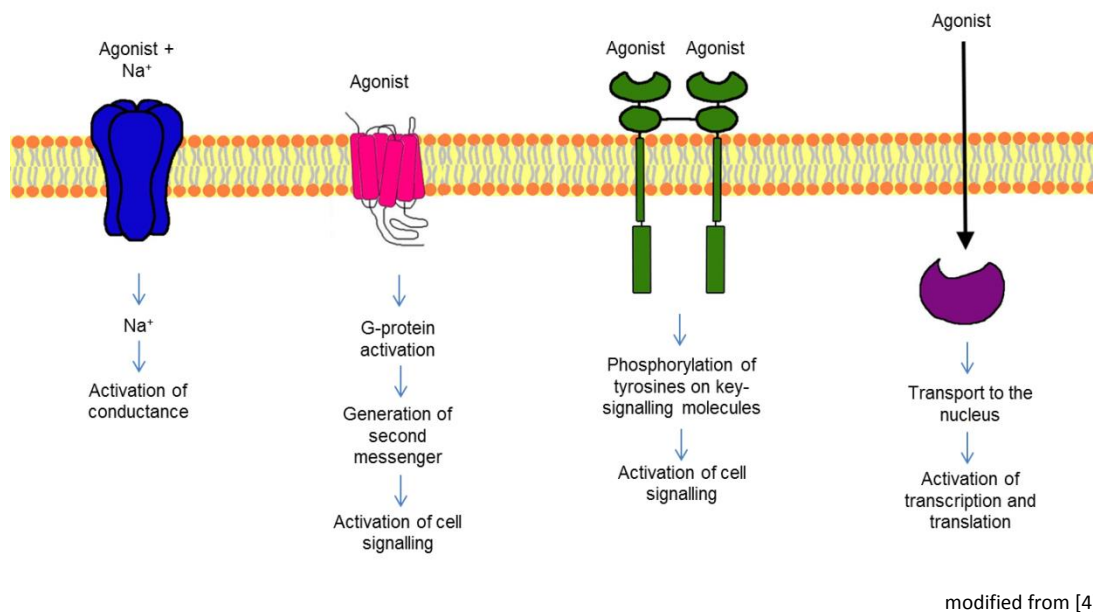


Figure 10: Illustration of the main receptor types and their function

However, some ligands block the receptor without inducing any response – these are called „antagonists“. Other substrates have the potential to bind to the receptor and trigger a response by the cell – these are called „agonists“. Depending on their behaviour to bind to the target region agonists as well as antagonists can be defined to be selective, affine or specific.

3.2 AGONIST

An agonist is a ligand or substrate which mimics the action of an endogenously generated substance and binds to the active site of a receptor protein and triggers a response by the cell – a cascade, which results in a physiological action. Agonists exist in a variety of different

characteristics (e.g. partial-, full-, inverse-, silent-, super-agonists) and have accordingly different responses, which could lead to contraction, relaxation, secretion, enzyme activation, etc.

3.3 ANTAGONIST

An antagonist is a type of receptor ligand which does not induce a biochemical response upon binding to the receptor, but prevents an agonist depended response of the cell. In pharmacology, antagonists have affinity but no efficacy for receptors and binding disrupts the receptor to be susceptible for any agonist. Agonists exert their effects by binding to the active site or the allosteric site of the receptor molecule. The activity of antagonists can be reversible or irreversible – depending on the duration and stability of the formed antagonist-receptor complex. The effectiveness of antagonists is determined by competing with endogenous ligands at the binding sites on receptors.

3.4 RECEPTOR CLASSIFICATION

The structural diversity of receptors divides them into three main categories:

3.4.1 Peripheral membrane receptors

This type of proteins is temporarily adhered to the side of the membrane to which they are associated. If they attach to the lipid-bilayer of the cell surface these proteins get embedded but do not cross. This reversible process has shown to regulate cell signalling [Pollard, 2007, Cafiso, 2005].

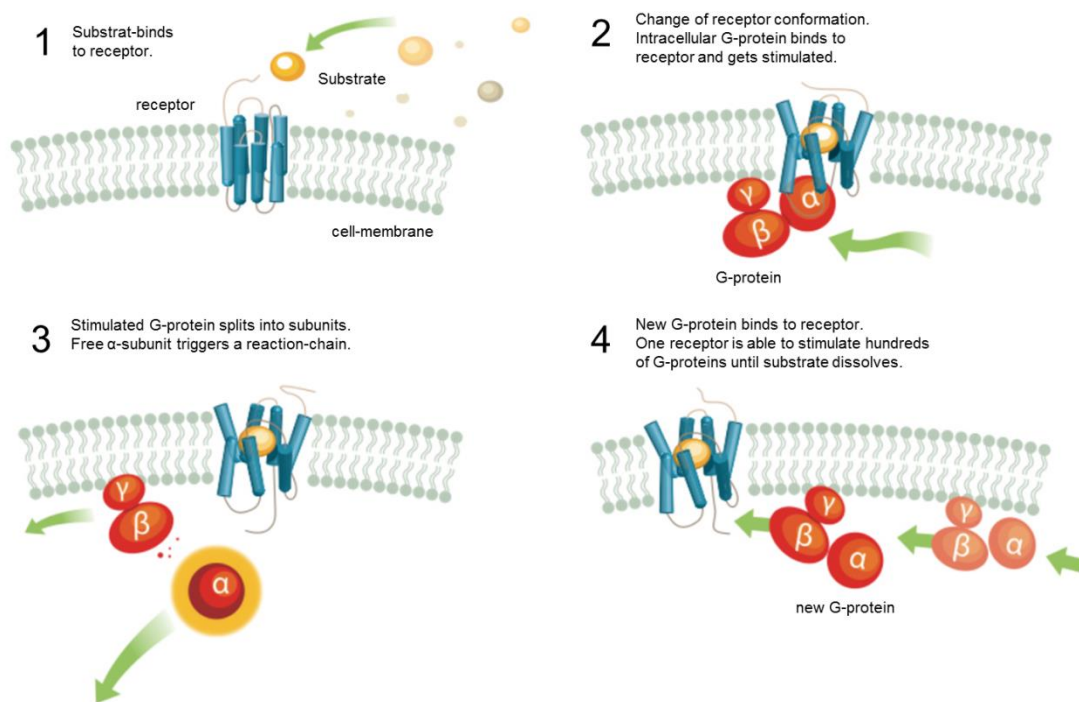
3.4.2 Trans-membrane receptors

These types of proteins penetrate the lipid bilayer of the cell and transmit information into the interior of the cell.

G-protein coupled receptors

G-protein coupled receptors (GPCRs) are also known as seven transmembrane receptors, since they possess seven transmembrane helices [Gobeil, 2006]. Once activated, GPCRs in turn induces an associated G-protein that in turn activates intracellular signalling cascades. This receptor type can be grouped into 6 classes based on their sequence homology and functional similarity [Kolakowski, 1994, Foord, 2005].

G-protein-coupled receptors are involved in many diseases, and are also the target of approximately 40% of all modern medicinal drugs [Filmore, 2004, Overington, 2006].



modified from [5]

Figure 11: Simplified illustration of the G-protein-coupled receptor pathway

Ligand-gated ion channels

This subtype, also known as ionotropic receptors consists of homomeric and heteromeric oligomers [Boron, 2005]. Binding of a drug to the receptor results in

opening of the channel and starting an ion flow whereas closing of the receptor stops the ion flow.

Enzyme linked receptors

These receptors are also known as catalytic receptors, where the binding of an extracellular ligand causes enzymatic activity on the intracellular side [Dudek, 2006]. The most common type is “receptor tyrosine kinase”.

3.4.3 Intracellular receptors

These types of proteins are mainly located inside of the cell. They could be found in cell nucleus, cytoplasm or at the endoplasmic reticulum. Ligands which bind to these receptors are usually intracellular second messenger molecules and extracellular lipophilic hormones.

3.5 PLASMA STABILITY

The stability of various drug candidates in plasma is of huge importance for maintaining acceptable drug concentrations and sustaining biological half-life in order to obtain desirable pharmacological effects. Chemicals which are unstable in plasma show a rapid degradation, short half-life and sometimes a poor *in-vivo* performance. This is mainly due to the ongoing metabolic process after blood samples were taken until their measurement. This behaviour pattern requires the presence of hydrolase inhibitors in plasma samples using standard components to inhibit degradation [Di, 2005].

The screening process for plasma metabolites provides useful information to prioritize drugs for *in-vivo* studies and to force researchers early enough to modify chemical structures in order to improve stability against metabolic degradation of existing catalytic plasma enzymes [Borthwick, 2002, 2003, Breitenlechner, 2004].

Several methods were established to verify plasma stability with main focus to improve efficiency of sample preparation and analysis such as direct injection of plasma using liquid chromatography with connected mass spectrometer(s) (LC-MS-(MS)) with restricted access

HPLC columns [Wang, 2002]. Other possibilities are the use of automated column switching methods [Peng, 1999] or robotic sample preparation [Linget, 1999].

4. Blood-Brain-Barrier

4.1 GENERAL

The continuous tight layer of the brain endothelium lining capillaries is commonly called the blood-brain-barrier (BBB) and acts as a barrier for drugs entering the central-nervous system (CNS). The penetration of hydrophilic drugs or xenobiotics into the brain depends on the restriction of the BBB [Yoon, 2006]. Lipophilic components including CNS-targeted drugs are able to reach the brain via passive diffusion. Chemicals can also enter the brain by active processes like influx transporters but as well back to periphery via active efflux systems [Pardridge, 1998, Tamai, 2000].

The BBB penetration potential of various chemical components which are potential drug candidates could be assessed by different *in-silico*, *in-vitro* and *in-vivo* methods. The octanol-water partition coefficient ($\log P$) [Pardridge, 1995] (also applicable as *in-vitro* assay using HPLC analysis), hydrogen-bond potential ($\Delta \log P$) [Young, 1988], molecular polar surface area (PSA) [Clark, 1999], linear free energy [Platts, 2001] and surface tension [Suomalainen, 2004] are physicochemical properties being included in *in-silico* assays. Micro-dialysis [Mano, 2002], cerebrospinal fluid sampling [Shen, 2004], autoradiography [Ross, 2004], nuclear magnetic resonance (NMR) [Ludemann, 2000] and positron emission spectroscopy [Luurtsema, 2005] are possible *in-vivo* approaches. The *in-vitro* applications are using isolated brain capillaries [Siakotos, 1969] and cultured cells such as bovine brain microvessel endothelial cells [Otis, 2001], brain capillary endothelial cells [Yamazaki, 1994], mouse brain endothelial cells⁴ [Murakami, 2000] and Colon carcinoma² cells (Caco2) [Faassen, 2003]. Other *in-vitro* methods to assess the membrane permeability are the parallel artificial membrane assay (PAMPA) [Di, 2003] and immobilized artificial membrane chromatography [Dash, 2003, Reichel, 1998].

4.2 IMMOBILIZED ARTIFICIAL MEMBRANE

Immobilized artificial membranes (IAM) mimic the lipid environment of a fluid cell membrane on a solid matrix. This is of interest for the prediction of drug permeation into biological membranes such as BBB [Ong, 1995, 1996].

Drug absorption, which is associated with activity, toxicity and distribution of a drug, often depends on drug membrane partitioning. Orally administered drugs are poorly absorbed by cell membranes within the gastrointestinal tract. If these drugs are able to pass the intestinal mucosa, one of the key mechanisms is passive diffusion [Artursson, 1991].

Solute-membrane interactions are characterized by the membrane partition coefficient (K_m) which represents the solute distribution related to all possible molecular interactions between the aqueous phase and the membrane [Ong, 1996, Tavares, 2012]. A synthetic phospholipid monolayer is connected covalently to silica particles and represents the packing material of a high-pressure-liquid chromatography (HPLC) column. Therefore with the HPLC technique it is possible to define partitioning solutes in an artificial membrane very quickly [Rhee, 1994, Pidgeon, 1989]. For drugs which are absorbed mainly via passive diffusion, the permeability (P_m) through the membrane is directly proportional to K_m [Stein, 1986].

For more than forty years, n-octanol-water partitioning systems (e.g. log P) have been used as a reference during the evaluation process of various drugs to determine lipophilicity. Large progress has been achieved towards prediction of log P using *in-silico* methods and, of course, the understanding of log P is a prerequisite to estimate the behaviour of solutes in lipophilic environments [Giaginis, 2008]. Log P values between 1 and 3.5 have been considered optimal for brain penetration of a compound (through passive diffusion) and the log P value is frequently used as a selection criterion to move compounds forward in the development process both for “classical” drug candidates and radioactive compounds [Waterhouse, 2003, Clark, 1999].

The stationary phase of an IAM column is prepared from phospholipids covalently bonded to a propylaminosilica supported material. The free propylamine residues are chemically

treated to minimize undesired basic function in the silica backbone [Giaginis, 2008]. The most frequently used IAM columns contain phosphatidylcholine as stationary phase. The use of double chain IAM surfaces mimics the natural occurring phospholipids in membranes even better and, therefore, a higher correlation to permeability data is obtained [Taillardat-Bertschinger, 2003, Markovich, 1989, Barbato, 2004].

IAM chromatography permits the use of mobile phases, which are free of organic solvents to directly measure $\log k_w$ (isocratic capacity factor) values. For aqueous solvents a phosphate buffered saline (PBS) and mainly a pH value of 7.0 are used to mimic nearly physiological conditions. This is applicable only for compounds which can be quantitatively dissolved in aqueous solutions. Substances, which are more lipophilic and therefore developing a higher affinity to the stationary phase, acetonitrile up to 30% (v/v) has to be added for an efficient retention. The $\log k_w$ values were obtained by linear extrapolation to 100% aqueous solvent by plotting them versus the percentage of organic compound [Morse, 2001, Rhee, 1994, Taillardat-Bertschinger, 2002].

5. Positron-Emission-Tomography

Positron emission tomography (PET) has become a powerful scientific and clinical tool for probing biochemical processes in the human body. The clinical application of PET has mushroomed in the last decade and it has proven to be needed in the evaluation and diagnosis of diseases [Schlyer, 2004]. The measurement of altered biochemical pathways and metabolic behaviour *in-vivo* in a non-invasive and quantitative manner is done routinely. The advantages of PET are sensitivity, versatility and the broad scope. That is why this powerful molecular imaging technique is suitable and valuable for clinical use.

PET is a nuclear medical imaging technique which produces three dimensional pictures of functional processes in the body. The tomographic system detects two simultaneous gamma-rays which are emitted in consequence of a positron emitting radionuclide. The gamma rays are emitted at an angle of nearly 180 degrees on basis of an annihilation reaction of a positron and an electron.

Routinely used radionuclides are e.g. fluorine-18 (F-18) and carbon-11 (C-11). Because C-11 is a short-lived radionuclide (half-life 20.3 minutes) the specific activity is critical and has to be monitored closely in the preparation of C-11- radiotracers [Schlyer, 2004].

5.1 C-11 CHEMISTRY

C-11 is produced in a cyclotron target using the $^{14}\text{N}(\text{p},\alpha)^{11}\text{C}$ nuclear reaction [Christman, 1975]. The target which is usually used is filled with high purity N_2 with a trace of oxygen (0.1-1.0%) to produce $[^{11}\text{C}]\text{CO}_2$. All precursor molecules require some synthetic manipulation during or after cyclotron bombardment. Today, the most widely used method for introducing C-11 into organic molecules is an alkylation with $[^{11}\text{C}]\text{methyl iodide}$ [Langstrom, 1976].

If this short-lived PET radioisotope is used, time is the critical parameter. Typically, 10-30 minutes are needed for radionuclide production, 40 minutes for tracer synthesis and quality control and, finally, up to 90 minutes for PET-imaging [Schlyer, 2004].

5.2 F-18 CHEMISTRY

A common way to receive F-18 using a cyclotron is the $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ nuclear reaction on O-18 enriched water which delivers $[^{18}\text{F}]\text{fluoride}$ of high molar activity. After irradiation, $[^{18}\text{F}]\text{F}^-$, which is isolated in the aqueous phase has to be recovered from the target material by passage through an anion exchange column [Schlyer, 2004]. $[^{18}\text{F}]\text{F}^-$ is then eluted using acetonitril which contains kryptofix 222 and potassium carbonate. After required azeotropic drying which has to be done under heat the “naked fluoride” is obtained. $[^{18}\text{F}]\text{F}^-$ is then activated for nucleophilic labelling to a variety of biomolecules.

6. Aim and outline of the present thesis

As prerequisite for newly developed drugs with special emphasis to positron-emission tomography tracers (PET-tracers), it is indispensable to deal specifically with preclinical evaluation. A suitable PET-radioligand for successful molecular imaging is characterized by high affinity, high selectivity, low non-specific binding and the absence of interfering radioactive metabolites in the target tissue. This requires a high metabolic stability against enzymatic degradation. Data which are obtained from metabolite studies are amongst mentioned parameters important to find the appropriate animal species during the preclinical process. Moreover, these preclinical data can help to explain the question on the efficacy of a drug or the lack of it.

Information about the metabolic *in-vitro* behaviour of a drug is essential for the knowledge about the catalytic activity of enzymes of phase I reactions. These enzymes, either single enzymes or whole enzyme families, can catalyze the conversion of molecules to a more hydrophilic form which makes them more easily excretable. Clarification of the possible penetration of a tracer designed for targets within the brain through the BBB has also to be evaluated in the developing process as early as possible. These important informations give the radiochemists the facility to re-design the structure of the molecule in an early stage.

To verify the *in-vivo* behaviour of a potential drug it is of importance to select the best suited animal model for the used drug and the corresponding research question. The data referring to the metabolic process in those animals give valuable and detailed information on the efficacy of selected radiotracers with respect to the expected metabolic behaviour in men.

The aim of this PhD-thesis is to provide an insight in the evaluation process of different PET-radiotracers with a main focus on metabolic stability testing. The overall topic is the implementation of a variability of enzymatic methods (*in-vitro* and *in-vivo*) using human tissue (enzymes and blood) to enhance the knowledge about the metabolic behaviour of investigated PET-tracers. In this way, a certain predictability could be enabled to guide

further development. Introduced methods will become standard procedures in the developing process to assess the metabolic status of new PET-radiotracers at the Vienna PET centre.

The **first aim** was to determine whether a molecule is stable against a main single enzyme of the phase I reaction. The selected enzyme of choice was carboxylesterase (CES), since its wide distribution in the organism, its broad substrate specificity and its ability to cleave ester functions of the selected radiotracers where the radiolabel was attached. Therefore I developed and evaluated an enzymatic method associated with radio-HPLC analyses to check the stability of selected radiotracers using Michaelis-Menten kinetics.

The **second aim** was to determine the metabolic behaviour against cytochrom P450 (CYP450). This enzyme family is distributed ubiquitarily in the human body and responsible for the enzymatic phase I metabolism of approximately 70% of all components in mammals. Hence, I developed an enzymatic method associated with radio-HPLC to evaluate the ratio of metabolites to intact parent compound.

Plasma stability testing of selected radiotracers was also carried out using chromatographic separation methods, like solid phase extraction (SPE) and radio-HPLC, respectively.

Gaining information about the penetration through BBB, which goes along with the lipophilicity of used tracers, was the **third aim** of this thesis. The better correlation of IAM data to the permeability of a tracer, in comparison with the questionable correlation of log P data to permeability, lead us to establish this method to get a deeper insight into the passive diffusion of radiotracers across the BBB. The IAM method also required an HPLC system for analysis.

The **fourth aim** of the thesis was to quantify the metabolic cleavage within a clinical study. A potent, rapid and robust method using radio-HPLC for analysis had to be developed since both arterial and venous blood samples were collected at different time-points.

Scientific part

7. Author's Contribution

I hereby declare to have significantly contributed to the realization of each of the five publications which are included in the present thesis.

First publication

FE@SUPPY and [¹⁸F]FE@SUPPY:2 - Metabolic considerations

Daniela Haeusler[#], Lukas Nics[#], Leonhard-Key Mien, Johanna Ungersboeck, Rupert R. Lanzenberger, Karem Shanab, Karoline M. Sindelar, Helmut Viernstein, Karl-Heinz Wagner, Robert Dudczak, Kurt Kletter, Wolfgang Wadsak Markus Mitterhauser

#...the authors contributed equally

I participated in the *ex-vivo* experiments at the AIT Seibersdorf and was responsible for the corresponding metabolite studies. Moreover, I performed the Carboxylesterase experiments, did the data analyses and participated in writing of the manuscript.

Second publication

The stability of methyl-, ethyl-, and fluoroethyl-esters against Carboxylesterases in-vitro: there is no difference

Lukas Nics, Daniela Haeusler, Wolfgang Wadsak Karl-Heinz Wagner, Robert Dudczak, Kurt Kletter, Markus Mitterhauser

I was mainly responsible for the study design, fully performed in the Carboxylesterase experiments and carried out the data analyses. Additionally, I took a significant part in the writing of the manuscript.

Third publication

Preparation and first preclinical evaluation of [^{18}F]FE@SNAP: a new PET tracer for the melanin concentrating hormone receptor 1 (MCHR1)

Cécile Philippe, Lukas Nics, Markus Zeilinger, Eva Schirmer, Helmut Spreitzer, Georgios Karanikas, Rupert Lanzenberger, Helmut Viernstein, Wolfgang Wadsak, Markus Mitterhauser

I was mainly responsible for the *in-vitro* metabolic experiments including carboxylesterase-, microsomes-, plasma-stability-, free-fraction- and immobilized artificial membrane studies. Moreover, I did metabolite data analyses and participated in writing of the manuscript.

Fourth publication

Development and automation of a novel NET-PET tracer: [^{11}C]Me@APPI

Christina Mark, Birgit Bornatowicz, Markus Mitterhauser, Matthias Hendl, Lukas Nics, Daniela Haeusler, Rupert Lanzenberger, Michael L. Berger, Helmut Spreitzer, Wolfgang Wadsak

I was mainly responsible for the *in-vitro* metabolic experiments including microsomes-, plasma-stability- and immobilized artificial membrane studies. Furthermore, I participated in analyses and interpretation of these data. I also participated in composing of the manuscript.

Fifth publication

Quantification of the radio-metabolites of the serotonin-1A receptor radioligand [*carbonyl*- ^{11}C]WAY-100635 in human plasma: An HPLC-assay which enables measurement of two patients in parallel

Lukas Nics, Andreas Hahn, Markus Zeilinger, Chrysoula Vraka, Johanna Ungersboeck, Daniela Haeusler, Sabine Hartmann, Karl-Heinz Wagner, Rupert R. Lanzenberger, Wolfgang Wadsak, Markus Mitterhauser

I conceived of the study, contributed to the whole methodological design and performed the experiments. Additionally, I carried out the analyses and interpretation of the data. I was mainly responsible for drafting of the manuscript.

Sixth publication

Combining image-derived and venous input functions enables quantification of serotonin-1A receptors with [*carbonyl*-¹¹C]WAY-100635 independent of arterial sampling

Andreas Hahn[#], Lukas Nics[#], Pia Baldinger, Johanna Ungersboeck, Peter Dolliner, Richard Frey, Wolfgang Birkfellner, Markus Mitterhauser, Wolfgang Wadsak, Georgios Karanikas, Siegfried Kasper, Rupert Lanzenberger

[#]...the authors contributed equally

I was mainly responsible for the *ex-vivo* metabolite experiments and carried out the data analyses. Moreover, I participated in writing and revising of the manuscript.

8. [¹⁸F]FE@SUPPY and [¹⁸F]FE@SUPPY:2 - Metabolic considerations

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Daniela Haeusler^{1,2#}, **Lukas Nics**^{1,3#}, Leonhard-Key Mien^{1,2}, Johanna Ungersboeck^{1,4}, Rupert R. Lanzenberger⁵, Karem Shanab⁶, Karoline M. Sindelar¹, Helmut Viernstein², Karl-Heinz Wagner³, Robert Dudczak¹, Kurt Kletter¹, Wolfgang Wadsak^{1,4} Markus Mitterhauser^{1,2,7*}

#... the authors contributed equally

¹ Dept of Nuclear Medicine, Medical University of Vienna, A-1090 Vienna, Austria

² Dept of Pharmaceutical Technology and Biopharmaceutics, University of Vienna, A-1090 Vienna, Austria

³ Dept of Nutritional Sciences, University of Vienna, A-1090 Vienna, Austria

⁴ Dept of Inorganic Chemistry, University of Vienna, A-1090 Vienna, Austria

⁵ Dept of Psychiatry and Psychotherapy, Medical University of Vienna, A-1090 Vienna, Austria

⁶ Dept of Drug and Natural Product Synthesis, University of Vienna, A-1090 Vienna, Austria

⁷ Hospital Pharmacy of the General Hospital of Vienna, A-1090 Vienna, Austria

ABSTRACT

Introduction

Recently, [¹⁸F]FE@SUPPY and [¹⁸F]FE@SUPPY:2 were introduced as the first PET-tracers for the adenosine A₃ receptor. Thus, aim of the present study was the metabolic characterisation of the two adenosine A₃ receptor PET tracers.

Methods

In-vitro carboxylesterase experiments were conducted using incubation mixtures containing of different concentrations of the two substrates, porcine carboxylesterase and phosphate buffered saline. Enzymatic reactions were stopped by adding acetonitrile/methanol (10:1) after various timepoints, and analyzed by a HPLC standard protocol. *In-vivo* experiments

were conducted in male wild type rats; tracers were injected through a tail vein. Rats were sacrificed after various timepoints (n=3) and blood and brain samples were collected. Sample clean-up was performed by a HPLC standard protocol.

Results

The rate of enzymatic hydrolysis by carboxylesterase demonstrated Michaelis-Menten constants in a μM range (FE@SUPPY: $20.12\mu\text{M}$ and FE@SUPPY:2: $13.11\mu\text{M}$) and limiting velocities of $0.038\mu\text{M}/\text{min}$ and $0.015\mu\text{M}/\text{min}$ for FE@SUPPY and FE@SUPPY:2, respectively. Degree of metabolism in blood showed: 15min p.i. 47.7% of [^{18}F]FE@SUPPY were intact compared to 33.1% of [^{18}F]FE@SUPPY:2; 30min p.i. 30.3% intact [^{18}F]FE@SUPPY were found compared to 15.6% [^{18}F]FE@SUPPY:2. In brain [^{18}F]FE@SUPPY:2 formed an early hydrophilic metabolite, whereas metabolism of [^{18}F]FE@SUPPY was not observed before 30 minutes p.i.

Conclusion

Knowing, that metabolism in rats is several times faster than in human, we conclude that [^{18}F]FE@SUPPY should be stable for the typical time span of a clinical investigation. As a consequence, from a metabolic point of view, one would tend to decide in favour of [^{18}F]FE@SUPPY.

KEYWORDS

PET, Adenosine A_3 Receptor, Fluorine-18, SUPPY, Metabolism, Carboxylesterase

INTRODUCTION

Adenosine is an important regulatory molecule which activates four receptors named A_1 , A_{2A} , A_{2B} , and A_3 (A_{1R} , A_{2AR} , A_{2BR} , A_{3R}), respectively, which all belong to the G-protein-coupled superfamily of receptors. There exists a lot of information about the A_1 and A_{2A} receptors, because good pharmacological tools - including radioligands - are available. In the case of the A_3 receptors, these tools have been presented lately, but there is still little information in literature regarding the distribution and density of these receptors in humans.

Yet, A3R agonists and antagonists are discussed for the treatment of pathophysiological conditions, such as asthma, neurodegenerative disorders and inflammatory diseases [1].

Moreover, it was repeatedly shown that A3Rs are highly expressed on the cell surface of various tumor cell lines [2, 3]. Hence, the A3R may also serve as a potential target for tumor growth inhibition and tumor imaging. The most suitable and accurate technique to gain information about receptors in the living organism is PET (positron emission tomography). As a prerequisite for molecular PET imaging, there is need for suitable radioligands displaying high affinity, high selectivity and low nonspecific binding.

The fluoroethylester FE@SUPPY (5-(2-fluoroethyl) 2,4-diethyl-3-(ethylsulfanyl-carbonyl)-6-phenylpyridine-5-carboxylate), which was evaluated by Li et al., displays high affinity ($K_i=4.22\text{nM}$) as well as excellent selectivity for the A3R (ratio $A_1/A_3=2700$) [4]. Recently, we introduced [^{18}F]FE@SUPPY as the first PET-tracer for the A3R [5, 6]. Meanwhile, a second potential PET-radiotracer has been presented, radiolabelled with the radionuclide bromine-76 [7].

Regarding possible metabolic pathways for [^{18}F]FE@SUPPY, it has to be pointed out that this diacyl-derivative carries two ester moieties, one carboxylic and one thiocarboxylic ester. Enzymes derived from the family of porcine carboxylesterases EC 3.1.1.1 (CES) would be expected to significantly contribute to cleavage of the ester function and thus to the degradation of this molecule [8]. [^{18}F]FE@SUPPY carries the [^{18}F]fluoroethyl-substituent on the carboxylic function, whereas the thiocarboxylic moiety carries an ethyl-ester group. It appeared promising to exchange these fluoroethyl and ethyl substituents within the molecule to generate a structural analogue which could be evaluated as a second potential PET-tracer.

Hence, we translated the fluoroethyl-ester [^{18}F]FE@SUPPY into the fluoroethyl-thioester [^{18}F]FE@SUPPY:2 (5-ethyl 2,4-diethyl-3-((2-[^{18}F]fluoroethyl) sulfanylcarbonyl)-6-phenylpyridine-5-carboxylate) [9, 10]. As far as assessable from literature, there is no conclusive data regarding differences in stability of carboxylic and thiocarboxylic ester moieties within one molecule. Interestingly, Azema et al. presented evidence for increased stability of a thioester function compared to a carboxylic ester group [11].

The radiosyntheses of both molecules were performed in simple one-pot, one-step reactions with good and reliable radiolabelling yields and good specific radioactivities [5, 9].

Biodistribution experiments revealed that the uptake pattern of the two tracers mainly followed the distribution pattern of the A3R mRNA [6, 10].

As a next step in the preclinical evaluation of these two radioligands, their metabolic profiles had to be examined to gain further information regarding their stability and degradation *in-vitro* and *ex-vivo*. Since radioactive metabolites – if present in the target tissue – could interfere with the signal of the intact molecule, distinct information regarding the amount of possible formation of these metabolites is crucial before application in humans. Thus, aims of the present study were the metabolic characterisation and the comparison of the two A3R PET tracers, [^{18}F]FE@SUPPY and [^{18}F]FE@SUPPY:2.

In detail we aimed for,

- (1) the assessment of the *in-vitro* stability of FE@SUPPY and FE@SUPPY:2 against Carboxylesterase and the metabolites;
- (2) the determination of the extent of metabolisation of [^{18}F]FE@SUPPY and [^{18}F]FE@SUPPY:2 in rodents; and
- (3) the comparison of the metabolic behaviour of both tracers.

MATERIALS AND METHODS

General

Radioanalytical thinlayer chromatography (radio-TLC) was performed for determination of radiochemical purity using TLC Silicagel 60 F₂₅₄ plates from Merck (Darmstadt, Germany). Analyses of radio-TLC plates were performed using a Canberra-Packard Instant Imager (Perkin Elmer, Watford, UK). Analytical high-performance liquid chromatography (HPLC) system was used for determination of specific radioactivity and identity consisting of a Merck-Hitachi LaChrom pump and UV-detector, as well as a NaI-radiodetector from Berthold Technologies (Bad Wildbach, Germany). The semi-preparative HPLC system (as part of the GE *TRACERlab Fx_{FN}* synthesizer) consisted of a Sykam S1021 pump (Sykam GmbH, Eresing, Germany), an UV detector and a radioactivity detector.

Solid phase extraction cartridges (SepPak[®] C18plus) were purchased from Waters Associates (Milford, USA). Anion exchange cartridges (PS-HCO₃) for [^{18}F]fluoride fixation were obtained

from Macherey-Nagel (Dueringen, Germany). All starting materials for precursor and reference standard syntheses were commercially available and used without further purification. Acetonitrile was purchased from Merck; methanol and carboxylesterase (E-3019) were purchased from Sigma Aldrich (Steinheim, Germany).

HPLC System1: Autosampler 1100, quaternary pump 1200 (at a flow rate of 3ml/min) and diode array detector 1100 (248 and 255nm) were purchased from Agilent (Böblingen, Germany). Chromolith® Performance column (RP-18e 100-4.6mm) and Chromolith® Guard Cartridge pre-column (RP-18e 5-4.6mm) as well as chemicals for mobile phase citrate buffer/acetonitrile 1:1 (0.034M sodium citrate tribasic dihydrate, pH 2.5) were purchased from Merck.

HPLC System2: Pump L-6220 (at a flow rate of 2ml/min) and UV-detector L-4000 were purchased from Merck-Hitachi. Radiomatic Flo-One Beta Flow Scintillation Analyzer was purchased from Packard, Radiomatic FlowBeta FSA 150 detector cell from PerkinElmer. Columns (see HPLC-System 1) and chemicals for mobile phase (60% acetonitrile and 40% water/acetic acid 97.5/2.5, 32mM ammonium acetate, pH 3.5) were purchased from Merck.

Chemistry and Radiochemistry

The preparations of the precursors, Tos@SUPPY and Tos@SUPPY:2, as well as the productions of the reference standards FE@SUPPY, FE@SUPPY:2, SUPPY:0, FE@SUPPY:11, FE@SUPPY:21, DFE@SUPPY and DFE@SUPPY:2 were described in detail elsewhere [9, 10, 12, 13] (for chemical structures see figure 1).

The automated preparations of both [¹⁸F]fluorinated FE@SUPPY analogues were performed on a GE *TRACERlab Fx_{FN}* synthesizer by radiofluorination of the corresponding tosylated precursor in a one-step, one-pot reaction, as already described [5, 9].

Enzyme reactions

Experiments (n=3) were performed using several pre-tested concentrations of the two tracers to get reasonable Michaelis-Menten kinetics. For FE@SUPPY 20, 50, 100, 200, 350µg/ml and for FE@SUPPY:2 5, 10, 20, 50, 200, 350µg/ml turned out to be the most

useful concentrations. Inkubations of the substrates were accomplished with constant quantity of porcine carboxylesterase: 80 I.U. in phosphate buffered saline (PBS) at 37°C. Enzymatic reactions were stopped by adding twice the amount of acetonitrile/methanol (10:1) after 0, 60, 120, 180, 240, 360min, respectively. After centrifugation (10000rpm, 3min) of the reaction mixtures, the obtained supernatant (~80µl) was analyzed by HPLC (injecting volume 20µl; System1).

Metabolite studies

In-vivo experiments were approved by the Austrian law on animal experiments and followed the protocol established in previous studies of our group [6]. Briefly, 21 male wild type rats (250-300g) were injected with $17\pm 1\text{MBq}$ of [^{18}F]FE@SUPPY and $14\pm 1\text{MBq}$ of [^{18}F]FE@SUPPY:2 through a tail vein. Subsequently, the rats were sacrificed after 2, 5, 10, 15, 30, 60, 120 minutes (n=3).

Blood samples (3ml) were collected from the abdominal aorta in sodium citrate buffered tubes and centrifuged (3000rpm, 5min) to separate cellular components. Sample clean-up was performed by vortexing plasma with the equivalent amount of methanol/acetonitrile (9:1) for 1min and by subsequent two step-centrifugation (3000rpm, 3min and 10000rpm, 3min) to remove precipitated proteins. The obtained supernatants were analyzed by HPLC (System2).

Brains were collected, weighed, homogenized and washed twice with phosphate buffered saline. After centrifugation (15000rpm; 5min), sample clean-up was performed as described for blood samples. Statistical calculations were performed with Microsoft® Excel and Graph Pad Prism®.

RESULTS

Radiochemistry

The radiopreparations for both A3R radioligands were completed within 70-80min. Starting from $51\pm 25\text{GBq}$ of [^{18}F]fluoride, $9.4\pm 3.6\text{GBq}$ ([^{18}F]FE@SUPPY) and $5.1\pm 4.2\text{GBq}$ ([^{18}F]FE@SUPPY:2) were formulated. Specific radioactivity (determined via radio-HPLC) was

70±26GBq/μmol for [¹⁸F]FE@SUPPY and 340±140GBq/μmol for [¹⁸F]FE@SUPPY:2 at the end of synthesis (EOS). Radiochemical purities always exceeded 98%.

Enzyme reactions

Results are presented in figure 2. The *in-vitro* assays showed Michaelis-Menten constants (K_M) of 20.12μM for FE@SUPPY and 13.11μM for FE@SUPPY:2. The limiting velocities (V_{Max}) were 0.038μM/min for FE@SUPPY and 0.015μM/min for FE@SUPPY:2. FE@SUPPY was cleaved to SUPPY:0, while FE@SUPPY:2 was cleaved to FE@SUPPY:21 (see figure 1). The HPLC assay was capable to qualitatively and quantitatively separate and resolve the potential metabolites. The detected radioactive metabolites are co-eluting with the corresponding reference standards (see table 1).

Metabolite studies

Extent of metabolism of [¹⁸F]FE@SUPPY and [¹⁸F]FE@SUPPY:2 in blood is presented in figure 3A. Rate of metabolism was different for [¹⁸F]FE@SUPPY and [¹⁸F]FE@SUPPY:2. 47.7% of [¹⁸F]FE@SUPPY were intact 15min after injection compared to 33.1% of [¹⁸F]FE@SUPPY:2; 30min p.i. 30.3% of intact [¹⁸F]FE@SUPPY were found compared to 15.6% of [¹⁸F]FE@SUPPY:2.

The radiolabeled metabolites in brain are presented in figure 3B. The point of time of the first detection of metabolites was different for [¹⁸F]FE@SUPPY and [¹⁸F]FE@SUPPY:2.

DISCUSSION

General

It appears undisputed, that there is a demand for a suitable PET-ligand for the A3R both for *in-vitro* and *in-vivo* use. The molecules, based on a diacyl-pyridine structure [¹⁸F]FE@SUPPY and [¹⁸F]FE@SUPPY:2, and later, xanthine-derivatives (radiolabelled with bromine-76) have been introduced for that purpose [5-7, 9, 10, 12]. All these molecules did not yet find their way into human application. In preclinical experiments with [¹⁸F]FE@SUPPY and [¹⁸F]FE@SUPPY:2, so far, the results indicate to focus on these molecules [5, 6, 9, 10]: Both

automated radiosyntheses were feasible; uptake was found in organs reliably expressing A3R mRNA and first brain autoradiographic slides showed good selectivity and specificity. The uptake pattern of [^{18}F]FE@SUPPY and [^{18}F]FE@SUPPY:2 was similar and comparable to that found by Kiesewetter et al. [7].

To find its way into clinical application a newly developed radiotracer has to show favourable metabolic characteristics: sufficient stability within the time period specified for the application and absence of excessive metabolites in the target tissue.

Enzyme reactions

Taking a closer look on the chemical structure of [^{18}F]FE@SUPPY and [^{18}F]FE@SUPPY:2, it can be expected, that, after passing the blood brain barrier or other tissue membranes, the molecules are cleaved by hydrolase (e.g. carboxylesterase) by forming 2- ^{18}F fluoroethanol and the respective corresponding acids (cf. figure 1). 2- ^{18}F Fluoroethanol would easily diffuse from tissue and would not contribute to receptor signal leading to lower non-specific accumulation compared to tracers keeping their radiolabel to their backbone. To obtain a first estimate of potential *in-vivo* degradation, *in-vitro* enzyme (e.g. CES) reactions can be a helpful tool. It can be derived from figure 2 and table 1, that the degradation paths and products of enzymatic cleavage of FE@SUPPY and FE@SUPPY:2 are different. Since we expect negligible differences because of structural isomeres and electronic effects within our two molecules, we are left without an explanation for this phenomenon. Transposing these findings into *in-vivo* conditions, [^{18}F]FE@SUPPY:2 would be expected to form a radioactive hydrophilic metabolite ([^{18}F]FE@SUPPY:21), potentially interacting with the target. Hence, from this point of view, [^{18}F]FE@SUPPY, forming no radioactive hydrophilic derivative by CES path, would be favourable.

Metabolite studies

In both, blood and brain, one hydrophilic metabolite was detected for [^{18}F]FE@SUPPY and [^{18}F]FE@SUPPY:2. As evident from figure 3A, differences in blood are between the time period of 15 and 30 minutes p.i. In this time period, [^{18}F]FE@SUPPY is more stable (after 15 minutes a factor of 1.42, after 30 minutes a factor of 1.93). This higher stability is in

agreement with our in-vitro findings (difference in K_M : factor 1.53). In brain, we found major differences: [^{18}F]FE@SUPPY:2 formed an early hydrophilic metabolite, whereas metabolism of [^{18}F]FE@SUPPY was not observed before 30 minutes p.i. A possible explanation for these findings could be the differences in metabolic pathways as observed in our previous carboxylesterase experiments.

CONCLUSION

Aim of the present study was the assessment of the *in-vitro* and *in-vivo* stability of [^{18}F]FE@SUPPY and [^{18}F]FE@SUPPY:2. Although being structurally closely related, the way of CES degradation was different: both in the degradation pathway and velocity. Metabolic characteristics both in rodent blood and brain were different too: for [^{18}F]FE@SUPPY, brain metabolites were not observed before 30 minutes p.i, and its metabolism in blood was more slowly. Knowing, that metabolism in rats is several times faster than in human, we conclude that [^{18}F]FE@SUPPY should be stable for the typical time span of a clinical investigation. Hence, from a metabolic point of view, for imaging of the density of the A3R one would tend to decide in favour of [^{18}F]FE@SUPPY.

ACKNOWLEDGEMENTS

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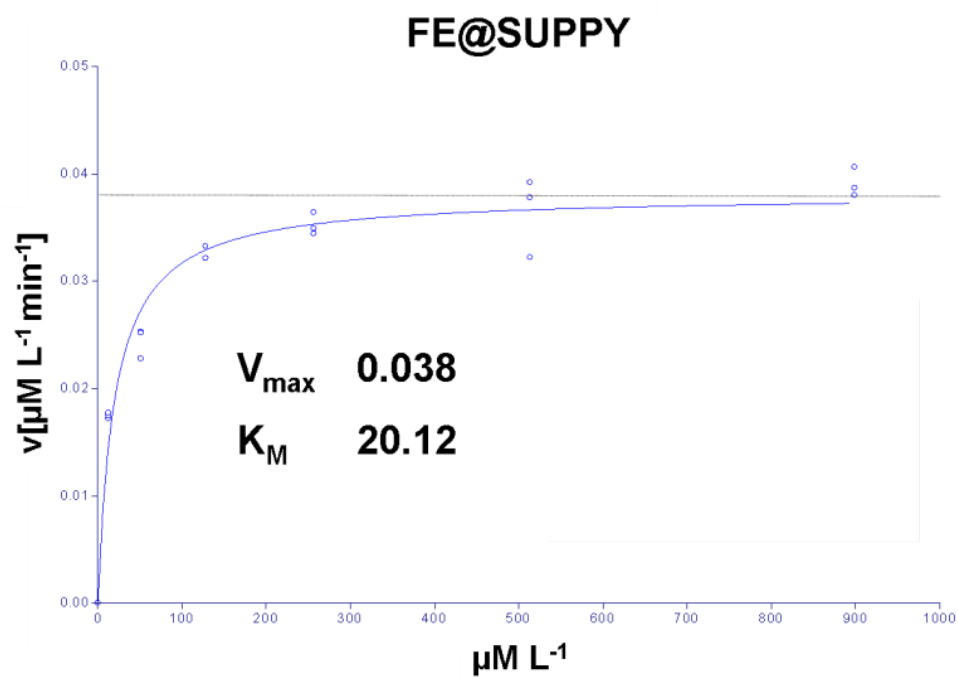
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- ### Figure 1



Figure 2

A



B

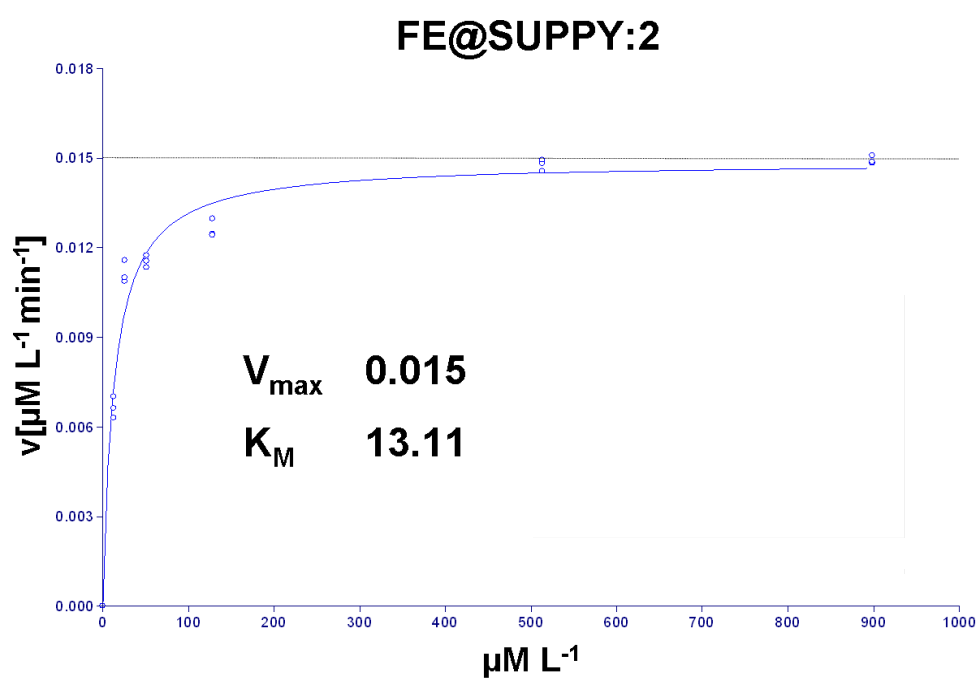


Figure 3

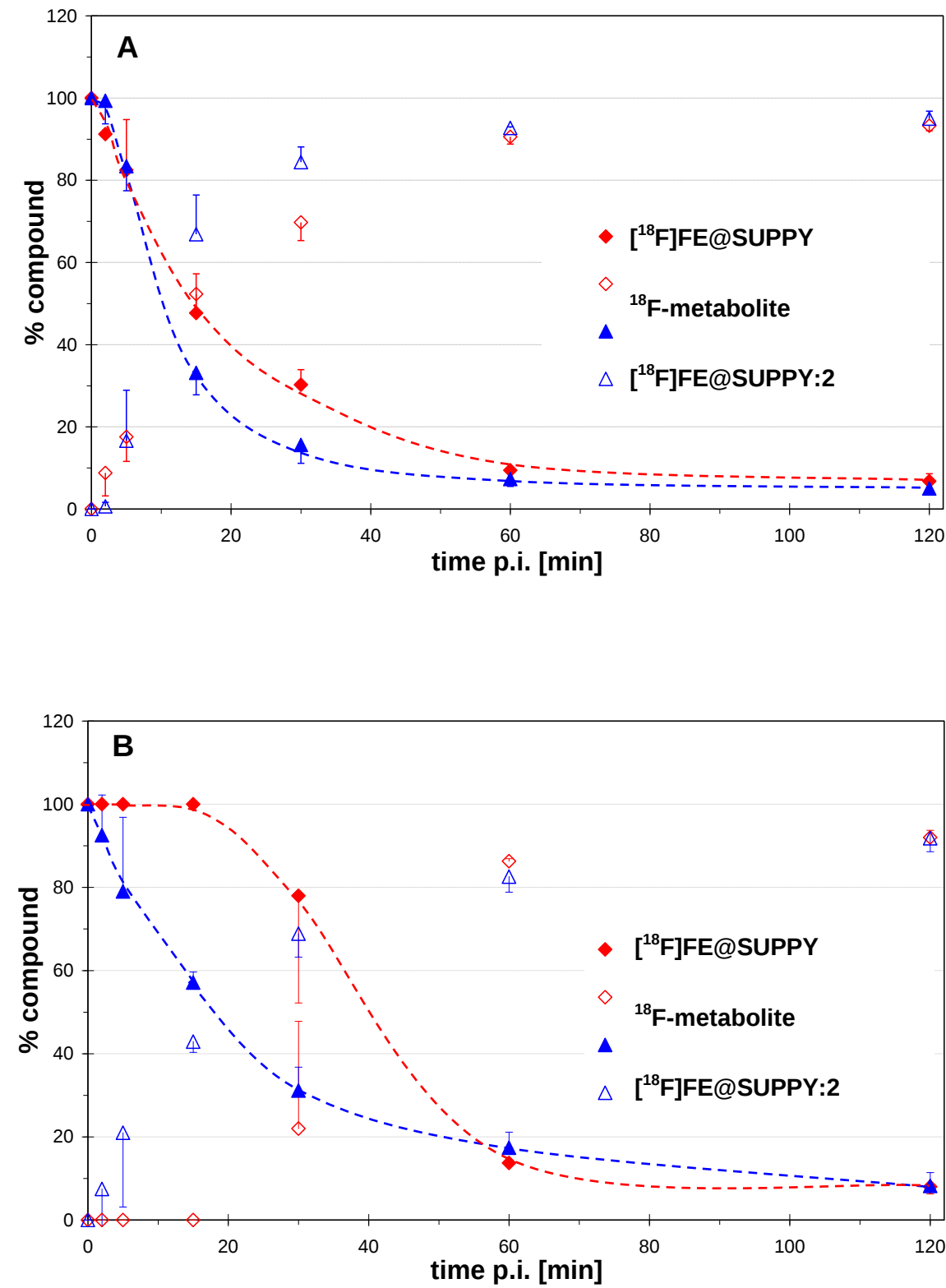


Table 1

A3R Ligands	defluoroethylated metabolite	fluoroethylated metabolite	final expected metabolite	found metabolite
<i>FE@SUPPY</i> 7.9±0.05	<i>DFE@SUPPY</i> X	<i>FE@SUPPY:11</i> 0.60±0.02	<i>SUPPY:0</i> 2.11±0.01	<i>SUPPY:0</i> 2.10±0.03
<i>FE@SUPPY:2</i> 7.89±0.04	<i>DFE@SUPPY:2</i> 1.08±0.01	<i>FE@SUPPY:21</i> 1.62±0.02	<i>SUPPY:0</i> 2.11±0.01	<i>FE@SUPPY:21</i> 1.57±0.02

CAPTIONS**Figure 1**

Potential metabolic pathways of [¹⁸F]FE@SUPPY and [¹⁸F]FE@SUPPY:2 cleaved by carboxylesterase (CES).

Figure 2

Michaelis-Menten kinetics and schematic Lineweaver-Burk illustration of (A) FE@SUPPY and (B) FE@SUPPY:2 – each single point represents the mean value of triplicate analyses. $v_{\max}$ is the limiting velocity and K_M is the Michaelis-Menten constant.

Figure 3

Scheme showing [¹⁸F]FE@SUPPY and [¹⁸F]FE@SUPPY:2 and their respective metabolites

A) in blood

B) in brain

Table 1

Retention times [min] of [¹⁸F]FE@SUPPY and [¹⁸F]FE@SUPPY:2 and their metabolites. Data represent arithmetic means ± SD.

9. The stability of methyl-, ethyl-, and fluoroethyl-esters against Carboxylesterases *in-vitro*: there is no difference

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Lukas Nics^{1,2}, Daniela Haeusler^{1,3}, Wolfgang Wadsak^{1,4}, Karl-Heinz Wagner², Robert Dudczak¹, Kurt Kletter¹, Markus Mitterhauser^{1,3,5}

¹Department of Nuclear Medicine, Medical University of Vienna, A-1090 Vienna, Austria

²Department of Nutritional Sciences, University of Vienna, A-1090 Vienna, Austria

³Department of Pharmaceutical Technology and Biopharmaceutics, University of Vienna, A-1090 Vienna, Austria

⁴Department of Inorganic Chemistry, University of Vienna, A-1090 Vienna, Austria

⁵Hospital Pharmacy of the General Hospital of Vienna, A-1090 Vienna, Austria

ABSTRACT

Introduction

Carboxylesterases play a very important role in the hydrophilic biotransformation of a huge number of structurally diverse drugs and especially play a leading part in the catabolic pathway of carboxylesters or thioesters. Hence, aim of the present study was the comparison of the in-vitro stability of methyl- and ethylesters with fluoroethylesters.

Methods

We incubated β -CIT/FE@CIT, MTO/ETO/FETO, FMZ/FFMZ, CFN/FE@CFN, and (Me)²@SUPPY/FE@SUPPY under physiological conditions. The enzymatic reactions were stopped at different time points and analysed by a standard protocol.

Results

The Michaelis-Menten constants (KM) and limiting velocities (VMax) are comparable. The statistical KM values were: β -CIT/FE@CIT $p > 0.05$; MTO/FETO $p > 0.06$; ETO/FETO $p > 0.09$; FMZ/FFMZ $p > 0.05$; CFN/FE@CFN $p > 0.9$; (Me)²@SUPPY/FE@SUPPY $p > 0.07$.

Conclusion

We found no statistical difference in stability against carboxylesterases *in-vitro*. These findings support the strategy to translate C-11-methyl-/ethylesters into their longer-lived F-18-fluoroethyl analogues.

KEYWORDS

metabolism, Michaelis-Menten kinetics, *in-vitro*, Carboxylesterase (CES), EC 3.1.1.1

INTRODUCTION

Carboxylesterases (CES) are α,β -hydrolase-fold proteins and belong to a multigene superfamily. They play a very important role in the hydrophilic biotransformation of a huge number of structurally diverse drugs and prodrugs. Within the body, CES are located in the Endoplasmatic Reticulum (ER) of many tissues like liver (highest CES activity), blood, skin, kidney, intestines, testes and lung, but also in the central nervous system [1, 2, 3]. These enzymes play a leading part in the catabolic pathway of carboxy-esters or thio-esters [3]. During saponification, the molecules are cleaved to an acid core and the corresponding leaving group. Furthermore, CES are capable of other catalyzing functions like acylation-reactions to nucleophilic acceptors or degradation of aromatic amides [3, 4].

Several Positron Emission Tomography (PET)-tracers are molecules bearing such carboxylic- or thiocarbonic- ester cores, being a target for CES degradation. Since, it is part of a suitable and complete evaluation of newly developed PET-tracers to fully characterize the metabolic fate, we established an *in-vitro* CES-assay for this purpose [5, 6, 7, 8]. This assay is based on the Michaelis-Menten kinetic (MMK) and delivers information about the quantitative stability of the evaluated carboxylic or thiocarboxylic esters against CES. The examined molecules comprised either methyl-, ethyl-, or fluoroethyl-ester moieties, respectively.

Hence, aim of the present study was the comparison of the stability of methyl- and ethylesters (as a basis for [^{11}C]-labeled tracers) with fluoroethylesters (as a basis for [^{18}F]-labeled tracers).

MATERIALS AND METHODS

General

Acetonitrile (LiChrosolv[®] hypergrade for LC-MS), columns and precolumns were purchased from Merck (Darmstadt, GER); methanol (CHROMASOLV[®]), ethanol (EMPROVE[®] exp), carboxylesterase (E-3019) were purchased from Sigma Aldrich (Steinheim, GER) and reference standards (β -CIT, FE@CIT, metomidate, etomidate, FETO, carfentanil, FE@CFN, flumazenil, fluoro-flumazenil) were purchased from advanced biochemical compounds GmbH (ABX, Radeberg, GER). FE@SUPPY and (Me)²@SUPPY were prepared as previously described [10]. All solvents were used without further purification.

Analytical high-performance liquid chromatography (HPLC) analyses were performed on an Agilent 1100/1200 series system (Böblingen, GER) equipped with: isocratic (G1310A) or quaternary pump (G1311A); degasser (G1322A); a diode-array detector (G1315A) with manual injection or using an autosampler (G1367A). Incubations of different amounts of the unlabeled substrates were accomplished with constant quantity of 80 International Units (I.U.) of porcine carboxylesterase under physiological conditions (phosphate buffered saline (PBS), pH 7.4, 37°C). The use of varying substrate concentrations, also partly within the groups, is based on an optimal choice to create MMK. Enzymatic reactions (n=3 for each of the three timepoints A, B, C) were stopped by adding the double amount of ice-cold acetonitrile/methanol (10:1) after 0, 60, 120, 180, 240, 360 min, respectively. After centrifugation of the reaction mixtures at 10.000 rpm for 5 minutes the obtained supernatant was analyzed by HPLC. Each analysis was performed in triplicate (i.e. A₁, A₂, A₃, etc.). Statistical calculations were performed with Microsoft[®] Excel 2007 (Microsoft[®] Cooperation, Redmond, USA) and Graph Pad Prism[®] 5.1 (GraphPad[®] Software, CA, USA).

β-CIT / FE@CIT

β-CIT (methyl 3β-(4-iodophenyl)tropane-2β-carboxylate; CAS-No. 135416-43-2) and FE@CIT (2-fluoroethyl 3β-(4-iodophenyl)tropane-2β-carboxylate; CAS-No. 398497-81-9) were analyzed using amounts of 60, 120, 180, 240 and 360 μmol of the respective substrate at 235 and 240 nm. HPLC assay: isocratic pump, manual injection, diode-array detector; stationary phase: LiChrospher® LiChroCART® 100 RP-18 column (250 × 4 mm, 5 μm), mobile phase: 63 % ammonium acetate, buffered at 0.03 M, pH 3.5; 30 % acetonitrile and 7 % ethanol at a flow rate of 1 ml/min.

MTO / ETO / FETO

MTO (metomidate; methyl 1-(1-phenylethyl)-1H-imidazole-5-carboxylate; CAS-No. 66392-64-2), ETO (etomidate; ethyl 1-(1-phenylethyl)-1H-imidazole-5-carboxylate; CAS-No. 33125-97-2) and FETO (2-fluoroethyl 1-(1-phenylethyl)-1H-imidazole-5-carboxylate; CAS-No. 960403-07-0) were analyzed using amounts of 9, 46, 116, 232, 463 and 926 μmol of the respective substrate at 235 and 242 nm; isocratic pump, manual injection, diode-array detector; stationary phase: LiChrospher® LiChroCART® 100 RP-18 column (250 × 4 mm, 5 μm); mobile phase: 60 % sodium phosphate, buffered at 0.095 M, pH 8.1; 25 % acetonitrile and 15 % methanol at a flow rate of 1 ml/min.

FMZ / FFMZ

FMZ (flumazenil; ethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4H-benzo-[f]imidazo[1,5-a]-[1,4]diazepine-3-carboxylate; CAS-No. 78755-81-4) and FFMZ (fluoro-flumazenil; 2-fluoroethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4H-benzo-[f]imidazo[1,5-a]-[1,4]diazepine-3-carboxylate; CAS-No. 676437-19-7) were analyzed using amounts of 30, 60, 150, 225, and 300 μmol of the respective substrate at 254 and 350 nm; isocratic pump, manual injection, diode-array detector; stationary phase: LiChrospher® LiChroCART® 100 RP-18 column (250 × 4 mm, 5 μm); mobile phase: 80 % phosphoric acid-solution, 0.073 M, pH 1.9; 20 % acetonitrile at a flow rate of 1 ml/min.

CFN / FE@CFN

CFN (carfentanil; methyl 1-phenylethyl-4-(N-propanoylanilino)piperidine-4-carboxylate; CAS-No. 59708-52-0) and FE@CFN (2-fluoroethyl 1-phenylethyl-4-(N-propanoylanilino)piperidine-4-carboxylate; CAS-No. 904892-57-5) were analyzed at 235 and 254 nm using amounts of CFN: 42, 103, 206, 413 and 1014 μmol , FE@CFN: 43, 108, 216, 432 μmol of the respective substrate; isocratic pump, autosampler, diode-array detector; stationary phase: Chromolith® Performance RP-18e column (100 x 4.6 mm, 5 μm), Chromolith® RP-18e Guard Cartridges (5 x 4.6 mm, 5 μm); mobile phase: 75 % ammonium acetate, buffered at 0.03 M, pH 3.5; 25 % acetonitrile at a flow rate of 1 ml/min.

(Me)²@SUPPY / FE@SUPPY

(Me)²@SUPPY (methyl 2,4-diethyl-3-methylsulfanylcarbonyl-6-phenylpyridine-5-carboxylate) and FE@SUPPY (2-fluorethyl 2,4-diethyl-3-ethylsulfanylcarbonyl-6-phenylpyridine-5-carboxylate), were analyzed using amounts of (Me)²@SUPPY: 15, 30, 58, 146, 582 μmol , FE@SUPPY: 13, 51, 128, 257, 514 and 900 μmol of the respective substrate at 248 and 254 nm; quaternary pump, degasser, autosampler, diode-array detector; stationary phase: Chromolith® Performance RP-18e column (100 x 4.6 mm, 5 μm), Chromolith® RP-18e Guard cartridge (5 x 4.6 mm, 5 μm); mobile phase: A: sodium citrate tribasic dehydrate, buffered at 0.034 M, pH 2.5; B: acetonitrile; (Me)²@SUPPY: gradient of 50 – 35 % A from 0 – 5.0 min and 35 – 50 % A from 7.0 – 9.0 min at a flow rate of 1 ml/min, FE@SUPPY 50 % A 50 % B at a flow rate of 1 ml/min.

RESULTS

β -CIT / FE@CIT

Results are presented in Table 1: K_M -values range from 175.1 to 182.6 ($p > 0.05$), V_{Max} -values range from 0.101 to 0.142.

MTO / ETO / FETO

Results are presented in Table 1: K_M -values range from 115.2 to 168.6 (MTO and FETO $p>0.06$; ETO and FETO $p>0.09$), V_{Max} -values range from 1.352 to 1.543.

FMZ / FFMZ

Results are presented in Table 1: K_M -values range from 39.8 to 54.4 ($p>0.05$), V_{Max} -values range from 0.208 to 0.245.

CFN / FE@CFN

Results are presented in Table 1: K_M -values range from 2503.0 to 2553.0 ($p>0.9$), V_{Max} -values range from 1.468 to 4.134.

(Me)²@SUPPY / FE@SUPPY

Results are presented in Table 1: K_M -values range from 20.2 to 24.7 ($p>0.07$), V_{Max} -values range from 0.021 to 0.038.

DISCUSSION

General

In the molecular modelling process of novel PET-tracers, there is a series of methods to find suitable fluorine-18 labeled molecules. F-18 can be introduced directly into aromatic rings or it can be attached to prosthetic or functional groups. Such groups can be alkyl rests, ethers, amines, amides or esters. In many cases, those groups can be derived from C-11 tracers, already evaluated for clinical purposes. These translations of existing C-11 tracers into the structurally related analogues bear general advantages: (1) longer half-lives allow longer measure protocols, (2) the distribution to PET-centers without on-site cyclotrons is possible, and (3) the close structural relation and available knowledge of the existing C-11 tracers simplifies the evaluation process [10]. In our case, we focused on the translation of C-11 esters into F-18 analogues. A major question in this context is the influence of this ester modification (methyl-/ethyl-ester $\rightarrow$ fluoroethyl-ester) on the stability against

enzymatic degradation. Hence, we developed a CES-model for the quantification of this ester stability [5, 6, 7, 8].

Michaelis-Menten kinetics (MMK)

For the quantitative comparison of the catabolic process, a MMK is the method of choice. Main deliveries from this equation are the Michaelis constant (K_M) and the limiting velocities (V_{MAX}). K_M approximates the affinity of enzyme for the substrate and is equivalent to the substrate concentration at which the rate of conversion is half of V_{MAX} . V_{MAX} represents the maximum rate of conversion at defined enzyme concentrations. The yielded values allow the stability-comparison of the investigated substrates. As depicted in table 1 and figure 1, K_M and V_{MAX} within the evaluated substance classes are comparable. Statistical analyses within the groups showed no significance (p-values ranged from 0.05 to 0.9). These findings are of interest, because fluoroethyl-substituents were even discussed as “protecting groups” for hetero atoms [11]. However, these findings were found under traditional chemical conditions. From the view of tracer development, our data support the metabolic equipollency of C-11 methyl-/ethyl-esters and fluoroethyl-esters. Due to this fact, from a metabolic point of view it is reasonable to follow a strategy of the translation of existing C-11 esters into F-18 fluoro-ethylated esters, as suggested for radiochemical reasons before [9].

CONCLUSION

Comparing structurally diverse methyl-/ethyl-/fluoroethyl-esters, we found no difference in stability against carboxylesterases *in-vitro*. The Michaelis-Menten constants are statistically similar. These findings support the strategy to translate C-11 methyl-/ethyl-esters into their longer-lived F-18 fluoroethyl analogues.

ACKNOWLEDGEMENTS

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Ettlinger, Karoline Sindelar, Hoda El-Samahi and Michael Machek for their support in performing the CES-experiments.

CAPTIONS

Table 1 shows the Michaelis-Menten constants (K_M) and limiting velocities (V_{Max}) of the calculated substrates (β -CIT/FE@CIT; MTO/ETO/FETO; FMZ/FFMZ; CFN/FE@CFN; $(Me)^2$ @SUPPLY/FE@SUPPLY); p-values are derived from 2-tailed unpaired t-test ($\alpha = 0.95$)

Figure 1 shows Michaelis-Menten saturation curves of the investigated CES-substrates. β -CIT/FE@CIT; MTO/ETO/FETO; FMZ/FFMZ; CFN/FE@CFN; $(Me)^2$ @SUPPLY/FE@SUPPLY

- (a) β -CIT / FE@CIT
- (b) MTO / ETO / FETO
- (c) FMZ / FFMZ
- (d) CFN / FE@CFN
- (e) $(Me)^2$ @SUPPLY / FE@SUPPLY

Figure 2 shows a structural overview of the investigated CES-substrates.

Table 1 shows the Michaelis-Menten constants (K_M) and limiting velocities (V_{Max}) of the calculated substrates (β -CIT/FE@CIT; MTO/ETO/FETO; FMZ/FFMZ; CFN/FE@CFN; $(Me)^2$ @SUPPLY/FE@SUPPLY); p-values are derived from 2-tailed unpaired t-test ($\alpha = 0.95$)

Substrate	$K_M[\mu\text{M}]$	$V_{\text{Max}}[\mu\text{M}/\text{min}]$	p-values
β -CIT	175.1	0.101	>0.05
FE@CIT	182.6	0.142	
MTO	162.0	1.471	] >0.06
FETO	115.1	1.543	
ETO	168.6	1.352	] >0.09
FMZ	39.8	0.208	>0.05
FFMZ	54.4	0.245	
CFN	2503.0	1.468	>0.9
FE@CFN	2553.0	4.134	
(Me) ² @SUPPY	24.7	0.021	>0.07
FE@SUPPY	20.2	0.038	

Figure 1 shows Michaelis-Menten saturation curves of the investigated CES-substrates. (a... β -CIT/FE@CIT; b...MTO/ETO/FETO; c...FMZ/FFMZ; d...CFN/FE@CFN; e...(Me)²@SUPPLY/FE@SUPPLY)

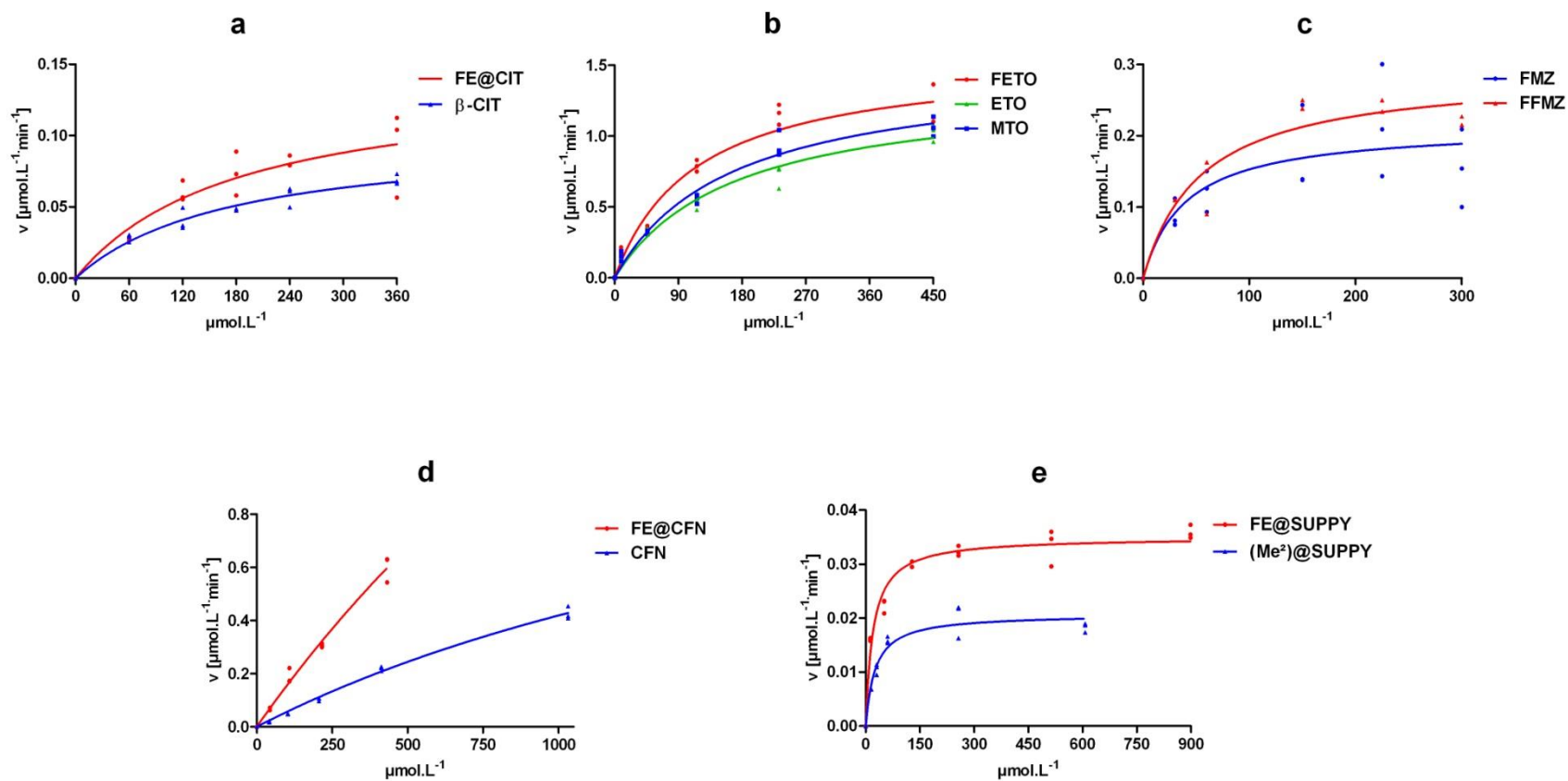
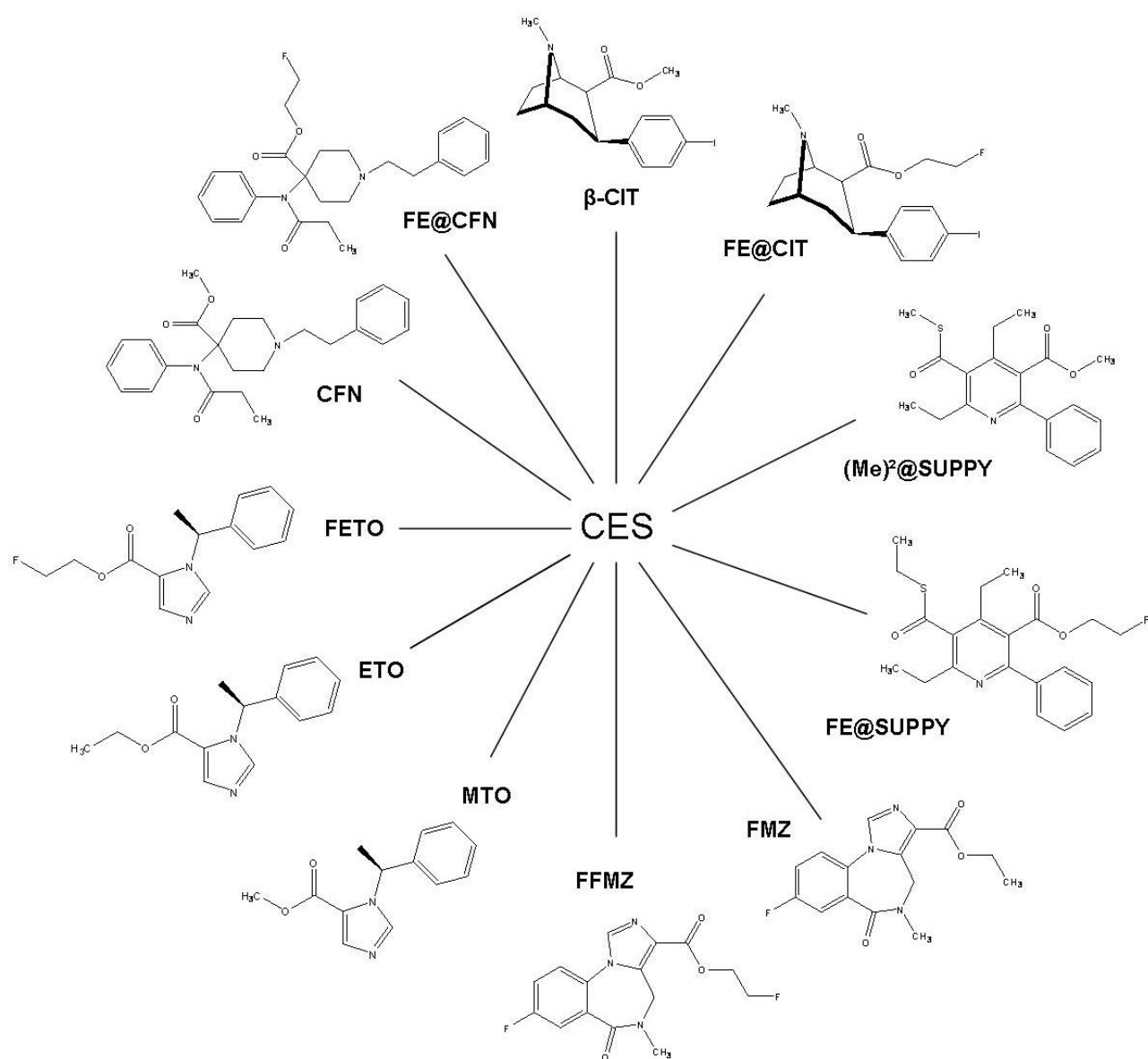


Figure 2 shows a structural overview of the investigated CES-substrates.



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10. Preparation and first preclinical evaluation of [¹⁸F]FE@SNAP: a new PET tracer for the melanin concentrating hormone receptor 1 (MCHR1)

Bioorganic and Medicinal Chemistry, submitted

Cécile Philippe^{a,b}, **Lukas Nics**^{a,c}, Markus Zeilinger^a, Eva Schirmer^d, Helmut Spreitzer^d, Georgios Karanikas^a, Rupert Lanzenberger^e, Helmut Viernstein^b, Wolfgang Wadsak^{a,f}, Markus Mitterhauser^{a,b,*}

^aRadiochemistry and Biomarker Development Unit, Department of Nuclear Medicine, Medical University of Vienna, 1090 Vienna, Austria

^bDepartment of Pharmaceutical Technology and Biopharmaceutics, University of Vienna, 1090 Vienna, Austria

^cDepartment of Nutritional Sciences, University of Vienna, 1090 Vienna, Austria

^dDepartment of Drug and Natural Product Synthesis, University of Vienna, 1090 Vienna, Austria

^eDepartment of Psychiatry and Psychotherapy, Medical University of Vienna, 1090 Vienna, Austria

^fDepartment of Inorganic Chemistry, University of Vienna, 1090 Vienna, Austria

ABSTRACT

The melanin concentrating hormone (MCH) system is a new target for the treatment of human disorders. Since the knowledge about the involvement of the MCH system in a variety of pathologies (obesity, diabetes, deregulation of metabolic feedback mechanism) bases on in vitro or preclinical studies, the development of a PET tracer is needed. We herein present the preparation and first preclinical evaluation of [¹⁸F]FE@SNAP – a new PET tracer for the MCH receptor 1.

The synthesis was performed using a microfluidic device. Preclinical evaluation included binding affinity, plasma stability, plasma free fraction, stability against the Cytochrom P-450 (CYP450) system using liver microsomes, stability against carboxylesterase as well as methods to assess the penetration of the blood brain barrier (BBB) such as logD analysis and immobilized artificial membrane (IAM) chromatography.

374 ± 202 MBq [¹⁸F]FE@SNAP were obtained after purification. The obtained K_d value of [¹⁸F]FE@SNAP was 2.9 ± 2.5 nM. [¹⁸F]FE@SNAP evinced high stability against carboxylesterase, CYP450 enzymes and in human plasma. LogD (3.83) and IAM chromatography results (P_m=0.51) were in the same range as for known BBB penetrating compounds.

The synthesis of [¹⁸F]FE@SNAP was reliable and successful. Due to high binding affinity, stability and suitable BBB characteristics, [¹⁸F]FE@SNAP is a promising tracer for the MCHR1.

KEYWORDS

MCHR1; Fluorine-18; PET; SNAP-7941; Radioligand

1. INTRODUCTION

Melanin concentrating hormone (MCH) is a cyclic nonadeca-peptide, predominantly expressed in the lateral hypothalamus and zona incerta.^{1,2} Beside, it is also found in peripheral organs and tissues, such as the pancreas,³ colonic epithelial cells⁴ or adipocytes.^{5,6} The biological function of MCH is mediated by two G-protein coupled receptors, MCH receptor 1 and 2 (MCHR1¹⁷⁻¹⁰ and MCHR2¹¹⁻¹⁴). MCH plays a key role in energy homeostasis, e.g. the control of food intake and body weight.^{15,16} Furthermore, it is involved in diabetes, gut inflammation and adiposity.³⁻⁶ The widespread distribution of MCH and its receptors and the involvement in a variety of pathologies makes the MCH system an interesting new target for the treatment of human disorders. Several MCHR1 antagonists were presented in the last decade; some of them have been entered in clinical trials for the treatment of obesity¹⁷ and some are in discussion of becoming anti-diabetic drugs.¹⁸ However, to enable confidence in preclinical to clinical translation of central MCHR1 pharmacology, a suitable positron emission tomography (PET) tracer needs to be developed. Borowsky et al.¹⁹ presented the evaluation of the very potent MCHR1 antagonist SNAP-7941 ((+)-methyl (4S)-3-[[[3-{4-[3-(acetylamino)phenyl]-1-piperidinyl}propyl)amino]carbonyl]-4-(3,4-difluorophenyl)-6-(methoxymethyl)-2-oxo-1,2,3,4-tetrahydro-5-pyrimidinecarboxylate hydrochloride, **1**, Fig.1) (K_d=0.18 nM, evaluated on Cos-7 cells expressing the human

MCHR1 (hMCHR1)).¹⁹ We previously reported the successful radiosyntheses of its radiolabelled analogues, [¹¹C]SNAP-7941²⁰ (**2**, Fig. 1) and [¹⁸F]FE@SNAP²¹ (**3**, Fig. 1). The present work focuses on:

1. the up-scaling, purification and formulation of [¹⁸F]FE@SNAP, and
2. on the *in-vitro* assessment of its potential as a PET tracer through preclinical evaluation of its main biological and physicochemical properties.

2. RESULTS

2.1. Radiochemistry

From a single synthesis in the microfluidic system 374 ± 202 MBq (range: 98-662 MBq) [¹⁸F]FE@SNAP were obtained after purification (n=6). Radiochemical purity always exceeded 99%. 3.1 ± 0.5 µg FE@SNAP were detected in the final product solution. Precursor mass was below the limit of detection (< 0.5 µg/mL). Specific radioactivity was 24.8 ± 12 GBq/µmol at the end of synthesis (EOS). Residual solvent analysis revealed < 10 ppm acetonitrile and no other impurities. Osmolality was 222 ± 4 mosmol/kg and pH was 7.4 ± 0.2 (n=3).

2.2. Biological evaluation

The binding experiments on the hMCHR1 revealed a K_d of 2.9 ± 2.5 nM (n=2) for [¹⁸F]FE@SNAP (Fig. 2). Preliminary competition binding experiments against [¹²⁵I]MCH on the hMCHR2 showed poor binding of FE@SNAP ($K_i > 1000$ nM).

The plasma free fraction (f_1) of [¹⁸F]FE@SNAP was $12.6 \pm 0.2\%$ in human plasma (n=3). Due to the fast metabolization of [¹⁸F]FE@SNAP in rat plasma, it was not possible to determine f_1 in that medium.

The degradation of [¹⁸F]FE@SNAP in human plasma (n=6) was $0.48 \pm 0.5\%$ after 0 min and $3.87 \pm 3.9\%$ after 120 min. In rat plasma (n=11) the degradation was $32.44 \pm 33.5\%$ initially and, after 120 min, [¹⁸F]FE@SNAP was completely metabolized. The formation of a radioactive hydrophilic metabolite could be observed.

The enzymatic degradation of [^{18}F]FE@SNAP by CYP450 after 60 min was $5.39 \pm 1.6\%$ using human liver microsomes (n=4) and $2.59 \pm 1.8\%$ using rat liver microsomes (n=4) (Fig. 3).

The Michaelis-Menten constant (K_m) of FE@SNAP was $347.3 \mu\text{M}$ and the limiting velocity (V_{max}) was $0.874 \mu\text{M}/\text{min}$ (Fig. 4).

2.3. Physicochemical parameters

The logD value of FE@SNAP was 3.83 (n=3). The P_m value was 0.51 (n=3).

3. DISCUSSION

Due to the low density of the MCH-receptors in the human brain ($B_{\text{max}}=5.8 \pm 0.3 \text{ fmol}/\text{mg}$),²² a high binding affinity in a low nanomolar range of [^{18}F]FE@SNAP is mandatory. [^{18}F]FE@SNAP evinced a high affinity in saturation binding assays ($K_d=2.9 \pm 2.5 \text{ nM}$). Moreover, FE@SNAP revealed high selectivity to the hMCHR1 (K_i on hMCHR2 $> 1000 \text{ nM}$) in competition binding assays.

Starting from $29 \pm 4 \text{ GBq}$ [^{18}F]fluoride, $374 \pm 202 \text{ MBq}$ ($2.6 \pm 1.5\%$ at the end of bombardment (EOB)) [^{18}F]FE@SNAP were obtained. 3 different circumstances led to this unexpected low radiochemical yield:

1. Due to incomplete priming of the solution into the loop to guarantee bubble-free filling (which is a systematic problem in the used microfluidic system), $16.1 \pm 0.3\%$ of activity remained in the concentrator vial of the microfluidic system after azeotropic drying and was not assessable for the synthesis.²³
2. Further $8.0 \pm 3.5\%$ remained in the lines and thereby were not accessible for the reaction.²³
3. The syntheses were performed in the discovery mode of the microfluidic system. There, approximately only half the amount of activity from the loop was used for the synthesis. The residual activity was kept as reserve in the loop to have the option for a second consecutive synthesis.

However, $374 \pm 202 \text{ MBq}$ [^{18}F]FE@SNAP were sufficient for any subsequent preclinical evaluation studies. Higher radiochemical yields will be achieved using the sequence mode of the microfluidic system.

The tested quality control parameters of the physiologically formulated [^{18}F]FE@SNAP solution were in accordance with the standards for human application. Specific activity was relatively low ($24.8 \pm 12 \text{ GBq}/\mu\text{mol}$). Higher specific activities are expected for future syntheses with higher yields. We note that no conversion of [^{18}F]FE@SNAP could be achieved using conventional synthesizing modules.²¹

We evaluated the stability of [^{18}F]FE@SNAP not only in human but also in rat tissues (plasma, liver microsomes) to be prepared for species differences in future small animal PET experiments. [^{18}F]FE@SNAP was highly stable against human and rat liver microsomes (consisting of the multi enzyme complex Cytochrom P-450: $5.39 \pm 1.6\%$ (human) and $2.59 \pm 1.8\%$ (rat) decomposition after 60 min) and in human plasma (only $3.87 \pm 3.9\%$ metabolization after 120 min). In contrast, [^{18}F]FE@SNAP was completely metabolized in rat plasma within 120 min.

The amount of unbound (free) [^{18}F]FE@SNAP ($f_1=12.6 \pm 0.2\%$) in the human plasma should be sufficient for potential future clinical PET-studies targeting the brain. For comparison: the 5HT_{1A} ligand [^{11}C]WAY 100635 has a plasma free fraction of $5.8 \pm 0.2\%$.²⁴

Porcine carboxylesterase was used to assess Michaelis Menten kinetics (MMK), due to its wide use as biochemical model for in vitro studies.²⁵ With a K_m of $347.3 \mu\text{M}$, FE@SNAP showed again very high stability.

For prediction of BBB penetration the lipophilicity expressed as logD was measured in a first step. Since logP/logD values were shown to be poor predictors for BBB penetration,²⁶ IAM chromatography was additionally performed. Under the modified conditions from Tavares et al.²⁷ FE@SNAP ($P_m=0.51$) is situated well in between of β -CIT ($P_m=0.31$) and DASB ($P_m=1.23$) – two known BBB penetrating compounds. Therefore, considering only the passive diffusion, a prediction of a BBB penetration seems reasonable.

4. CONCLUSION

The synthesis of [^{18}F]FE@SNAP yielded sufficient amounts ($374 \pm 202 \text{ MBq}$) for subsequent preclinical evaluations. Our main criteria to pursue the evaluation of [^{18}F]FE@SNAP as a PET tracer for the MCHR1 were:

- a high binding affinity to the MCHR1 in a low nanomolar range,
- high metabolic stability to assure enough intact tracer for visualization of MCHR1 specific tissues and
- reasonable lipophilicity to expect BBB penetration.

FE@SNAP binds to hMCHR1 in a nanomolar range ($K_d=2.9 \pm 2.5$ nM) and is highly selective to this receptor subtype. It showed very high stability against porcine carboxylesterase, the Cytochrom P-450 fraction of human and rat liver microsomes and in human plasma. Furthermore, the human plasma free fraction ($f_1=12.6 \pm 0.2\%$) is high enough for potential brain imaging. The fact, that decomposition in rat plasma is complete within 120 min, has to be considered for further preclinical studies in rats. As IAM chromatography experiments showed comparable behaviour to known BBB penetrating compounds, BBB penetration is expectable. Collectively, [^{18}F]FE@SNAP is a promising tracer for the MCHR1 and further preclinical evaluation steps (autoradiography, small animal PET) will elucidate its potential.

5. EXPERIMENTAL SECTION

5.1. General

5.1.1. Materials

[^{18}F]fluoride was produced via the $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ reaction in a GE PETtrace cyclotron (16.5-MeV protons; GE Medical Systems, Uppsala, Sweden). H_2^{18}O (HYOX18; > 98%) was purchased from Rotem Europe (Leipzig, Germany). Typical beam currents were 48-52 μA and the irradiation was stopped as soon as the desired activity level was reached (approx. 25-30 GBq). Anion-exchange cartridges (PS- HCO_3) for [^{18}F]fluoride trapping were obtained from Macherey-Nagel (Dueren, Germany). The precursor compound (Tos@SNAP; **4**, Fig. 1) and the reference standard (FE@SNAP) were synthesized in cooperation with the Department of Drug and Natural Product Synthesis of the University of Vienna (Austria).^{28,29} Solid phase extraction (SPE) cartridges SepPak® C18-plus and Oasis HLB 6cc Vac (200 mg) were purchased from Waters (Waters® Associates, Milford, MA, USA). Sterile water Ecotainer® and 0.9% saline solution were purchased from B. Braun (Melsungen, Germany). 3% saline solution was obtained from

a local pharmacy (Landesapotheker Salzburg, Austria). 125 mM phosphate buffer was prepared by dissolving 0.224g sodium dihydrogenphosphate-monohydrate and 1.935g disodiumhydrogenphosphate-dihydrate (both from Merck, Darmstadt, Germany) in 100 mL sterile water. Phosphate buffered saline (PBS) concentrate (10:1) was obtained from Morphisto (Frankfurt, Germany). NADPH regenerating system solution-A and solution-B were obtained from BD Biosciences (Bedford, MA, USA). Acetonitrile, acetic acid, tetrahydrofuran (THF) anhydrous, methanol, ethylenediaminetetraacetic acid (EDTA), bacitracin, bovine serum albumin and porcine liver carboxylesterase (EC 3.1.1.1) were purchased from Sigma Aldrich (Vienna, Austria). Ammonium acetate, acetonitrile (for DNA synthesis, ≤ 10 ppm H₂O), Kryptofix 2.2.2, K₂CO₃, MgCl₂, Tris(hydroxymethyl)-aminomethane (Tris), triphenylene, toluol and ethanol were purchased from Merck (Darmstadt, Germany). Pooled human liver microsomes (Lot No. 34689), pooled male rat liver microsomes (Lot No. 85157) and pooled female rat liver microsomes (Lot No. 59232) (both from Sprague Dawley rats) were purchased from BD Biosciences (Woburn, MA, USA). The male and female rat liver microsomes were homogenized. Pooled lithium-heparinised human plasma (No. IPLA-N) and pooled lithium-heparinised rat plasma (No. IRT-N) were purchased from Innovative Research (Novi, MI, USA). Centrifugal Filter Units (Centrifree®-30K) were purchased from Merck Millipore (Tullagreen, Ireland). [¹²⁵I]MCH and CHO-K1 cell membranes expressing the hMCHR1/hMCHR2 were purchased from PerkinElmer (Waltham, MA, USA). The vials for the binding affinity assay were purchased from Beckman Coulter Inc. (Brea, CA, USA; Bio-Vial™, 4 mL, 14 × 55 mm) and from Seton Scientific (Petaluma, CA, USA; Open-Top Centrifuge Tubes Polyclear, 13 × 64 mm). Semi-preparative high-performance liquid chromatography (HPLC) column (Chromolith® SemiPrep RP-18e; 100-4.6 mm), analytical HPLC column (LiChroCART® 250-4 mm) and the column for metabolic stability testing (Chromolith® Performance RP-18e; 100-4.6 mm precolumn: Chromolith® Guard Cartridge RP-18e; 5-4.6 mm) were purchased from Merck (Darmstadt, Germany). Gas chromatography capillary column (forte GC Capillary Column ID-BP20; 12 m × 0.22 mm × 0.25 µm) was purchased from SGE Analytical Science Pty. Ltd. (Victoria, Australia). IAM (immobilized artificial membrane) chromatography was performed using a IAM.PC.DD2 column (15 cm × 4.6 mm) (Regis

Technologies Inc., Morton Grove, IL, USA). The ODP-50 column for logD measurement was purchased from Shodex™ (Showa Denko Europe GmbH, Munich, Germany).

5.1.2. Instrumentation

The radiosynthesis of [^{18}F]FE@SNAP was carried out within an Advion NanoTek® unit (Ithaca, NY, USA) comprising a concentrator unit (CE) and a liquid flow reaction unit (LF) with dedicated control software (Advion, version 1.4). Microreactors were made of fused silica tubing (ID, 0.1 μm ; length 2.0 m), wound-up and held in a brass ring filled with a thermoresistant polymer to hold the tubing in its place. The purification of the resulting crude product solution and the final formulation of [^{18}F]FE@SNAP was carried out within an Nuclear Interface® PET synthesizer (GE Medical Systems, Uppsala, Sweden) remotely controlled via GINastar software (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany) installed on a standard PC. Analytical HPLC was performed using an Agilent system (Boeblingen, Germany) consisting of an autosampler 1100, a quaternary pump 1200, a diode array detector 1200 (operated at 254 nm) and a lead-shielded BGO-radiodetector. The osmolality was measured using a Wescor osmometer Vapro® 5600 (Sanova Medical Systems, Vienna, Austria), pH was measured using a WTW inoLab 740 pH meter (WTW, Weilheim, Germany). Gas chromatography was performed using a 430-GC system (Burker Daltonik GmbH, Bremen, Germany). For binding experiments a Sorvall Ultracentrifuge Combi OTD (Thermo Fisher Scientific Inc, Waltham, MA, USA) and a 2480 WIZARD² Automatic Gamma Counter (PerkinElmer, Waltham, MA, USA) were used. For stability experiments sample incubation was conducted within a Thermomixer compact from Eppendorf® (Vienna, Austria) and sample centrifugation with a Universal 30 RF centrifuge (Hettich, Tuttlingen, Germany). The same centrifuge was used for determination of the plasma free fraction.

5.2. Radiochemistry

5.2.1. Radiosynthesis

The azeotropic drying of cyclotron produced [^{18}F]fluoride and the radiosynthesis of [^{18}F]FE@SNAP were carried out within a microfluidic system (Advion NanoTek®) as

described in detail elsewhere.²¹ Briefly, n.c.a [¹⁸F]fluoride (25-30 GBq) was trapped on an anion exchange cartridge (PS-HCO₃) and released with a solution containing Kryptofix 2.2.2 (4,7,13,16,21,24-hexaoxa-1,10-diaza-bi-cyclo[8.8.8]hexacosane; 10 mg, 26.6 μmol) and potassium carbonate (2.25 mg, 16.6 μmol) in acetonitrile/water (70/30 v/v; V=0.5 mL). Iterative azeotropic drying was performed at 110°C by addition of 3 times 300 μL of dry acetonitrile. Subsequently, the dried [¹⁸F]fluoride-aminopolyether was dissolved in 500 μL acetonitrile. 150 – 200 μL of Tos@SNAP (6 mg/mL in acetonitrile (for DNA synthesis)) and the same volume of the [¹⁸F]fluoride-aminopolyether in acetonitrile (final precursor concentration: 3mg/mL) were simultaneously pushed through the microreactor at 170°C with a total flow rate of 170 μL/min. Subsequently, the crude product solution was swept out of the microreactor with a defined volume of 200 μL acetonitrile. The crude product solution was transferred into the Nuclear Interface® synthesizer unit, quenched with 1 mL water and subsequently injected onto the semi-preparative HPLC column (mobile phase: (water/acetic acid 97.5/2.5 v/v; 2.5 g/L ammonium acetate; pH 3.5)/acetonitrile 75/25 v/v; flow: 8 mL/min, after 9 min: 10 mL/min). Chromatograms were registered using an UV-detector (245 nm) and a NaI radioactivity detector in series. The retention times were 2'20-3'10 (*k'*=0'16-0'63) for Tos@SNAP and 14'05-16'35 min (*k'*=5'11-6'11) for [¹⁸F]FE@SNAP (Fig. 5). The [¹⁸F]FE@SNAP fraction was cut and diluted with 100 mL water. This aqueous product solution was then pushed through a C18 SPE cartridge. After washing with 10 mL water, the pure product was eluted with 1.5 mL ethanol and 5 mL 0.9% saline solution. Formulation was done with an additional 9 mL physiological saline (0.9%), 1 mL of saline solution (3%) and 1 mL phosphate buffer (125 nM). Hence, the final total volume was 17.5 mL. For stability and binding affinity experiments, [¹⁸F]FE@SNAP was eluted from the SPE cartridge with only 1 mL ethanol and 0.5 mL water in order to enhance the activity concentration in the product solution.

5.2.2. Quality control

Chemical and radiochemical impurities were detected using analytical HPLC (mobile phase: 0.1 M ammonium acetate/acetonitrile 60/40 v/v; flow: 1 mL/min). The retention time of [¹⁸F]FE@SNAP was 11.8-12.5 min (*k'*=4.9-5.3). The chemical identity

of [^{18}F]FE@SNAP was determined by co-injection of the unlabeled reference compound, FE@SNAP. The physiological formulated product solutions were further checked on residual solvents (analyzed by GC), osmolality and pH (checked with dedicated equipment).

5.3. Biological evaluation

5.3.1. Binding affinity

The method used was conducted according to Mashiko et al.³⁰ with minor modifications. CHO-K1 cell membranes expressing the hMCHR1 (10 $\mu\text{g}/\text{mL}$) were dissolved in 500 μL 50 mM Tris buffer (pH 7.4) (containing 10 mM MgCl_2 , 2 mM EDTA, 0.1% bacitracin and 0.2% BSA). For the evaluation of the equilibrium dissociation constant (K_d) of [^{18}F]FE@SNAP, several concentrations (0-500 nM) of [^{18}F]FE@SNAP were added. The membranes were incubated in vials at room temperature for 120 min. Bound and free fractions of radioligand were separated by centrifugation at $40.000 \times g$ for 20 min. The supernatants were removed into new vials. The pellets were washed with 800 μL ice cold Tris buffer, which was added to the supernatant and the pellets were dissolved in 1300 μL Tris buffer. The radioactivity in the vials was measured in a Gamma Counter. The K_d values were calculated by using GraphPad Prism software Version 5.0 (La Jolla, CA, USA).

5.3.2. Plasma stability

Stability of [^{18}F]FE@SNAP in human and rat plasma was determined according to Nics et al.³¹ 1800 μL lithium-heparinized plasma (rat and human, respectively) were pre-incubated under physiological conditions (PBS, pH 7.4, 37°C) in a shaking incubator for 5 minutes. 36 μL [^{18}F]FE@SNAP (corresponding to 2% ethanol v/v in the total volume) were added and the plasma vial was vortexed for at least 10 seconds. After defined time points (0 and 120 min) 500 μL of the incubation-mixture were added to a preconditioned (with 5 mL methanol followed by 5 mL water) SPE-cartridge (Oasis). The cartridge was then eluted into a collection tube, washed with 5 mL of 5% methanol in water (v/v) into a second tube and eluted with 3 mL of THF into a third tube. 20 μL of the eluate-solution of tube two and three were injected into analytical

HPLC (mobile phase: (water/ acetic acid 97.5/2.5 v/v; 2.5 g/L ammonium acetate; pH 3.5)/acetonitrile 70/30 v/v; flow: 2mL/min).

5.3.3. Plasma free fraction

The method used was modified from Parsey et al.²⁴ 1 mL heparinized plasma (rat and human, respectively) were mixed with 10-50 μL [^{18}F]FE@SNAP. 200 μL aliquots were pipetted into centrifugal filter units and the total radioactivity was measured in a Gamma Counter. After the centrifugation step ($2.000 \times g$, 50 min) 50 μL of the obtained filtrate was back-measured for radioactivity. For determination of f_1 the ratio of filtrate to total activity concentration was calculated.

5.3.4. Stability against liver microsomes (CYP450)

The method used was described by Nics et al.³¹ Briefly, liver microsomes (pooled from human or rat origin) were pre-incubated under physiological conditions (PBS, pH 7.4, 37°C) with a NADPH-generating system (solution-A: NADP⁺, Glucose-6-phosphate and magnesium-chloride in H₂O and solution-B: Glucose-6-phosphate dehydrogenase in sodium citrate) for 5 min. 6 μL of [^{18}F]FE@SNAP, which correspond to 2% ethanol (v/v) in the total volume, were added. Enzymatic reactions were stopped after defined time points (0, 2, 5, 10, 20, 40 and 60 min) by adding the same amount of ice-cold acetonitrile/methanol (10:1). The mixtures were vortexed, followed by a centrifugation step ($23.000 \times g$, 5 min). Aliquots of the obtained supernatant were analyzed by analytical HPLC (for conditions see 5.3.2. Plasma stability).

5.3.5. Stability against carboxylesterase

The method used was slightly modified from Nics et al.³² Incubations of different amounts (10, 30, 50, 70, 100, 200 $\mu\text{g}/\text{ml}$) of FE@SNAP were accomplished with constant quantity of 80 International Units (I.U.) of porcine carboxylesterase under physiological conditions (PBS, pH 7.4, 37°C). The use of selected concentrations of FE@SNAP was based on an optimal choice to create MMK. 35 μL of the incubation-mixture were stopped after defined time points (0, 60, 120, 180 and 240 min) by adding the same amount of ice-cold acetonitrile/methanol (10:1) and vortexed. After

centrifugation of the reaction mixtures ($23.000 \times g$, 5 min), 20 μ L of the obtained supernatant were analyzed by analytical HPLC (for conditions see 5.3.2. Plasma stability). The MMK of FE@SNAP was calculated by using GraphPad Prism software Version 5.0 (La Jolla, CA, USA).

5.5. Physicochemical properties

5.5.1. logD analysis

LogD values were determined using an HPLC based assay according to Donovan and Pescatore.³³ A cocktail of two internal standards (toluene and triphenylene) with known logD and k' values and FE@SNAP in methanol was injected onto a short polymeric ODP-50 column. A linear gradient from 10% methanol/90% phosphate buffer (pH 7.4) to 100% methanol within 9.4 min at a flow rate of 2 mL/min was applied. Detection was performed at 260 nm and 285 nm.

5.5.2. IAM chromatography

IAM chromatography was modified from Tavares et al.²⁷ 0.01 M phosphate buffer (pH 7.0) and acetonitrile (ranging from 50% to 35%, v/v) were used as mobile phase at a flow rate of 1 mL/min. FE@SNAP was injected onto the IAM column. As result the permeability through the membrane (P_m) was calculated and compared with the P_m of known BBB penetrating compounds (DASB, β -CIT) as external standards.

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Captions:

Figure 1. SNAP-7941 derivatives **1-4** (**1**: SNAP-7941; **2**: [^{11}C]SNAP-7941; **3**: [^{18}F]FE@SNAP ; **4**: Tos@SNAP)

Figure 2. Total binding and calculated unspecific binding of [^{18}F]FE@SNAP. Saturation binding experiment was performed on CHO-K1 cell membranes expressing the hMCHR1.

Figure 3. Degradation of [^{18}F]FE@SNAP by rat and human liver microsomes.

Figure 4. Michaelis-Menten saturation curve of FE@SNAP against carboxylesterase.

Figure 5. Representative semi-preparative HPLC chromatogram of the reaction solution of [^{18}F]FE@SNAP.

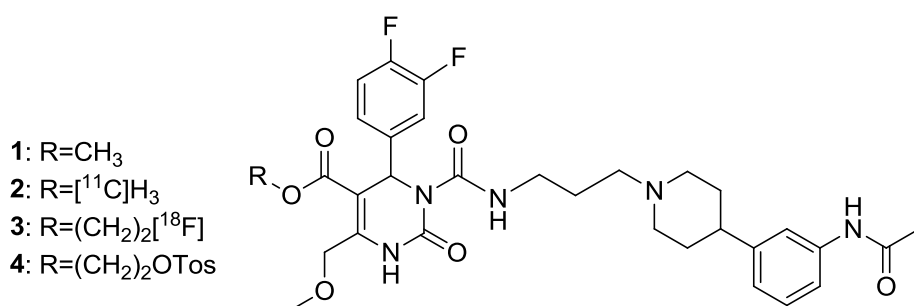


Figure. 1. SNAP-7941 derivatives **1-4** (**1**: SNAP-7941; **2**: [^{11}C]SNAP-7941; **3**: [^{18}F]FE@SNAP ; **4**: Tos@SNAP)

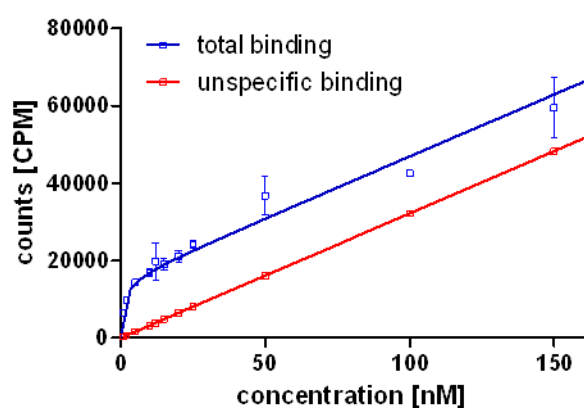


Figure 2. Total binding and calculated unspecific binding of [^{18}F]FE@SNAP. Saturation binding experiment was performed on CHO-K1 cell membranes expressing the hMCHR1.

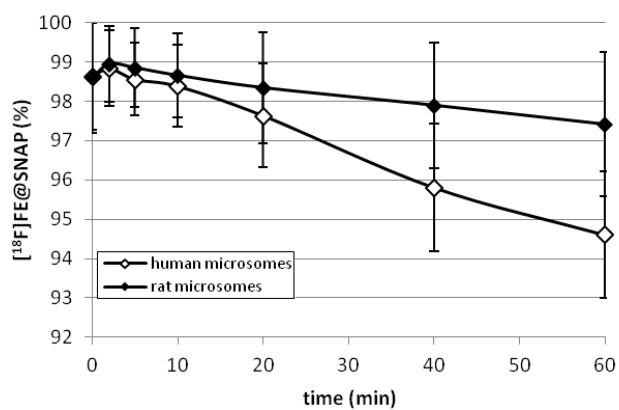


Figure 3. Degradation of [^{18}F]FE@SNAP by rat and human liver microsomes.

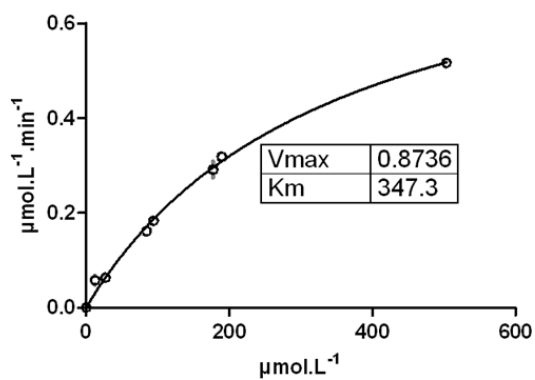


Figure 4. Michaelis-Menten saturation curve of FE@SNAP against carboxylesterase.

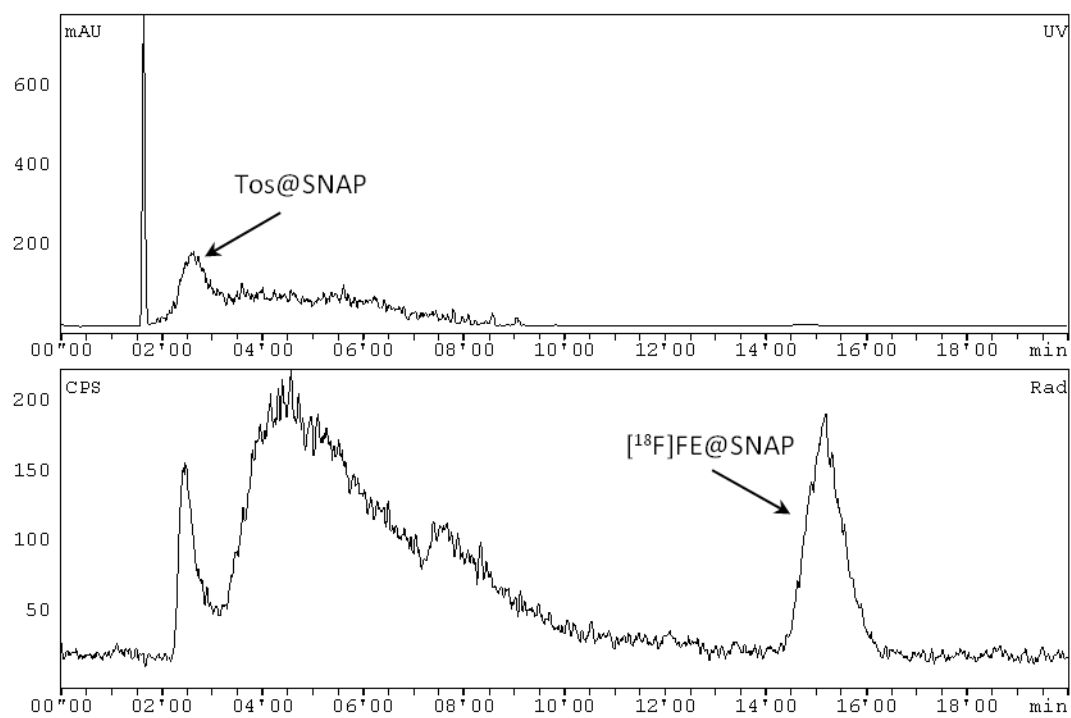


Figure 5. Representative semi-preparative HPLC chromatogram of the reaction solution of [¹⁸F]FE@SNAP.

11. Development and automation of a novel NET-PET tracer: [¹¹C]Me@APPI

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Christina Mark^{a,b}, Birgit Bornatowicz^c, Markus Mitterhauser^{a,d}, Matthias Hendl^a, **Lukas Nics**^{a,f}, Daniela Haeusler^a, Rupert Lanzenberger^g, Michael L. Berger^h, Helmut Spreitzer^c, Wolfgang Wadsak^{a,b}

^aRadiochemistry and Biomarker Development Unit, Department of Nuclear Medicine, Medical University of Vienna, Austria

^bDepartment of Inorganic Chemistry, University of Vienna, Austria

^cDepartment of Drug and Natural Product Synthesis, University of Vienna, Austria

^dHospital Pharmacy of the General Hospital of Vienna, Austria

^fDepartment of Nutritional Sciences, University of Vienna, Austria

^gDepartment of Psychiatry and Psychotherapy, Medical University of Vienna, Austria

^hCenter for Brain Research, Department of Biochemistry and Molecular Biology, Medical University of Vienna, Austria

KEYWORDS

transporter, noradrenaline, PET, Me@APPI, Carbon-11, radiosynthesis

ABSTRACT

Introduction

The norepinephrine transporter (NET) is an important target for research in neurology and psychology and is involved in the pathophysiology of many neurodegenerative diseases such as Alzheimer's disease and attention deficient hyperactivity disorder. For visualization of NET abundance and deregulation, a novel PET tracer - [¹¹C]Me@APPI - has been developed.

Methods

For precursor synthesis, a 4-step synthesis starting from N-phenyl-o-phenylenediamine was set up. Radiosynthesis was established and optimized using standard methods and subsequently automated in a GE TRACERlab Fx C Pro synthesizer. Preclinical testing was performed comprising affinity and selectivity testing on human membranes as well as stability and blood-brain-barrier-penetration using *in-vitro* models.

Results

Precursor molecule (APPI:O) and reference compound (Me@APPI) were synthesized with 26.5% and 21.4% overall yield, respectively. So far, 1.25 ± 0.72 GBq [^{11}C]Me@APPI with 54.35 ± 7.80 GBq/ μmol specific activity were produced ($n=11$). Affinity of reference compounds was determined as 8.08 ± 1.75 nM for Me@APPI and 19.31 ± 2.91 nM for APPI:O, respectively ($n \geq 9$). IAM-chromatography experiments ($n=3$) revealed a K_m value of 1.85 ± 0.05 for Me@APPI. Stability testing using human liver microsomes revealed that 99.5% of the tracer was found to be still intact after 60 minutes ($n=4$).

Conclusion: Present data indicate that [^{11}C]Me@APPI has promising properties to become a clinically useful NET-PET-tracer. Further *in-vitro* and *in-vivo* evaluations are currently under way.

INTRODUCTION

In the central nervous system monoamines such as norepinephrine (NE, noradrenaline), dopamine (DA) and serotonin (5-HT) play an important modulatory role in neurotransmission, and are involved in various (patho-)physiological functions and processes; including depression, attention deficient hyperactivity disorder (ADHD), anxiety and personality disorders, Alzheimer's Disease (AD) and substance abuse. [1] Specifically, dysregulation of NE – and, therefore, also of the norepinephrine transporter (NET) - plays a pivotal role in mood and affective disorders. Thus many selective monoamine transporter inhibitors have been developed for specific treatment. In major depression, perturbed regulation/expression of NET has been reported. [2] The most significant evidence for an increase in synaptic NE

concentration in the treatment of depression originates from data gained using reboxetine, a selective NET-inhibitor. [3] In autoradiographic studies (using [³H]-nisoxetine) high levels of NET were found in locus ceruleus (LC), while there was a significant decrease in NET availability in LC in major depressed patients. [4][5]

To understand this dysregulation of the NE system and its role in depression and other (psychiatric) disorders, it is crucial to gain information about the receptor abundance and density in healthy and pathological brains *in-vivo*. For that purpose, non-invasive molecular imaging using specific radioligands is the method of choice. Moreover, imaging of receptor and transporter dynamics before and after treatment of depression would be important to visualize and quantify molecular changes in the human brain and measure therapeutic outcome. Hence, the availability of suitable radioligands for positron emission tomography (PET) is crucial for gaining insight in molecular changes in the noradrenergic system. As a consequence selective NET-PET-ligands must be developed and evaluated.

So far, radiolabeled reboxetine analogs [¹¹C]MeNER ([¹¹C]MRB, ((S,S)-2-(α-(2-[¹¹C]methoxyphenoxy)benzyl)morpholine) and [¹⁸F]FMeNER-D₂ ((S,S)-2-(α-(2-[¹⁸F]Fluoro[²H₂] methoxyphenoxy)benzyl) morpholine) have been described [5-8]. Since both compounds display certain limitations (e.g. metabolic stability, late equilibrium, complex radiosynthesis) [6, 9], which put some constraints on their applicability in clinical trials, better suitable NET-PET ligands are still of interest.

Thus, the rationale of this and future work is the use of the recently described lead structure 1-(3-(methylamino)-1-phenylpropyl)-3-phenyl-1H-benzo[d]imidazol-2(3H)-one (=Me@APPI, figure 1) as starting point for novel NET-PET ligands.[10] The authors evaluated Me@APPI, which is not derived from reboxetine, *in-vitro* and found very promising affinity (hNET IC₅₀= 9 nM) and selectivity towards NET (hSERT /hNET = 333; hDAT= 33 % inh. at 10 μM Mazindol).[10]

Hence, main objectives of the presented investigations were:

- the preparation and characterization of a suitable labelling precursor, APPI:0 (1-(3-amino-1-phenylpropyl)-3-phenyl-1H-benzo[d]imidazol-2(3H)-one);

- the establishment of a radiosynthetic procedure for the preparation of the carbon-11 labelled analogue, [^{11}C]Me@APPI and its optimization;
- up-scaling and set-up of a fully automated preparation of [^{11}C]Me@APPI, including purification and formulation;
- set-up of a suitable quality control and;
- *in-vitro* evaluation, including binding studies for determination of affinity and selectivity of both Me@APPI and APPI:O towards NET using NET, SERT and DAT expressing membranes; metabolic stability testing *in-vitro* against selective enzymes; logP analysis and IAM chromatography for indirect measurement of blood-brain-barrier penetration.

MATERIALS AND METHODS

Materials

Precursor, 1-(3-amino-1-phenylpropyl)-3-phenyl-1H-benzo[d]imidazol-2(3H)-one (APPI:O), and cold reference compound 1-(3-(methylamino)-1-phenylpropyl)-3-phenyl-1H-benzo[d]imidazol-2(3H)-one (Me@APPI) were synthesized at the Department of Drug and Natural Product Synthesis, Faculty of Life Sciences, University of Vienna (for details see 'Methods' section).

Acetonitrile (ACN for synthesis of DNA, $\geq 99.9\%$ (GC) and ACN HPLC grade), tetrabutylammonium hydroxide 30-hydrate (TBAH), methanol (MeOH, CHROMASOLV[®], for HPLC, $\geq 99.9\%$), ammonium formate, and ethanol (absolute) were purchased from Sigma Aldrich (Vienna, Austria). Iodine (sublimated grade for analysis; ACS, Pharm.Eur.) was obtained from Merck (Darmstadt, Germany). Silver triflate impregnated carbon was prepared by dissolving 1 g of silver trifluoromethanesulfonate (Sigma Aldrich, Vienna, Austria) in 20 mL ACN and adding 3 g of Graphpac-GC (80/100 mesh, Alltech, Deerfield, USA). The suspension was stirred under protection from light for 30 min, the solvent was removed and the powder was dried for further 2 h under reduced pressure and protection from light.

For formulation of the product 0.9% saline solution from B. Braun (Melsungen, Germany), 3% saline solution (Landesapotheker Salzburg, Austria) and sodium dihydrogenphosphate-monohydrate and disodiumhydrogenphosphate-dihydrate (both from Merck, Darmstadt, Germany) were used. Sterile water was purchased from

Meditrade Medicare Medizinprodukte (Kufstein, Austria). Phosphate buffer (125 mM) was prepared by dissolving 0.224 g sodium dihydrogenphosphate-monohydrate and 1.935 g disodiumhydrogenphosphate-dihydrate in 100 mL sterile water. For solid phase extraction C8 plus SepPak[®] cartridges were purchased from Waters (Waters[®] Associates Milford, USA). Low-protein binding Millex[®] GS 0.22 µm sterile filters were obtained from Millipore (Bedford, USA).

All other chemicals and solvents for the radiosyntheses were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich (Vienna, Austria) with at least analytical grade and used without further purification.

Instrumentation

¹H- and ¹³C-NMR spectra were recorded on a Bruker Avance DPX-200 spectrometer at 27°C (200.13 MHz for ¹H, 50.32 MHz for ¹³C). Mass spectra were obtained using a SHIMADZU mass spectrometer (GC/MS-Q95050 GC-17A SHIMADZU), high resolution mass spectra were recorded on a Finnigan MAT 8230 using EI at 70 eV and on a Finnigan MAT 900 S using ESI at 4 kV (3 µA CH₃CN/MeOH). Chemical shifts δ are given in ppm relative to the residual peaks of the deuterated solvent used. Spectra measured in CDCl₃ are referenced to 7.24 ppm (¹H) and 77.00 ppm (¹³C). Coupling patterns are designated as s (singlet), d (doublet), m (multiplet) and b (broad). The ¹³C spectra were recorded in a j-modulated mode. Signals are assigned as C, CH, CH₂ and CH₃. Coupling constants J are given in Hz.

[¹¹C]CO₂ was produced within a GE PET trace cyclotron (General Electric Medical System, Uppsala, Sweden) by a ¹⁴N(p,α)¹¹C nuclear reaction under irradiation of a gas target (Aluminium) filled with N₂ (+1% O₂) (Messer Gases, Vienna, Austria). The production of [¹¹C]CH₃I and [¹¹C]CH₃OTf was performed within a TRACERlab[™] FX C Pro synthesizer (GE Healthcare, Uppsala, Sweden). Evaluation of reaction conditions was

performed manually in a lead-shielded hood with small quantities of radioactivity (<1 GBq). After optimization, [^{11}C]Me@APPI-synthesis was automated in the TRACERlab™ FX C Pro synthesizer, remotely controlled by a standard laptop with suitable processing software.

[^{11}C]Me@APPI was purified by semi-preparative reversed phase HPLC using the built-in semi-preparative HPLC system equipped with a radioactivity and a UV detector (Linear Instruments Model 200 Detector UV/VIS) and a LaPrep HPLC pump (VWR International, Radnor, USA). A Phenomenex® Gemini, C-18 with TMS endcapping, 10 μm , 250x10 mm (Phenomenex®, Aschaffenburg, Germany) column with a mobile phase of MeOH/0.1 M ammonium formate 70/30 v/v% +1 % NEt_3 at a flow rate of 8 mL/min was used for purification.

Analytical HPLC was performed on Merck-Hitachi LaChrom HPLC system (L-7100 pump; LaChrom L-7400 UV detector at 254 nm) and a NaI radio-detector (Bertholdt Technologies, Bad Wildbach, Germany) using Raytest software (Raytest, Straubenhardt, Germany). A Phenomenex® Prodigy, Phenyl-3(PH-3), 5 μm , 250x4.6 mm (Phenomenex®, Aschaffenburg, Germany) column with a mobile phase consisting of ACN/0.1 M ammonium formate 50/50 v/v% at a flow rate of 2 mL/min was used.

The osmolality was measured with a Wescor osmometer Vapro® 5600 (Sanova Medical Systems, Vienna, Austria) and pH was measured using a WTW inoLab 740 pH meter (WTW, Weilheim, Germany).

Methods

Precursor chemistry (APPI:0) and reference standard (Me@APPI)

The synthesis scheme of APPI:0 is outlined in figure 2 based on Zhang et al.[10]

A solution of 3-chloro-1-phenylpropan-1-one (**1**, 5.00 g, 29.65 mmol) in 25 mL THF and 35 mL EtOH was cooled to -10°C and sodium borohydride (1.17 g, 31.12 mmol) was slowly added. After stirring at -5°C for 10 min, the solution was poured into a mixture of 80 mL saturated aqueous ammonium chloride and 40 g of ice. After extraction with

diethylether, the organic layers were separated, dried over sodium sulfate and evaporated to give 4.78 g (94.45%) of a pale yellow oil **2**. After addition of 40 mL of 48% aqueous hydrobromic acid to chloride **2** (2.09 g, 12.24 mmol), the mixture was stirred at room temperature for 3 h and then carefully poured into a mixture of 11 g potassium carbonate in 70 g ice. After neutralization, the product was extracted with diethylether. The combined organic extracts were dried (MgSO₄) and evaporated to give 1.74 g (60.84%) of **3** as a pale yellow oil.[11]

N-phenyl-*o*-phenylenediamine (1.00 g, 5.43 mmol) and 1,1-carbonyldiimidazol (CDI) (1.23 g, 7.60 mmol) were stirred in THF over night at room temperature. After evaporation of the solvent the crude mixture was purified by chromatography (ligroin/ethylacetate 1/1) yielding 845 mg (74.05%) of pink crystals **5**. [10] Subsequently, 0.84 g (4.00 mmol) of intermediate **5** and 1.04 g (8.00 mmol) potassium carbonate were suspended in DMF. After stirring for 30 min at room temperature, synthon **3** (1.03 g, 6.00 mmol) was added and the mixture stirred overnight. The mixture was poured into ethylacetate and H₂O, extracted with ethylacetate and the combined organic layers were dried over MgSO₄. After evaporation and silica gel chromatography (ligroin/ethylacetate 9/1) of the residue 1.15 g (79.38%) of chloride **6** were obtained as colorless solid.[12]

A solution of chloride **6** (2.30 g, 6.34 mmol) and sodium iodide (1.90 g, 12.69 mmol) in acetone was refluxed for 24 h. The crude product was filtered and dried under reduced pressure to give 2.74 g (95.08%) of **7** as a yellow solid. After heating iodide **7** (0.20 g, 0.44 mmol) and 22 mL of 2 M ammonia in isopropanol to 80°C for 3 h in a sealed tube, the solvent was removed under reduced pressure and the crude product purified by chromatography (dichloromethane/methanol 9/1). After recrystallization (dichloromethane/hexane) 72 mg (47.62%) of APPI:O (**8**) were obtained as white solid.

NMR analysis (¹H and ¹³C) of intermediates were in full accordance with the literature.

6: ¹H-NMR (200 MHz, CDCl₃): δ (ppm) 7.54 (m, 6 H), 7.38 (m, 4H), 7.07 (m, 4H), 5.78 (m, 1H), 3.65 (m, 2H), 3.09 (dm, 2H); ¹³C-NMR (50 MHz, CDCl₃): δ (ppm) 153.1, 138.4, 134.3, 129.3, 128.7, 128.6, 127.9, 127.5, 127.3, 125.9, 121.8, 121.4, 108.8, 108.7, 54.3,

41.9, 34.1; **MS**: m/z (%): 362 (M^+ 18%), 211 (19), 210 (100), 181 (15), 167 (20), 115 (10), 91 (58), 77 (18), 51 (9); **HRMS**: m/z calculated for $C_{22}H_{19}ClN_2O$ Na ($M^+ + Na$): 385.1084. Found: 385.1077.

7: **1H -NMR** (200 MHz, $CDCl_3$): δ (ppm) 7.55 (m, 6H), 7.36 (m, 4H), 7.07 (m, 4H), 5.72 (m, 1H), 3.64 (m, 1H), 3.22 (m, 2H), 2.86 (m, 1H); **^{13}C -NMR** (50 MHz, $CDCl_3$): δ (ppm) 153.3, 138.3, 134.4, 129.5, 128.9, 128.6, 128.1, 127.7, 127.4, 126.0, 122.0, 121.5, 109.1, 108.9, 57.5, 35.3, 2.5; **MS**: m/z (%): 454 (M^+ 13%), 211 (16), 210 (100), 167 (22), 117 (45), 115 (17), 91 (48), 77 (27), 51 (15); **HRMS**: m/z calculated for $C_{22}H_{19}IN_2O$ Na ($M^+ + Na$): 477.0440. Found: 477.0457.

8: **1H -NMR** (200 MHz, $CDCl_3$): δ (ppm) 7.51 (m, 6H), 7.30 (m, 4H), 6.95 (m, 4H), 5.82 (m, 1H), 5.15 (s, 2H), 2.84 (m, 2H), 2.73 (m, 2H); **^{13}C -NMR** (50 MHz, $CDCl_3$): δ (ppm) 154.0, 137.5, 134.0, 129.7, 129.5, 128.9, 128.1, 127.6, 127.3, 126.4, 122.2, 121.9, 110.2, 109.1, 53.1, 37.9, 30.6; **MS**: m/z (%): 343 (M^+ 20 %), 313 (11), 300 (12), 210 (84), 181 (24), 126 (29), 77 (31), 60 (38), 58 (31), 45 (91), 43 (100), 42 (34), 41 (31); **HRMS**: m/z calculated for $C_{22}H_{21}N_3O$ ($M^+ + 1$): 344.1685. Found: 344.1755.

Reference standard:

A mixture of iodide **7** (1.026 g, 2.26 mmol) and 28.25 ml 8 M methylamine in ethanol was heated at 80°C for 3 h in a sealed tube. Evaporation of the solvent and subsequent column chromatography (dichloromethane/methanol 9/1) yielded 311 mg (38.52%) of Me@APPI **9** as yellow solid.

9: **1H -NMR** (200 MHz, $CDCl_3$): δ (ppm) 7.52 (m, 6H), 7.35 (m, 4H), 7.03 (m, 3H), 6.73 (d, $J = 7.46$ Hz), 5.79 (m, 1H), 3.26 (m, 2H), 2.93 (m, 2H), 2.59 (s, 3H); **^{13}C -NMR** (50 MHz, $CDCl_3$): δ (ppm) 153.6, 138.3, 134.4, 129.5, 128.8, 128.0, 127.9, 127.8, 127.3, 126.1, 122.0, 121.5, 109.8, 108.9, 53.8, 48.0, 35.2, 29.6; **MS**: m/z (%): 357 (M^+ 8%), 210 (20), 97 (26), 81 (22), 71 (44), 69 (56), 67 (27), 57 (100), 55 (72), 44 (58), 43 (89), 41 (37). **HRMS**: m/z calculated for $C_{23}H_{23}N_3O$ ($M^+ + 1$): 358.1841. Found: 358.1930.

Radiochemistry

Production of [^{11}C]CH₃I and [^{11}C]CH₃OTf

[^{11}C]CO₂ production was stopped as soon as the desired activity (40-50 GBq) at currents between 45 and 54 μA was achieved (10-15 min). [^{11}C]CH₃I was produced using a gas phase conversion described by Larsen et al. [13] within the GE TRACERlabTM FX C Pro synthesizer adopting modifications described by Kniess et al. [14] Briefly, [^{11}C]CO₂ was trapped on a molecular sieve (4 Å) within the module and subsequently converted into [^{11}C]CH₄ by a Ni-catalysed reduction with H₂ at 400°C. The resulting [^{11}C]CH₄ was reacted in a recirculating process for 4 min with sublimated iodine at 720°C to give [^{11}C]CH₃I. The produced [^{11}C]CH₃I was trapped on-line on a Porapak[®] N column and finally released by heating the trap to 190°C. [^{11}C]CH₃OTf was prepared on-line at the passage of [^{11}C]CH₃I through a pre-heated (220°C) column containing 300 mg silver-triflate impregnated graphitized carbon. [15]

Set up of small-scale reaction and optimization

For small-scale examinations [^{11}C]CH₃I or [^{11}C]CH₃OTf, respectively, was trapped in 500 μL of ACN and divided for further experiments. If not stated otherwise all experiments were performed in triplicates. All evaluation reactions were performed manually (shielded hood; starting activity <1 GBq). The influence of reaction time (1, 2, 5 and 10 min), reaction temperature (RT, 50°C, 75°C), base (triethylamine and TBAH) and precursor concentration was investigated. Final reaction volumes of small-scale reactions were 100 - 200 μL . In figure 3 the reaction scheme is presented.

Automation of radiosynthesis

The automation of the radiosynthesis was done on the TRACERlabTM FX C Pro (GE Healthcare) [16], a schematic flowchart is depicted in figure 4.

Produced CH₃OTf was trapped at RT within the glass reactor (2 mL) containing APPI:O (1 mg, 2,9 μmol) and 1 μL of an aqueous TBAH-solution (2 mg/ μL) in 500 μL ACN. After heating the sealed reaction vessel to 75°C for 5 min, the mixture was cooled to 25°, the reaction was quenched and diluted by addition of 1 mL HPLC eluent (V2) and the whole volume was transferred to the injection loop. The crude mixture was

automatically (fluid detector controlled) injected onto the semi-preparative HPLC column. The [^{11}C]Me@APPI peak was cut into a bulb, and subsequently diluted with 80 mL water. The aqueous product solution was subjected to solid phase extraction by transferring over a preconditioned (10 mL EtOH, air, 20 mL water, air) C8 SPE cartridge. After rinsing the C8 SepPak[®] with water (V6), the pure product was eluted with 1.5 mL EtOH (V5) and the cartridge and transfer lines washed with 5 mL 0.9% saline. After formulation with further 4 mL 0.9% saline, 1 mL 3% saline and 1 mL 125 mM phosphate buffer, sterile filtration (0.22 μm) was performed under aseptic conditions (laminar air flow hot cell, class A) to avoid microbial contamination.

Quality control

Chemical and radiochemical impurities were identified using radio-HPLC according to the monograph in the European Pharmacopoeia. [17] Osmolality and pH were tested with designated equipment. Radiochemical identity and purity were assessed via analytical radio-HPLC by comparison of retention times with authentic samples. Radiochemical purity always exceeded 98%. Specific radioactivity was determined by quantification of the non-radioactive product (HPLC UV channel at 254 nm) and determination of overall radiochemical yield (GBq at end of synthesis). Sterility, presence of endotoxins, pH, osmolality and residual solvents were determined by standard procedures routinely performed at the PET Centre of the Vienna General Hospital/ Medical University of Vienna.

Statistical analysis

All quantitative data (both in text and figures) are given as arithmetic mean $\pm$ standard deviation. A student t-test (two-tailed) with $\alpha=0.05$ was performed for determination of significance; i.e. P values of < 0.05 were considered to be significant.

NET-expressing membrane binding studies

The affinity of new radiolabeled ligands was tested in a NET-expressing membrane binding protocol. [18, 19] Effects of sodium concentration, temperature and incubation times were investigated. The competitive binding experiments were performed in glass test tubes, filled with 350 μL of the new 'cold' (=non-radioactive)

reference compounds, 100 μ L of the membrane suspension (in assay buffer; 3 μ g protein/unit, human Norepinephrine Transporter RBHNETM400AU, Perkin Elmer) and 50 μ L of a 2 nM 3 H-nisoxetine*HCl solution (in assay buffer, 70-87 Ci/mmol, NET1084; Perkin Elmer). For unspecific binding 10 μ M reboxetine (Sigma-Aldrich, Vienna, Austria) was used; and for total binding (control) only 3 H-nisoxetine*HCl, buffer and membrane suspension were incubated. After incubation time, binding was quenched with ice cold buffer, and membrane bound radioactivity was recovered by rapid vacuum filtration through GF/C glass fiber filters (Whatman[®] Inc., Clifton, USA) presoaked in assay buffer containing 0.3% polyethylene imine (PEI). Filters were washed with 3 times with 4 mL assay buffer and transferred into β -counting vials. After addition of a β -scintillation cocktail (2 mL Ultima GoldTM, biodegradeable, Perkin Elmer), the tubes were shaken for 20 min and then counted. Data from the competition plots was analyzed; IC₅₀ and K_i values were calculated using GraphPad Prism[®] software (San Diego, USA). (Arithmetic means of values derived from three different assays, each in triplicate for each compound.)

To determine the selectivity of the tested compounds towards NET in comparison to DAT and SERT, respectively, assays similar to those described for NET were performed. DAT and SERT expressing membranes were used instead of NET-membranes (hSERT: 9 μ g protein/unit, RBHSTM400UA, Perkin Elmer and hDAT: 12.7 μ g protein/unit, RBHDATM400UA, Perkin Elmer, Waltham, USA).

IC₅₀ and K_i values were obtained in analogy to NET experiments. Ratios DAT/NET and SERT/NET were determined.

LogD analysis, IAM chromatography and blood-brain-barrier-penetration

LogD values were determined using a HPLC based assay according to Donovan and Pescatore. [20] All compounds (as cold references) were injected in a mix of two known compounds with known logD and k' values according to a standard protocol. A short polymeric ODP-50 column (Shodex[®], Showa Denko Europe GmbH, Munich, Germany) was used; a linear gradient from 10% MeOH 90% 25 mM Phosphate buffer to 100% methanol within 9.4 min at a flow-rate of 2 mL/min was applied. Internal

standards were triphenylene and toluene; detection was performed at 260 nm and 285 nm.

Since logP or logD values were shown to be poor predictors for BBB-penetration other *in-vitro* methods have been described [21, 22], such as immobilized artificial membrane (IAM) chromatography and parallel artificial membrane permeability assays. IAM chromatography was performed using a Redistech IAM.PC.DD2 (Regis Technologies Inc., Morton Grove, USA) column (15 cm x 4.6 mm) according to Tavares et al. [23] For analysis, 0.01 M phosphate buffer (pH 7.4) and ACN (in different ratios) were used as mobile phase at a flow rates of 1 mL/min isocratically. Resulting K_m (membrane partition coefficient) and P_m (permeability) were obtained after data analysis and compared with known BBB penetrating compounds. The data were compared with those derived from DASB (3-amino-4-[2-[(di(methyl)amino) methyl]phenyl]sulfanylbenzonitrile), a compound known to penetrate BBB, as external standard.

Metabolic stability testing

Pooled human liver microsomes (BD Biosciences, Woburn, 20 mg/mL in sucrose) are subcellular fractions (mainly endoplasmatic reticulum) that contain many drug-metabolizing enzymes, e.g. cytochrome P450s, flavinmonooxygenases and epoxide hydrolase. To investigate the metabolic fate of [^{11}C]Me@APPI, i.e. the metabolic stability and *in vitro* intrinsic clearance [$V_{\max}/K_m$], microsomal incubations were performed. Briefly, microsome solution (in sucrose) were pre-incubated under physiological conditions (phosphate buffer, pH 7.4, 37°C) with a NADPH-generating system (solution-A: NADP^+ , Glucose-6-phosphate and magnesium-chloride in H_2O and solution-B: Glucose-6-phosphate dehydrogenase in sodium citrate) for 5 min. After adding 6 μL of [^{11}C]Me@APPI, the final volume of the mixture was 300 μL . [24] Enzymatic reactions were stopped by adding one volume of an ice-cold methanol/ACN mixture (10/1 v/v). The mixtures were vortexed, followed by a centrifugation step (23.000g, 5 min, RT). Aliquots of the obtained supernatant were analysed by HPLC (pH 7.4, 37°C). As results, both the percentage of test compound metabolized after a certain time and the biological half-life were determined.

RESULTS

Chemistry

Successful preparation of precursor APPI:O and reference compound Me@APPI was achieved. APPI:O was prepared in a 4-step synthesis starting from N-phenyl-o-phenylenediamine (**4**) in 26.5% overall yield. Reference compound Me@APPI was synthesised likewise in 21.4% overall yield.

Radiochemistry

Radiochemical incorporation yields (RCIY) of [^{11}C]Me@APPI were below 0.5% for all examined conditions using [^{11}C]CH₃I as methylation agent. Using [^{11}C]CH₃OTf for methylation, the influence of precursor concentration, reaction time and temperature as well as amount of base were investigated (figure 5a-c). RCIYs ranged from $9.04 \pm 0.23\%$ for low precursor concentrations (0.25 $\mu\text{g/mL}$) at RT up to $32.81 \pm 1.8\%$ for reaction with 1 mg/mL precursor concentration. Addition of triethylamine instead of aqueous TBAH solution lead to formation of an unknown side product in high amounts. Increasing reaction temperature from RT to 75°C lead to increased RCIYs.

Optimum conditions were used for large scale preparations within a fully automated procedure. In table 1, synthesis steps, conversion and yields are outlined. For SPE procedure C-18 plus, C-18 light and C-8 plus cartridges were evaluated. Using the frequently used C-18 plus SPE cartridge 35.9% of the pure product was lost on the SepPak[®] after elution of the product. Changing to a C-18 light SepPak[®] revealed 32.0% of product bound to the cartridge; moreover addition of another 2 mL of EtOH could not achieve quantitative elution of the product, either. Therefore, a C-8 plus SPE cartridge was used; hereby only 6.3% of the product was lost due to retention on the cartridge.

So far, 11 large scale radio-syntheses have been performed, yielding 1.25 ± 0.72 GBq ($8.79 \pm 4.34\%$ EOB, corrected for decay) of formulated [^{11}C]Me@APPI within less than 40 min. Specific activity (SA) was found to be 54.35 ± 7.80 GBq/ μmol .

Purification of [^{11}C]Me@APPI

A typical semi-preparative radio-RP-HPLC chromatogram is shown in figure 6. The retention times were 1.9-2.1 min ($k'=0-0.1$) for [^{11}C]CH₃OTf, 3.5-3.8 min ($k'=0.84-1.0$) for [^{11}C]CH₃I, 4.5-5.2 min ($k'=1.4-1.7$) for precursor APPI:0 and 6.5-7.5 min ($k'=2.4-2.9$) for [^{11}C]Me@APPI.

Quality control

Quality control was performed directly after synthesis within 7 min. In figure 7 a typical analytical HPLC chromatogram is shown. The retention times in the analytical HPLC assay were 6.0-6.3 min ($k'=4-4.3$) for precursor (APPI:0), 1.7-1.9 min ($k'=0.4-0.6$) for [^{11}C]MeOH, 2.7-2.8 min ($k'=1.2-1.3$) for [^{11}C]CH₃OTf and 3.1-3.2 min ($k'=1.6-1.7$) for [^{11}C]CH₃I, respectively. The product [^{11}C]Me@APPI was eluted at a retention time of 7.6-8.1 min ($k'=5.3-5.8$). Osmolality and pH values were found to be in a physiological range. Residual solvent analysis revealed ACN <5 ppm and methanol <20 ppm, besides 8.5% ethanol present in the formulation (total product volume: 17.5 mL).

Affinity and Selectivity

Modification of testing conditions had a high impact on the feasibility of the experiments in terms of determined B_{max} , binding equilibrium and unspecific binding. The tested conditions for the NET-membranes were: 1) influence of Na⁺ and Cl⁻ concentration: 120 mM and 300 mM; 2) temperature: 4°C and 25°C; 3) incubation time: 1 h, 2 h and 4 h. Testing at 4°C lead to low B_{max} values, therefore incubation time was elongated from 1 h to 4 h. Thereby, an increase in B_{max} and a decrease of unspecific binding was observed. Generally, binding was rather low at 4°C, as compared to experiments at 25°C, where binding equilibrium was reached, resulting in high B_{max} values and low unspecific binding. Moreover, experiments at higher temperature (25°C) better represent the *in-vivo* situation (37°C). Hence, optimum conditions were obtained using incubation at 25°C for 1 h in a buffer containing 300 mM NaCl, 50 mM TRIS and 5 mM KCl.

Affinity of reference compounds (Me@APPI, APPI:0 and reboxetine) was determined using the optimum conditions as 8.08 ± 1.75 nM for Me@APPI, 19.31 ± 2.91 nM for APPI:0 and 3.29 ± 0.43 nM for reboxetine, respectively ($n \geq 9$).

For determination of selectivity, the affinity of Me@APPI and APPI:0 towards DAT and SERT was assessed and revealed >5 μ M for both compounds for DAT and 670 ± 210 nM (Me@APPI) and 485 ± 105 nM (APPI:0) towards SERT, respectively, ($n \geq 6$). Therefore, Me@APPI was found to display 83-fold affinity towards NET as compared to SERT.

LogD and BBB-penetration

LogD values were 3.08 ± 0.01 for APPI:0 and 3.14 ± 0.01 for Me@APPI, respectively. BBB-penetration experiments revealed that Me@APPI shows a similar behaviour to DASB, a SERT-PET-tracer known to be able to penetrate BBB sufficiently for *in-vivo* imaging. IAM-chromatography experiments ($n=3$) revealed K_m values of 1.85 ± 0.05 for Me@APPI and 1.78 ± 0.09 for DASB. The permeability P_m of Me@APPI was calculated as 1.47.

Metabolic stability

Stability testing using human liver microsomes ($n=4$), revealed no significant metabolism of [11 C]Me@APPI so far. After 1 h, 99.5% of the tracer was found to be still intact.

DISCUSSION

Synthesis of precursor APPI:0 and of cold reference compound Me@APPI were successfully established. Satisfactory preparation of [11 C]Me@APPI was achieved in small-scale reactions, hereby determining the ideal reaction conditions. Furthermore, up-scaling and automation of radiosynthesis was accomplished, and also purification via semi-preparative HPLC and subsequent SPE succeeded. The proposed assay guarantees the (almost) quantitative separation of APPI:0 from the product peak which is essential since the precursor molecule also displays considerable binding towards NET.

Methylation attempts of APPI:0 with [^{11}C]methyl iodide showed only poor radiochemical incorporation yields (<0.5%), whereas [^{11}C]methylation with [^{11}C]MeOTf yielded 32.8% in optimum conditions. The use of triethylamine as base lead predominantly to the formation of an unknown by-product, hence only small amounts of product were obtained. Using TBAH, satisfying amounts of [^{11}C]Me@APPI were achieved and no undesired by-product formation occurred.

Interestingly, we found when using a C-18 plus SPE cartridge for final product purification more than a third of the product was retained on the column irreversibly. Changing to a C-18 light cartridge, the amount of product bound after elution was decreased by 11%, but still unsatisfactory high amounts of irreversibly bound radioactivity were observed. Fortunately, the product could be eluted almost quantitatively using a C-8 plus cartridge. After sterile filtration, satisfying amounts of radioactivity with acceptable specific activity were obtained. Strict radiopharmaceutical quality control was passed for all produced batches allowing for preclinical testing and future in-vivo-applications.

When determining affinity and selectivity using membranes expressing the respective human transporter we found K_i -values of 8.08 ± 1.75 nM for Me@APPI, 19.31 ± 2.91 nM for APPI:0 and 3.29 ± 0.43 nM for reboxetine, respectively, towards hNET. The value for Me@APPI is well in line with the one already published by Zhang et al. (hNET IC_{50} = 9 nM). [10]

For determination of metabolic stability human liver microsomes were used. These experiments revealed excellent stability of the title compound, showing no significant degradation after 1 h incubation time for [^{11}C]Me@APPI (<0.05%). Setup of further tests to examine also phase-two-metabolism is currently in progress. In contrast, other widely used brain-PET tracers, e.g. [^{11}C -carbonyl]WAY-100635 [25], [^{11}C]DASB [26, 27] and [^{11}C]MeNER [9], display significant metabolic degradation. This highlights the outstanding stability of this novel PET-ligand.

In consideration of a possible application of [^{11}C]Me@APPI as NET-PET-tracer in human brain, BBB-penetration was tested as well. In IAM-chromatography experiments

Me@APPI showed comparable behaviour to DASB. Since all preliminary data indicate [¹¹C]Me@APPI's suitability for clinical use, further *in-vitro* and *in-vivo* experiments including autoradiography and small-animal-PET examinations are planned.

CONCLUSION

Automated radiosynthesis of the novel NET-PET-tracer, [¹¹C]Me@APPI, was established, leading to satisfying yields and specific radioactivities. So far, 1.25 ± 0.72 GBq with 54.35 ± 7.80 GBq/ μ mol SA were produced (n=11). Furthermore, all tested preclinical parameters such as selectivity, affinity, metabolic degradation, BBB-penetration and lipophilicity clearly indicate the suitability of [¹¹C]Me@APPI to become a NET-PET-tracer in clinical application. Further *in-vitro* and *in-vivo* tests will be performed to strengthen the presented findings.

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CAPTIONS:

TABLE 1 Fully automated preparation of ^{11}C Me@APPI with $n \geq 5$ (*at end of synthesis)

FIGURE 1 Structure of noradrenaline (NE), reboxetine, precursor APPI:O and Me@APPI

FIGURE 2 Synthesis of precursor molecule and cold reference compound

a) NaBH_4 , 5 min, -10°C ; b) HBr , 3 h, RT; c) CDI , overnight, RT; d) K_2CO_3 , overnight, 65°C ; e) NaI , 24 h, reflux; f) $\text{NH}_3/\text{MeNH}_2$, 80°C , 3 h;

FIGURE 3 Radio-synthesis of ^{11}C Me@APPI

FIGURE 4 Scheme of the synthesizer for the radiosynthesis and purification of ^{11}C Me@APPI

FIGURE 5 Dependence of the radiochemical incorporation yield of ^{11}C Me@APPI ($n \geq 2$) on (a) amount of precursor (RT, 5 min), (b) reaction temperature (1 mg/mL, 5 min) (c) base (75°C , 5 min). All reactions for a) and b) were done with TBAH as base. If not visible, error bars are within the margin of the symbols.

FIGURE 6 Semi-preparative radio-HPLC chromatogram

FIGURE 7 Analytical HPLC chromatogram

TABLE:

n≥5	GBq	% of initial activity (corr. for decay)	time from EOB [min]
[¹¹ C]CO ₂ , target activity	46.7 ± 7.4	100	0
[¹¹ C]CH ₄ trapped on mol.sieve	35.6 ± 6.2	95.0 ± 17.9	6 ± 1
[¹¹ C]CH ₃ I trapped on porapak	26.3 ± 5.2	79.89 ± 12.3	10 ± 1
[¹¹ C]CH ₃ OTf trapped in reactor	16.6 ± 7.8	57.0 ± 23.3	14 ± 1
residual in reactor (after transfer to HPLC)	1.0 ± 0.5	4.6 ± 1.9	22 ± 1
residual in HPLC injection loop waste	0.3 ± 0.2	1.1 ± 0.6	23 ± 1
[¹¹C]Me@APPI final product yield	1.25 ± 0.7	8.8 ± 4.3	37 ± 2
specific activity *	54.35 ± 7.8 GBq/μmol		

TABLE 2 Fully automated preparation of [¹¹C]Me@APPI with n≥5 (*at end of synthesis)

FIGURES

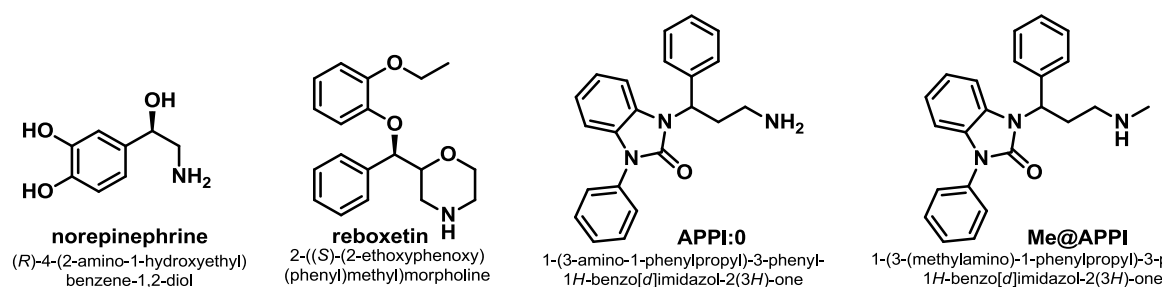


FIGURE 1: Structure of noradrenaline (NE), reboxetine, precursor APPI:0 and Me@APPI

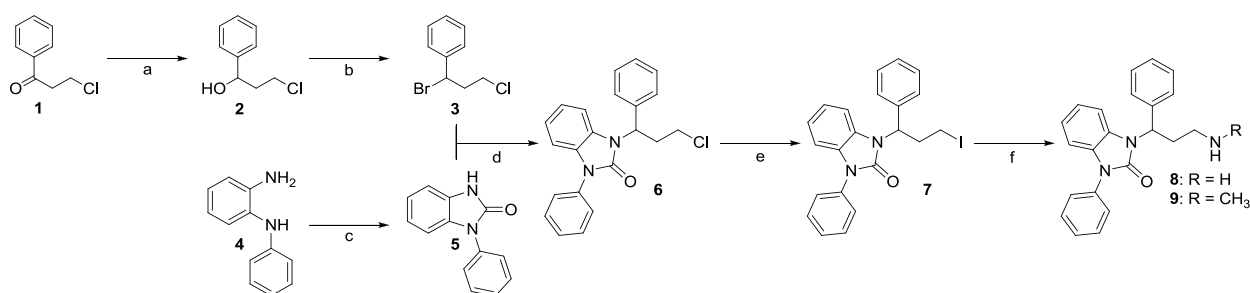


FIGURE 2: Synthesis of precursor molecule and cold reference compound

- a) NaBH₄, 5 min, -10°C; b) HBr, 3 h, RT; c) CDI, overnight, RT; d) K₂CO₃, overnight, 65°C;
 e) NaI, 24 h, reflux;
 f) NH₃/MeNH₂, 80°C, 3 h;

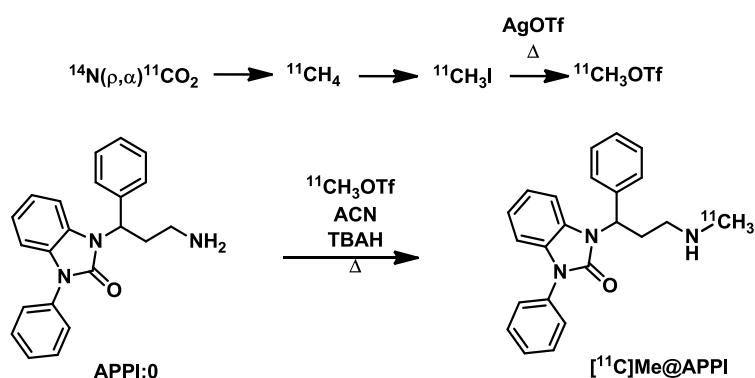


FIGURE 3 Radio-synthesis of [¹¹C]Me@APPI

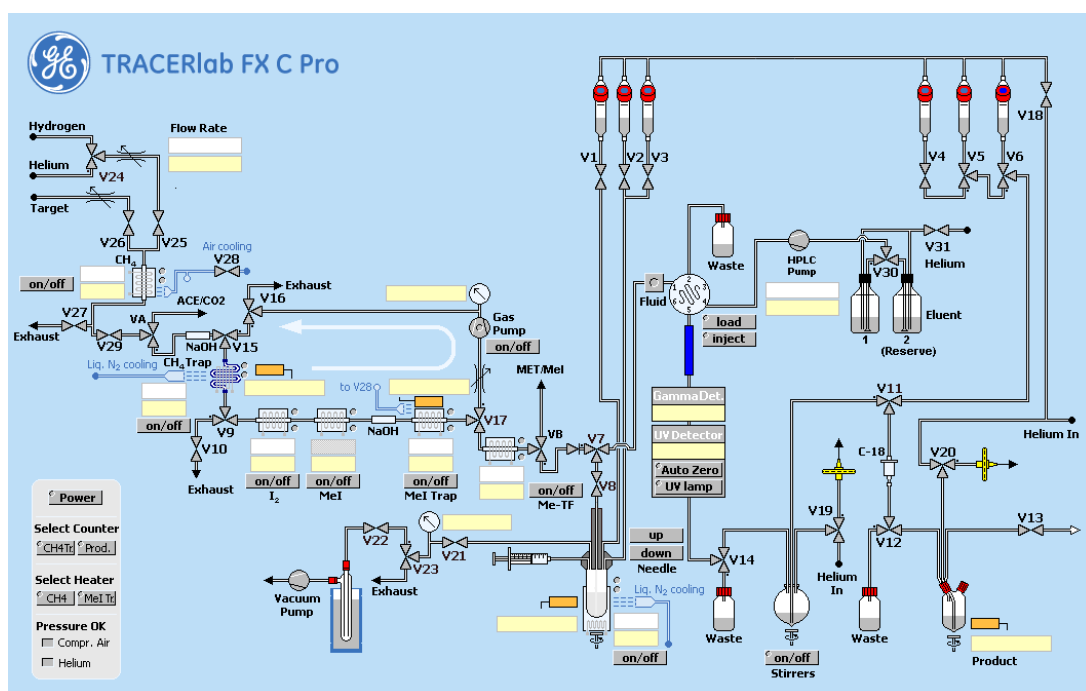
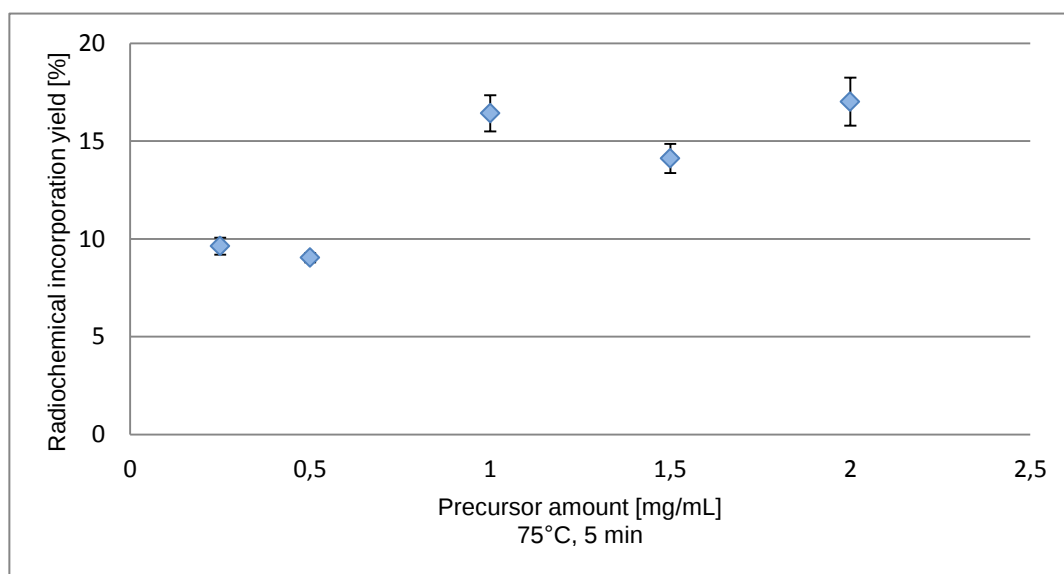
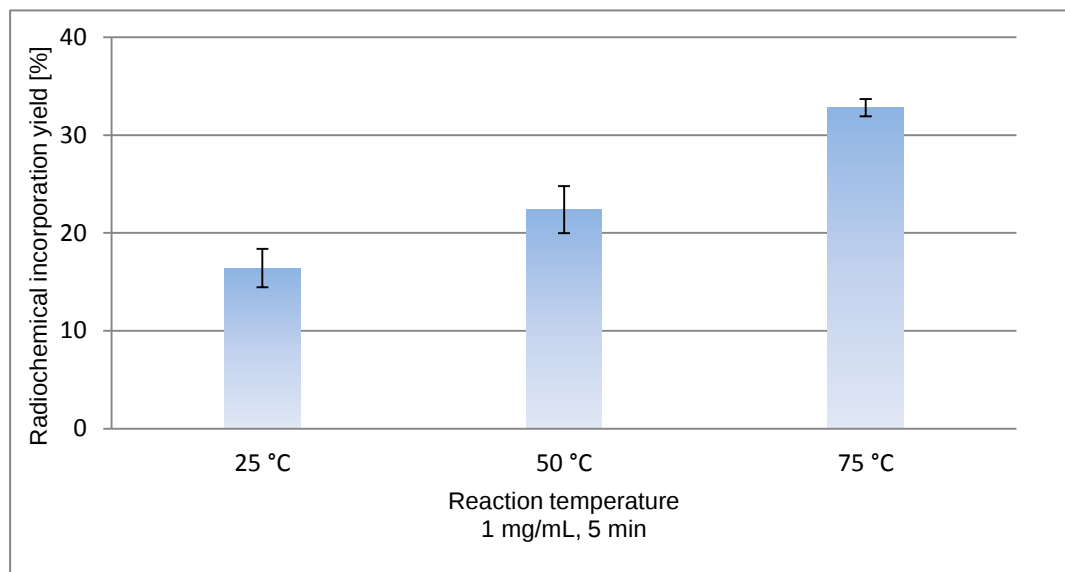


FIGURE 4 Scheme of the synthesizer for the radiosynthesis and purification of $[^{11}\text{C}]\text{Me@APPI}$

a)



b)



c)

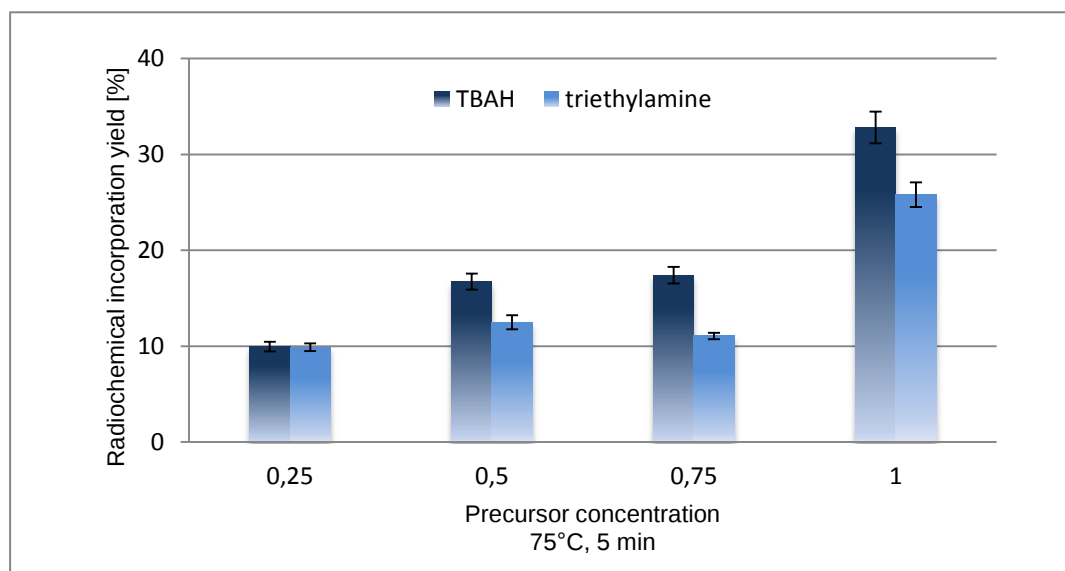


FIGURE 5 Dependence of the radiochemical incorporation yield of [^{11}C]Me@APPI ($n \geq 2$) on (a) amount of precursor (RT, 5 min), (b) reaction temperature (1 mg/mL, 5 min) (c) base (75 °C, 5 min). All reactions for a) and b) were done with TBAH as base. If not visible, error bars are within the margin of the symbols.

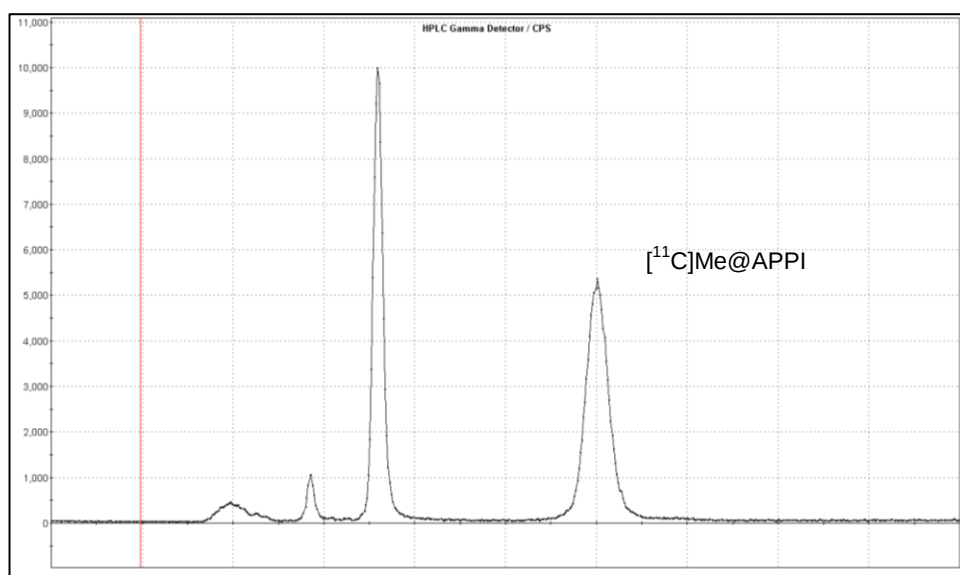


FIGURE 6 Semi-preparative radio-HPLC chromatogram

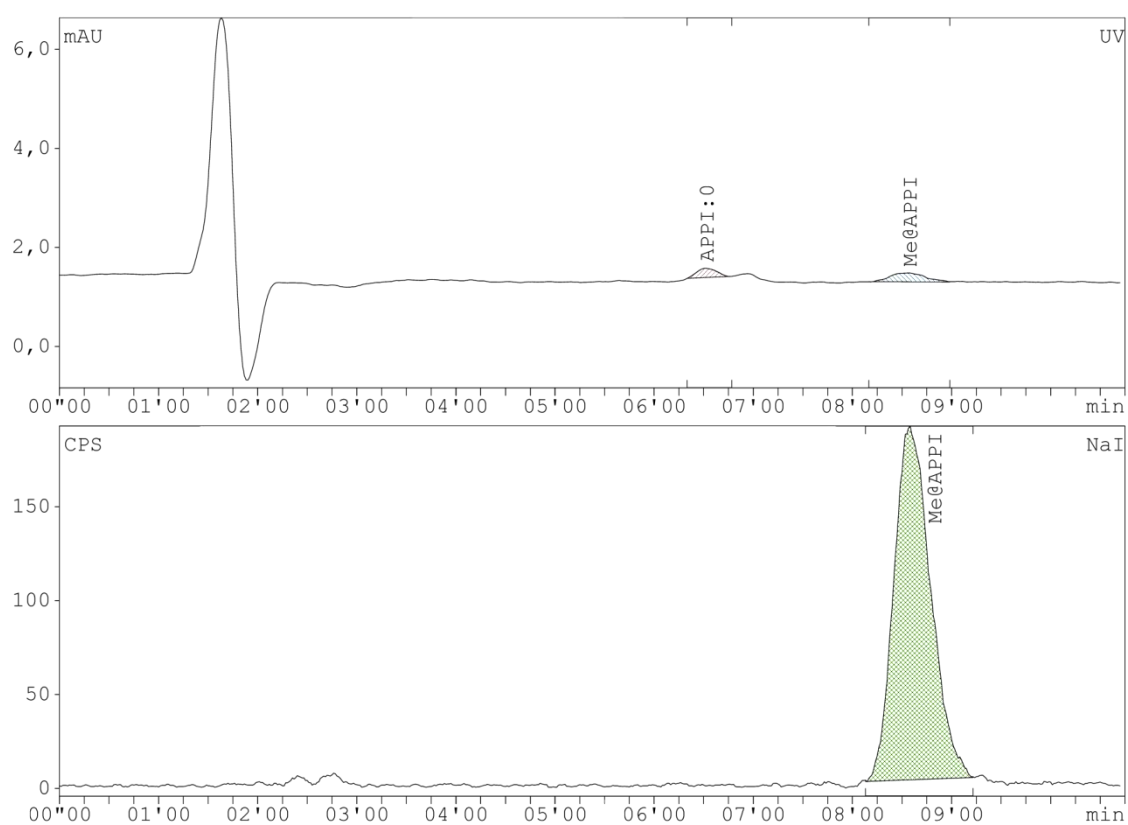


FIGURE 7 Analytical HPLC chromatogram

12. Quantification of the radio-metabolites of the serotonin-1A receptor radioligand [*carbonyl*-¹¹C]WAY-100635 in human plasma: An HPLC-assay which enables measurement of two patients in parallel

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Lukas Nics^{1,2}, Andreas Hahn³, Markus Zeilinger^{1,4}, Chrysoula Vraka^{1,2}, Johanna Ungersboeck^{1,5}, Daniela Haeusler¹, Sabine Hartmann¹, Karl-Heinz Wagner², Rupert R. Lanzenberger³, Wolfgang Wadsak^{1,5}, Markus Mitterhauser,^{1,6 *}

¹ Department of Nuclear Medicine, Medical University of Vienna, Austria

² Department of Nutritional Sciences, University of Vienna, Austria

³ Department of Psychiatry and Psychotherapy, Medical University of Vienna, Austria

⁴ University of Applied Sciences, Wiener Neustadt, Austria

⁵ Department of Inorganic Chemistry, University of Vienna, Austria

⁶ Department of Pharmaceutical Technology and Biopharmaceutics, University of Vienna, Austria

KEYWORDS

WAY100635, 5-HT, serotonin-1A, carbon-11, PET, metabolites

ABSTRACT

[*Carbonyl*-¹¹C]WAY-100635 is a potent and effective antagonist for the 5-HT_{1A} receptor subtype. We aimed to assess the status of [*carbonyl*-¹¹C]WAY-100635 and its main radio-metabolites, [*carbonyl*-¹¹C]desmethyl-WAY-100635 and [*carbonyl*-¹¹C]Cyclohexanecarboxylic acid, on the basis of an improved radio-HPLC method. Common methods were characterized by preparative HPLC columns with long runtimes and/or high flow rates. Considering the short half-life of C-11, we developed a more rapid and solvent saving HPLC assay, allowing a fast, efficient and reliable quantification of these major metabolites.

INTRODUCTION

The serotonin-1A receptor (5-HT_{1A}) shows highest densities in the cerebral cortex, hippocampus, amygdala and in the raphe nuclei (Hall et al., 1997; Ito et al., 1999). With this receptor subtype being the predominant inhibitory receptor it has been gaining considerable interest in the field of pathophysiology of neuropsychiatric disorders such as anxiety (Gunn et al., 1998; Lanzenberger et al., 2007), schizophrenia and depression (Hirvonen et al., 2007; Parsey et al., 2005). For the quantitative assessment of these receptors with positron emission tomography (PET), a kinetic input function is required. Therefore, an accurate determination and subsequently the correction of radioactive metabolites is essential. [Carbonyl-¹¹C]WAY-100635 (N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)cyclohexane[¹¹C]carboxamide) is an antagonist of the 5-HT_{1A} receptor and is particularly suitable for its quantification (Forster et al., 1995; Gunn et al., 1998; Ito et al., 1999; Parsey et al., 2000; Pike et al., 2000; Sargent et al., 2000). This tracer displays rapid degradation (Pike et al., 1996; Wu et al., 2007) to non radioactive WAY-100634 (N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)pyridin-2-amine) as well as [carbonyl-¹¹C]cyclohexanecarboxylic acid and other polar metabolites in high amounts. Moreover, minor tendency to form [carbonyl-¹¹C]desmethyl-WAY-100635 (N-(2-(4-(2-hydroxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)cyclohexane [¹¹C]carbox-amide) and methanol is observed (see figure 1). Additionally, due to the short half-life of C-11 (20.3 min), it is a daunting challenge to achieve robust data with sufficient counting statistics; this is especially true for late time points (> 20min), which are still important for adequate calculation of 5-HT_{1A} binding potentials. Although accurate methods for metabolite analysis have been introduced (Farde et al., 1998; Hirvonen et al., 2007; Osman et al., 1998; Parsey et al., 2000), they may be limited to few samples due to long runtimes of the high-performance liquid-chromatography (HPLC) system. Hence, the aim was to introduce a simple HPLC-method allowing for rapid, sensitive and reliable quantification of radiometabolites of [carbonyl-¹¹C]WAY-100635.

METHODS

Materials

All chemicals and solvents were received from commercial sources with analytical grade and used without further purification. Acetonitrile (ACN) CHROMASOLV[®] (for HPLC), methanol CHROMASOLV[®] (for HPLC), ammonium formate (for HPLC), triethylamine (TEA), thionyl chloride, tetrahydrofuran (THF) anhydrous, cyclohexyl magnesium chloride in diethylether were purchased from Sigma Aldrich (Vienna, Austria). Sodium dihydrogenphosphat monohydrat (for molecular biology), and cyclohexanecarboxylic acid (for synthesis), were obtained from Merck (Darmstadt, Germany) and disodiumhydrogenphosphate dihydrate (puriss) from Fluka (Neu-Ulm, Germany). WAY-100635 (reference standard for [*carbonyl*-¹¹C]WAY-100635), desmethyl-WAY-100635 (reference standard for radio-metabolite [*carbonyl*-¹¹C]desmethyl-WAY-100635) and WAY-100634 (precursor for radiosynthesis of [*carbonyl*-¹¹C]WAY-100635) were all purchased from ABX (Radeberg, Germany). Preparative HPLC column Phenomenex[®] Gemini C18, 250x10 mm (10 µm) was obtained from Phenomenex, Inc. (Torrance, USA) and analytical HPLC columns LiChrospher[®] LichroCART[®] 125x4 mm, RP-18 (5 µm) and pre-column Lichrospher[®] LichroCART[®] 4x4mm, RP-18 (5 µm) from Merck (Darmstadt, Germany).

Radiochemical Preparation

Radiosynthesis was performed according to an optimized procedure as described elsewhere (Wadsak et al., 2007). Briefly, [¹¹C]CO₂ (68.9-87.7 GBq) was obtained from a GE PET trace cyclotron (General Electric Medical System, Uppsala, Sweden) via the ¹⁴N(p,α)¹¹C nuclear reaction and passed through a synthesis loop containing cyclohexylmagnesium chloride in THF. [¹¹C]cyclohexylcarboxylic acid was eluted with thionyl chloride and added to 3.5 mg of WAY100634 in TEA and THF. After 4 min at 70°C, the product was purified by preparative HPLC (column: Phenomenex[®] Gemini C18, 250x10 mm (10 µm); mobile phase: 0.1 N ammonium formate, methanol, 30:70 with 0.3% triethylamine; flow at 8 ml/min; UV detection at 254 nm) and solid phase

extraction (SPE). Yields were 4.3 ± 2.7 GBq with specific radioactivities of 292 ± 130 GBq/ μ mol (n=16).

Instruments

All analytical HPLC analyses were performed on a single Merck Hitachi system equipped with an Interface D-6000, Intelligent Pump L-6220 and a UV- Detector L-4000 adjusted at 220 nm. The online radio-detection was obtained using a Packard flow scintillation analyzer with a BGO crystal, Radiomatic Flo-One Beta 150TR (Canberra, Canada). Solvent fractions were separated using a fraction-collector Teledyne ISCO Foxy Jr. (Lincoln, USA). Measurement of all activities was performed using an automatic Wizard² 3" 2480 (Waltham, USA) gamma-counter from Perkin Elmer.

Metabolite analysis

After radiotracer injection, blood samples were collected at 2, 5, 10, 20, 35 and 50 minutes. Three millilitres of whole blood were first measured in the gamma-counter, followed by a centrifugation step (1800 g, 7 min, 14°C) to separate plasma and hematocrit. Plasma aliquots of 830 μ l were removed and ACN (spiked with standards of WAY-100635, desmethyl-WAY-100635 and cyclohexanecarboxylic acid) was added to achieve a final plasma:ACN ratio of 42:58 (Parsey et al., 2000). Plasma activity was measured in another gamma-counting step and the precipitate was obtained by renewed centrifugation (23.000 g, 4 min, 20°C). Ultimately two millilitres of the supernatant were injected into the radio-HPLC. The separation of the components was carried out by an analytical HPLC column.

The mobile phase was prepared by mixing ACN and 25 mM phosphate buffer (pH 7.0) in a ratio of 58:42, degassed by an ultrasonic system. An isocratic HPLC method was established with a flow of 2 ml/min and the UV detector set at 220 nm.

Following HPLC quantification, two fractions with intact [*carbonyl*-¹¹C]WAY-100635 and its metabolites were collected by a fraction collector and re-measured in the Gamma-Counter to obtain an even more accurate assessment of metabolites.

Data processing

Fractions were then corrected for radioactive decay and background activity. For each sample, the parent fraction was calculated as the ratio of intact [*carbonyl*-¹¹C]WAY-100635 to total activity from both fractions. The final profile of the unmetabolized radioligand over time was obtained by fitting a Hill-type function (Gunn et al., 1998) to the six parent fractions (see figure 2):

$$\text{parent fraction (t)} = 1 - \frac{\alpha * t^{\beta}}{t^{\beta} + \gamma}$$

The model parameters α , β and γ were estimated with a standard non-linear least squares fitting procedure implemented in Matlab R2010a (The MathWorks, Natick, MA, USA).

RESULTS

The amount of intact [*carbonyl*-¹¹C]WAY-100635 and metabolites derived from blood samples as well as retention times and retention factors are given in table 1 (n=16). The compounds eluted from the HPLC-column in the following order: [*carbonyl*-¹¹C]cyclohexanecarboxylic acid and other polar metabolites (1.6 ± 0.05 min), followed by [*carbonyl*-¹¹C]desmethyl-WAY-100635 (3.0 ± 0.01 min) and [*carbonyl*-¹¹C]WAY-100635 (4.1 ± 0.12 min, see figure 4). Metabolism was very rapid: after 5min already $74 \pm 10.33\%$ of the parent compounds were metabolized (see table 2 and figure 2). The total analysis time of the HPLC was 5.3 minutes per sample. The suitability of the method could be proven by 16 patients so far. No failures due to technical or analytical malfunctions were observed.

DISCUSSION

We have introduced an improved HPLC-assay to quantify the metabolic fate of the 5-HT_{1A} antagonist [*carbonyl*-¹¹C]WAY-100635 *in vivo*. The method allows for the determination of 12 blood samples in less than 115 minutes using a single HPLC-system.

The arterial input function represents the gold standard for quantification of neuronal receptors and transporters with PET (Innis et al., 2007). This implies exact determination of the metabolite fraction across time.

In the case of [*carbonyl*- ^{11}C]WAY-100635 it is known that metabolism is fast. Four major methods (Farde et al., 1998; Hirvonen et al., 2007; Osman et al., 1998; Parsey et al., 2000) have been introduced so far to quantify the metabolites of this radioligand. All described approaches as well as the one introduced here were capable of distinguishing between [*carbonyl*- ^{11}C]WAY-100635 and [*carbonyl*- ^{11}C]desmethyl-WAY-100635.

In-vitro, several more hydrophilic metabolites were found in microsomal experiments using LC-MS/MS for detection (Laven et al., 2006). Furthermore, Osman et al. observed another significant polar metabolite, [*carbonyl*- ^{11}C]cyclohexanecarboxylic acid along with some other metabolites that were even more hydrophilic in human studies (Osman et al., 1998). Still, Wu et al concluded that, with the exception of [*carbonyl*- ^{11}C]desmethyl-WAY-100635, no metabolites are expected to bind specifically to the 5-HT_{1A} receptor (Wu et al., 2007).

Confronted with the requirement to establish such a method in our institution, we have compared it to the methods suggested in the literature so far, and observed that we had to adjust the setup to our prerequisites: analyses of blood samples derived from two patients simultaneously (parallel analyses). However, the fastest assays so far (Farde et al., 1998; Osman et al., 1998) have been used with gradient HPLC-methods. Analysing 7 samples (Farde et al., 1998) would take 52.5 min (HPLC runtime excluding workup). We determined to use an isocratic HPLC method because of the far better signal to noise ratio, less baseline-drift (both not applicable to radio-channel) and the possibility of immediately injecting the follow-up-sample to the system without having to wait for column equilibration. The use of two HPLC systems simultaneously would minimize the overall analysis time but may, in turn, lead to different results between the systems. Although cross-calibration limits internal differences across systems, even small errors result in considerable inaccuracies, especially at late time points characterized by low counting rates. In comparison to the

fastest isocratic method, we were able to finish the same amount of samples in only 37 ± 1.03 min (see figure 3). Hence, the fastest assay described so far is about 40% slower than the method described in this manuscript. This enables us to analyse 10 samples (i.e., 10 different time points) within the same timeframe, allowing for a more reliable curve fitting and hence input function modelling. When taking the blood samples of two patients (parallel analyses, 6 samples per patient), our assay is still fast enough to ensure that the radiosignals of the final samples have a sufficient counting statistic for robust curve fitting. This improvement is essential because the counting rate at late time points is strongly diminished due to rapid radioactive decay of C-11 labelled compounds; but it is absolutely pivotal to get sufficient signal for accurate unmetabolized radioligand quantification. A brief comparison of our method to the four standard approaches is also given in figure 3.

Apart from HPLC, some other methods, such as solid phase extraction (SPE) and liquid-liquid extraction, were described to assess the metabolism of F-18 [*carbonyl*- ^{11}C]WAY-100635 analogues (Ma et al., 2003; Ma et al., 2001). Since the major human *in-vivo* metabolites of [^{18}F]FCWAY are two very hydrophilic compounds, namely [^{18}F]parafluorocyclohexylcarboxylic acid and [^{18}F]fluoride, they can be easily separated using SPE methods. However, with [*carbonyl*- ^{11}C]WAY-100635, we have to focus on the lipophilic metabolite [*carbonyl*- ^{11}C]desmethyl-WAY-100635, which is known to bind to the 5-HT_{1A} receptor. Using these published SPE methods (Ma et al., 2003; Ma et al., 2001), we were not able to isolate [*carbonyl*- ^{11}C]WAY-100635 and [*carbonyl*- ^{11}C]desmethyl-WAY-100635 quantitatively due to the lower separating capacity of the cartridges. Hence, SPE was only effective to separate the main hydrophilic metabolite, [*carbonyl*- ^{11}C]cyclohexanecarboxylic acid, but was not able to distinguish between [*carbonyl*- ^{11}C]WAY-100635 and [*carbonyl*- ^{11}C]desmethyl-WAY-100635. Therefore, an additional HPLC would have been required, yielding even longer analytical protocols.

On the basis of a liquid-liquid extraction method (Ma et al., 2003), we found out that overall expenditure for a single sample (including sample preparation and gamma counting) demanded 5.0 ± 0.5 min more analysis time compared to our HPLC method.

Moreover, the quantitative separation of [*carbonyl*-¹¹C]WAY-100635 and [*carbonyl*-¹¹C]desmethyl-WAY-100635 was also unsatisfactory.

This motivated us to consider a method efficient enough to separate [*carbonyl*-¹¹C]WAY-100635, [*carbonyl*-¹¹C]desmethyl-WAY-100635 and [*carbonyl*-¹¹C]cyclohexanecarboxylic acid quantitatively in a minimum period of time. Based on these experiences, HPLC evinced as the only suitable method in our case (NB: this must not be generalized; using other tracers, e.g. [¹⁸F]FCWAY, [¹⁸F]MPPF, different methods might still be applicable). Hence, the direct comparison with other methods (see figure 3) was limited to the published HPLC-approaches.

Total number of analyses

The method in the literature allowing the highest number of analyses (based on HPLC time consumption, excluding workup) is given by Osman et al. (1998), allowing 8 analyses in 128 minutes, followed by Farde et al. (1998) with 7 analyses in 52.5 minutes. Since we had to analyze 12 samples, these methods may not allow for a sufficient signal-to-noise ratio at late time points. Additionally and in contrast to the approaches available, we administered a maximum of 3.08 ± 0.16 MBq/kg body weight (patient total amount: 206.01 ± 28.79 MBq compared to 325.6-391 MBq (Hirvonen et al., 2007; Osman et al., 1998; Parsey et al., 2000) due to regulatory issues regarding radiation protection for repetitive patient scans. Hence, our injected activity was considerably lower (36.7 to 47.4%) compared to the previously described methods (Hirvonen et al., 2007; Osman et al., 1998; Parsey et al., 2000). Thus, we had to establish a faster method compensating for the lower available activities in blood samples especially at late time points.

Methods based on preparative HPLC systems are characterized by larger column size (Farde et al., 1998; Hirvonen et al., 2007; Osman et al., 1998; Parsey et al., 2000) and higher flow rates (Farde et al., 1998; Hirvonen et al., 2007; Osman et al., 1998) (see figure 3). Our aim was to use a conventional single analytical HPLC system also widely used for routine quality control of radiopharmaceuticals. The use of a single HPLC-system provides the advantage of avoiding potential systemic errors, which can occur

when using two or more systems in parallel, even if they are identical in construction and cross-calibrated. This could have significant effects on results. The appropriateness and good resolution of the system is shown in figures 4a+b. Radiometabolites are baseline-separated and easily integrated/collected by the fraction collector.

CONCLUSION

The proposed HPLC system allows for the reliable quantification of the metabolic status of [carbonyl-¹¹C]WAY-100635 of two parallel patients simultaneously. The assay is realized with an analytical HPLC system with baseline separated metabolites. Total analysis time of two parallel patients (12 samples) is <64 minutes (excluding workup) and <115 minutes (including workup). The fastest assay so far (Farde et al., 1998; Osman et al., 1998) is about 40% slower in comparison to the herewith presented method. This improvement provides the advantage of achieving more accurate counting statistics with late samples for robustly modelling the metabolite-corrected input function.

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LEGENDS

Figure 1: Main radiometabolites and non-radioactive metabolites of [*carbonyl*-¹¹C]WAY-100635

Figure 2: profile of the unmetabolized radioligand over time, obtained by a fitted Hill-type function. Bars show mean±SD (n=16).

Figure 3: comparison of five major HPLC-methods (isocratic and gradient) previously described and our optimized method showing the runtime of a single sample, the HPLC-pump-flow of the mobile phase and the number of samples analyzed during one experimental setup

Figure 4: HPLC-Chromatogram 2 minutes (A) and 5 minutes (B) after radiotracer injection

Table 1: retention times (Rt), retention factors (k') of intact tracer and metabolites of all blood samples over time (mean±SD, n=16)

Table 2: metabolic status of radiotracer and metabolites after time (minutes past injection (mean±SD, n=16))

Table 3: overview of HPLC-systemic parameters of five major HPLC-methods (isocratic and gradient) showing stationary and mobile phase

FIGURES

Figure 1

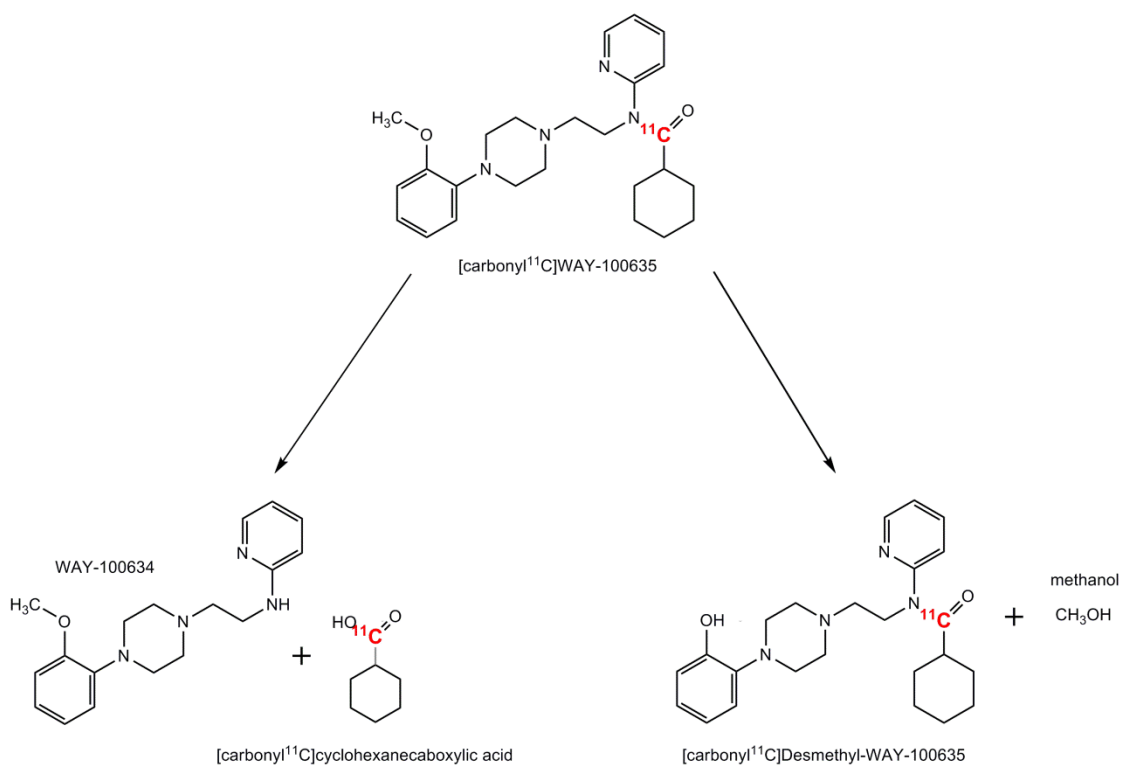


Figure 2

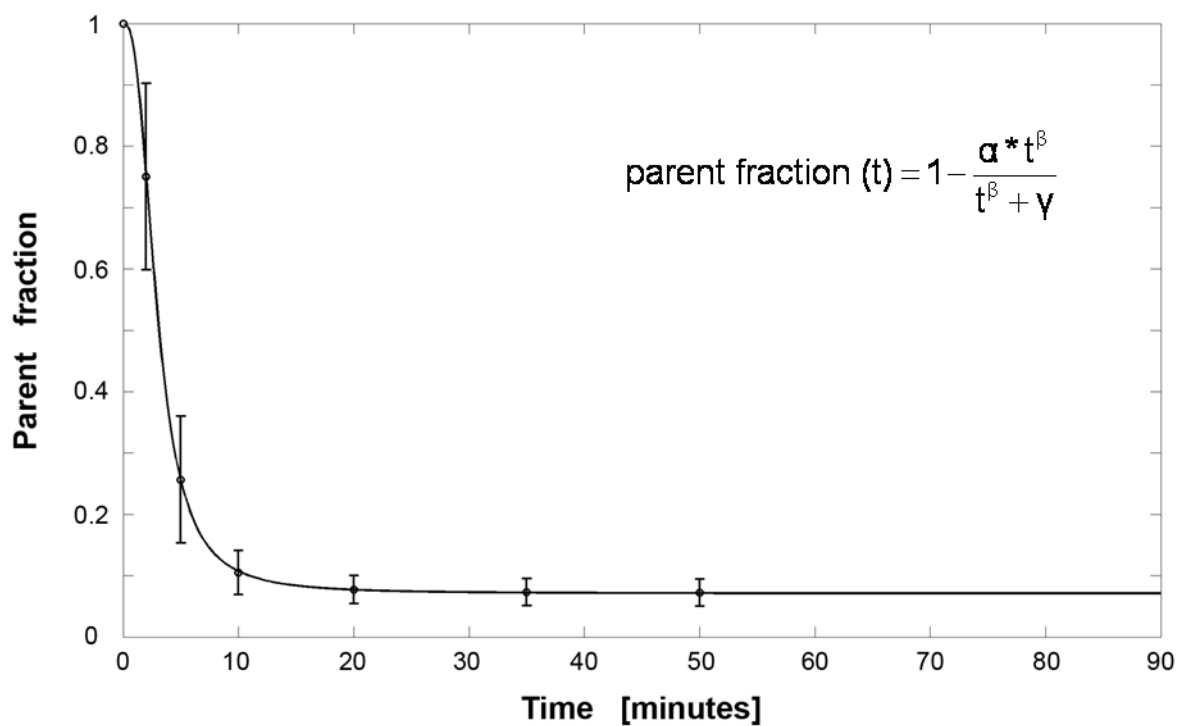


Figure 3

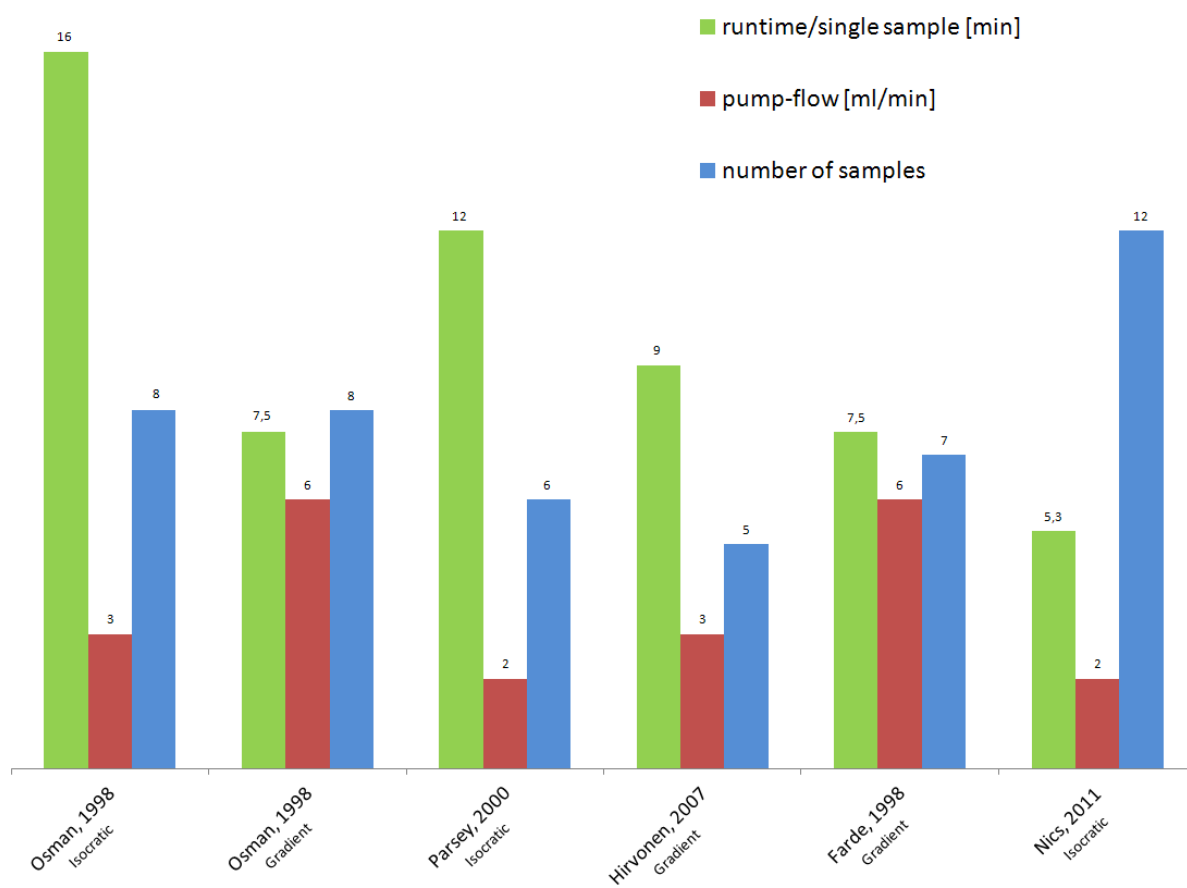
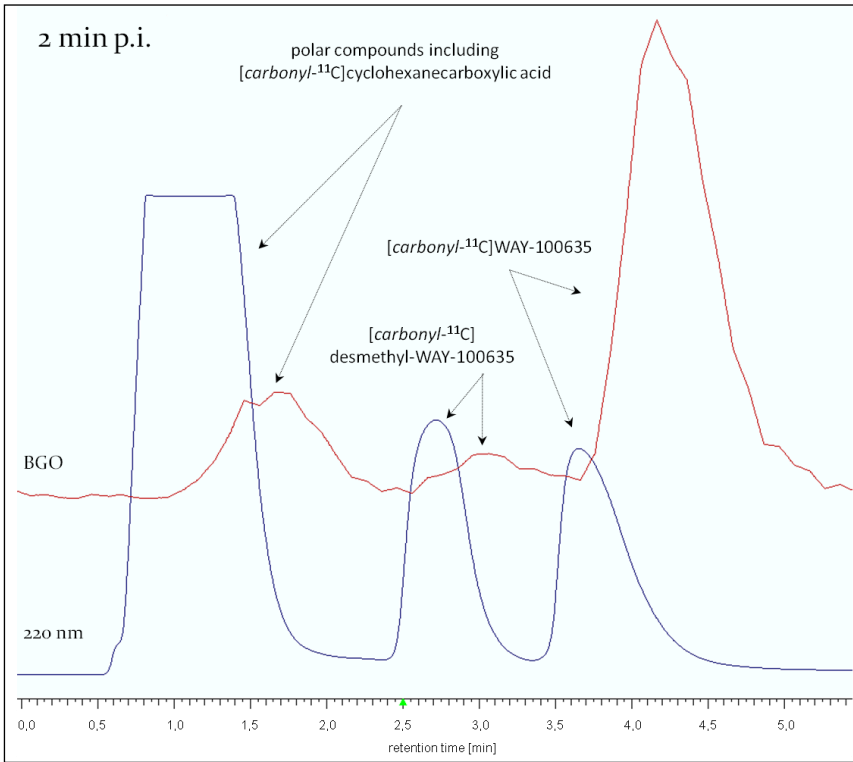
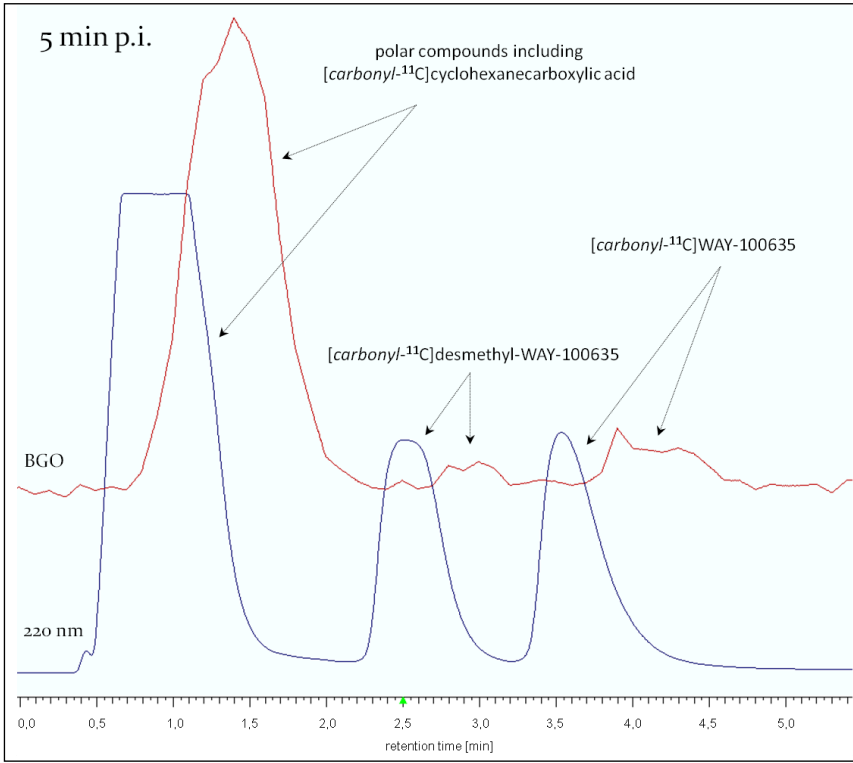


Figure 4

A



B



Tables

Table 1

Literature	System	column	mobile phase
Osman 1998	isocratic	μBondapak 300 x 7.8 mm, 10 μm partiel size (Waters Corporation, Milford U.S.A.)	0.1 M ammonium formate; Methanol (35/65)
	gradient	μBondapak 300 x 7.8 mm, 10 μm partiel size (Waters Corporation, Milford U.S.A.)	0.01 N phosphoric acid; ACN
Parsey 2000	isocratic	Phenomenex C18 ODS 250 x 4.6 mm (Phenomenex, Torrance, U.S.A)	0.1 M ammonium formate; ACN (50:50)
Hirvonen 2007	gradient	μBondapak 300 x 7.8 mm, 10 μm partiel size (Waters Corporation, Milford U.S.A.)	0.01 N phosphoric acid; ACN
Farde 1998	gradient	μBondapak 300 x 7.8 mm, 10 μm partiel size (Waters Corporation, Milford U.S.A.)	0.01 N phosphoric acid; ACN
Nics 2011	isocratic	LiChrospher LichroCART 125 x 4 mm, RP-18, 5 μm partiel size (Merck, Darmstadt, Germany)	25 mM phosphate buffer (pH 7.0); ACN (42/58)

Table 2

	molecule	% 2min p.i.	% 5min p.i.	% 10min p.i.	% 20min p.i.	% 35min p.i.	% 50min p.i.
metabolites	[carbonyl- ¹¹ C]cyclohexanecarboxylic acid	24.98±15.18	74.35±10.33	89.47±3.59	92.29±2.28	92.70±2.19	92.78±2.19
	[carbonyl- ¹¹ C]desmethyl-WAY-100635						
radiotracer	[carbonyl- ¹¹ C]WAY-100635	75.02±15.18	25.65±10.33	10.53±3.59	7.71±2.28		7.22±2.19

* [carbonyl-¹¹C]Desmethyl-WAY-100635 was detected only in 2 of 16 patients after 2min (1.33±0.55 % of total amount) and 5min (3.50±0.66 % of total amount)

Table 3

	molecule	Rt (min)	k'
hydrophilic	polar compounds including		
metabolites	[carbonyl- ¹¹ C]cyclohexanecarboxylic acid	1.6±0.05	1.7±0.03
metabolite	[carbonyl- ¹¹ C]desmethyl-WAY-100635	3.0±0.01	3.6±0.16
radiotracer	[carbonyl- ¹¹ C]WAY-100635	4.1±0.12	5.3±0.25

13. Combining image-derived and venous input functions enables quantification of serotonin-1A receptors with [*carbonyl*-¹¹C]WAY-100635 independent of arterial sampling

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Andreas Hahn^{a,#}, **Lukas Nics**^{b,d,#}, Pia Baldinger^a, Johanna Ungersboeck^b,
Peter Dolliner^b, Richard Frey^a, Wolfgang Birkfellner^c, Markus Mitterhauser^b,
Wolfgang Wadsak^b, Georgios Karanikas^b, Siegfried Kasper^a, Rupert Lanzenberger^a

#...the authors contributed equally

^a Department of Psychiatry and Psychotherapy, Medical University of Vienna, Austria

^b Department of Nuclear Medicine, Medical University of Vienna, Austria

^c Center for Medical Physics and Biomedical Engineering, Medical University of Vienna, Austria

^d Department of Nutritional Sciences, University of Vienna, Austria

Abstract

Image-derived input functions (IDIFs) represent a promising technique for a simpler and less invasive quantification of PET studies as compared to arterial cannulation. However, a number of limitations complicate the routine use of IDIFs in clinical research protocols and the full substitution of manual arterial samples by venous ones has hardly been evaluated. This study aims for a direct validation of IDIFs and venous data for the quantification of serotonin-1A receptor binding (5-HT_{1A}) with [*carbonyl*-¹¹C]WAY-100635 before and after hormone treatment.

Methods

Fifteen PET measurements with arterial and venous blood sampling were obtained from 10 healthy women, 8 scans before and 7 after eight weeks of hormone replacement therapy. Image-derived input functions were derived automatically from cerebral blood vessels, corrected for partial volume effects and combined with venous

manual samples from 10min onwards (IDIF+VIF). Corrections for plasma/whole-blood ratio and metabolites were done separately with arterial and venous samples. 5-HT_{1A} receptor quantification was achieved with arterial input functions (AIF) and IDIF+VIF using a two-tissue compartment model.

Results

Comparison between arterial and venous manual blood samples yielded excellent reproducibility. Variability (VAR) was less than 10% for whole-blood activity ($p>0.4$) and below 2% for plasma to whole-blood ratios ($p>0.4$). Variability was slightly higher for parent fractions (VAR_{max}=24% at 5min, $p<0.05$ and VAR<13% after 20min, $p>0.1$) but still within previously reported values. IDIFs after partial volume correction had peak values comparable to AIFs (mean difference $\Delta=-7.6\pm16.9$ kBq/ml, $p>0.1$), whereas AIFs exhibited a delay ($\Delta=4\pm6.4$ s, $p<0.05$) and higher peak width ($\Delta=15.9\pm5.2$ s, $p<0.001$). Linear regression analysis showed strong agreement for 5-HT_{1A} binding as obtained with AIF and IDIF+VIF at baseline ($R^2=0.95$), after treatment ($R^2=0.93$) and when pooling all scans ($R^2=0.93$), with slopes and intercepts in the range 0.97 to 1.07 and -0.05 to 0.16, respectively. In addition to the region of interest analysis, the approach yielded virtually identical results for voxel-wise quantification as compared to the AIF.

Conclusions

Despite the fast metabolism of the radioligand, manual arterial blood samples can be substituted by venous ones for parent fractions and plasma to whole-blood ratios. Moreover, the combination of image-derived and venous input functions provides a reliable quantification of 5-HT_{1A} receptors. This holds true for 5-HT_{1A} binding estimates before and after treatment for both regions of interest-based and voxel-wise modeling. Taken together, the approach provides less invasive receptor quantification by full independence of arterial cannulation. This offers great potential for the routine use in clinical research protocols and encourages further investigation for other radioligands with different kinetics characteristics.

KEYWORDS

image-derived input function, [*carbonyl*-¹¹C]WAY-100635, serotonin-1A, arterial input function, venous blood, kinetic analysis

INTRODUCTION

Positron emission tomography (PET) provides an excellent opportunity to quantify neuronal receptors in the living human brain. In addition to insights in basic human brain function, this has led to important investigations of psychiatric and neurological disorders such as depression (Drevets et al., 2007; Hirvonen et al., 2008; Parsey et al., 2010), anxiety disorders (Lanzenberger et al., 2007; Spindelegger et al., 2009) and Alzheimer's disease (Devanand et al., 2010). The gold standard for receptor quantification is the arterial input function, describing the amount of intact radioligand in plasma which is available for transport into tissue (Ichise et al., 2001; Mintun et al., 1984; Slifstein and Laruelle, 2001). However, arterial cannulation is an invasive and technically demanding technique, which discourages study participation and may lead to drop-out if cannulation fails or clots (Ogden et al., 2010; Zanotti-Fregonara et al., 2011a).

Several non-invasive techniques have been introduced on the basis of reference regions, which are devoid of specific receptor binding (Hume et al., 1992; Ichise et al., 2003; Lammertsma and Hume, 1996; Logan et al., 1996). These methods have been applied with great success in the majority of PET receptor studies. Still, the applicability of these models may be limited by the underlying assumptions and to tracers where a reliable reference region exists (Ichise et al., 2001; Slifstein and Laruelle, 2001). For instance, previous work reported reduced clinical sensitivity (Hammers et al., 2008) and differences in outcome parameters when using non-invasive models (Parsey et al., 2010). Notably, the two studies mention a high intersubject variability in the reference region regarding blood flow and/or low but considerable specific receptor binding in the reference region. Especially for the serotonin-1A receptor antagonist [*carbonyl*-¹¹C]WAY-100635, the violation of modeling assumptions may cause a bias in binding estimates (Parsey et al., 2000; Slifstein et al., 2000).

Recently, image-derived input functions (IDIFs) have received growing attention (Zanotti-Fregonara et al., 2011a). Extracted directly from the PET images, IDIFs represent a non-invasive alternative to arterial blood sampling while being independent from a reference region. The use of IDIFs has been evaluated for several radioligands to quantify receptors (Liptrot et al., 2004) and other neuronal binding sites (Mourik et al., 2009; Sanabria-Bohorquez et al., 2003; Zanotti-Fregonara et al., 2011b), but only in a few scenarios has this become a routine clinical tool (Zanotti-Fregonara et al., 2011a). Image-derived input functions represent the activity in whole-blood and hence, the majority of radioligands require correction for metabolites and plasma to whole-blood ratio. This implies that IDIFs only result in a less-invasive procedure for the patient if manual arterial blood samples for these corrections can be avoided. The most obvious alternative would be the substitution by venous samples (Zanotti-Fregonara et al., 2011a), but despite several suggestions of this kind (Liptrot et al., 2004; Mourik et al., 2009; Sanabria-Bohorquez et al., 2003), corrections with venous blood have hardly been used or even validated. Few exceptions include the application in a brain study using [^{18}F]FDG (Chen et al., 2007) as well as abdomen (Visvikis et al., 2004) and animal studies (Iida et al., 1992; Schroeder et al., 2007). In addition, differences between arterial and venous samples in whole-blood activity and parent fraction may further complicate successful substitution (Zanotti-Fregonara et al., 2011a). The validation of venous blood may be an even more important issue for radioligands with fast metabolism, such as [^{11}C]-PBR28 (Zanotti-Fregonara et al., 2011b) and [*carbonyl*- ^{11}C]WAY-100635 (Gunn et al., 1998).

Therefore, the aims of this study were, i) to evaluate the combination of image-derived and venous input functions for the quantification of serotonin-1A (5-HT_{1A}) receptor binding with [*carbonyl*- ^{11}C]WAY-100635. ii) To obtain a less-invasive method for quantification, the required corrections of the IDIFs for plasma to whole-blood ratio and metabolites were carried out using only venous samples. iii) The validation of the introduced approach was assessed by direct comparison with arterial blood sampling. iv) Finally, the applicability of the method was further evaluated in two independent

measurement series of healthy subjects at baseline and after 8 weeks of hormone treatment.

MATERIALS and METHODS

Subjects and treatment

Ten healthy women from a clinical project participated in this study (mean age $\pm$ sd = 54.5 $\pm$ 3.1 years). All subjects underwent standard medical examinations, electrocardiogram, routine laboratory tests and the Structural Clinical Interview for DSM-IV Diagnoses (SCID) to rule out physical, psychiatric and neurological disorders. Further exclusion criteria were past or current substance abuse, intake of psychotropic medication or hormonal treatment. All participants were postmenopausal women, since the clinical project assessed serotonin-1A binding before and after hormone replacement therapy in a randomized, double-blind, longitudinal study. Hence, subjects underwent PET before and after 8 weeks of treatment with either A) 17 β -estradiol and progesterone, B) 17 β -estradiol or C) placebo. For this study, 15 PET scans with full blood sampling were available, 8 before and 7 after treatment. According to the randomization of the clinical project, 3, 1 and 3 subjects with post-treatment scans received A, B and C, respectively. The treatment was monitored by experienced physicians and hormonal status was confirmed by blood tests at the screening visit and the days of the PET measurements. Subjects provided written informed consent after detailed explanation of the study protocol and received reimbursement for their participation. This study was approved by the Ethics Committee of the Medical University of Vienna and the General Hospital of Vienna.

Positron emission tomography (PET)

All measurements were carried out with a GE Advance PET scanner (General Electric Medical Systems, Waukesha, WI, USA) at the Department of Nuclear Medicine, Medical University of Vienna, and are essentially described elsewhere (Hahn et al., 2010a; Lanzenberger et al., 2007; Spindelegger et al., 2009). Briefly, the subject's head was placed in a cushioned polyurethane mold and head movement was minimized

with additional straps around the forehead. Following a 5min tissue attenuation scan (retractable ^{68}Ge rod sources, segmented attenuation approach), the 3D dynamic emission measurement started synchronously with the bolus injection of the radioligand [*carbonyl*- ^{11}C]WAY-100635 (mean injected dose $\pm$ sd = 210.2 $\pm$ 28.1 MBq, for synthesis see (Wadsak et al., 2007)). In order to capture the initial peak of the image-derived input function, 50 consecutive frames were acquired with short timing at the beginning (12x5s, 6x10s, 3x20s, 6x30s, 4x1min, 5x2min, 14x5min). Total scan time was 90min and reconstructed images comprised a spatial resolution of 4.36mm full-width at half-maximum (FWHM, determined by manufacturer's performance procedure according to NEMA NU 2-1994 standard) at the center of the field of view (matrix 128x128, 35 slices, voxel size = 3.125 x 3.125 x 4.25mm).

Blood sampling

Arterial blood samples were taken automatically for the first 3 minutes using an automated blood sampling system (ABSS, Allogg, Mariefred, Sweden). Pump rate was 4ml/min and a pre-existing cross-calibration measured according to manufacturer's recommendations was used. Manual arterial (a. radialis) and venous (v. cubitalis) blood samples were taken simultaneously by two staff members (physician supported by nurse/medical technician) at 2, 5, 10, 20, 35 and 50min post injection, 3ml each.

All manual blood samples were analyzed by two chemists/pharmacists as described previously (Nics et al., 2011). After measuring whole-blood activity in a gamma-counter (Wizard² 3" 2480, Perkin Elmer, Waltham, MA, USA), plasma was separated by centrifugation (1800g, 7min). As the plasma to whole-blood ratio is assumed to be temporally invariant for WAY-100635 (Parsey 2010, personal communication), plasma activity was measured for 3 samples (5, 10, 20min) to calculate plasma to whole-blood ratio, which were averaged for further analysis. Plasma aliquots of 830 μl were then precipitated with acetonitrile (Parsey et al., 2000) and spiked with standards of WAY-100635 and its main metabolites, Desmethyl-WAY-100635 and Cyclohexanecarboxylic acid. Following a second centrifugation (23000g, 4min), 2ml of the supernatant were injected into a high performance liquid chromatography system (HPLC, Merck Hitachi,

Sigma-Aldrich, St. Louis, MO, USA) equipped with a BGO-crystal and a UV-detector. To enable analysis of 12 blood samples (i.e., 6 arterial and 6 venous), an optimized HPLC assay was used with two main improvements regarding the HPLC-column and the mobile phase (Nics et al., 2011). To obtain a robust assessment of radioactive metabolites, two fractions containing intact WAY-100635 and its metabolites were collected with a fraction collector and measured in the gamma counter. All activity measurements were corrected for radioactive decay and background activity.

The parent fraction of [*carbonyl*- ^{11}C]WAY-100635 was then calculated as the ratio of intact radioligand to the total activity of both fractions. The time course of the unmetabolized radioligand was interpolated by fitting a Hill-type function (Gunn et al., 1998) to the six parent fractions for arterial and venous samples separately. The model parameters were estimated with a standard non-linear least squares algorithm as implemented in MatlabR2010a (The MathWorks, Natick, MA, USA). Since the radioligand experiences rapid metabolism during the first minutes, manual venous samples were preponed 15s in the analysis in order to account for different arrival times of the metabolites at different cannulation sites (a. radialis vs. v. cubitalis) (Ooue et al., 2007).

To obtain the arterial input function (AIF), automatic and manual whole-blood samples were combined and corrected for plasma to whole-blood ratio, radioactive metabolites and delay using only arterial samples. The delay (mean $\pm$ SD = 12.1 $\pm$ 4.9s) was determined by fitting the input function to the cerebellar white matter time activity curve for each subject and minimizing the sum of squared differences (Meyer, 1989; Ruotsalainen et al., 1997). The final plasma input function was interpolated with a sum of 3 exponentials from the peak onwards (Parsey et al., 2005).

Image-derived input functions (IDIFs)

Image-derived input functions were obtained from the original (i.e., not spatially normalized) PET scans in several automated steps. First, cerebral blood vessels were delineated using linear discriminant analysis (LDA) (Hahn et al., 2010b). LDA is a

supervised classification method which derives a discriminant function from a training set and assigns a (tissue) class to each voxel of the test set (i.e., the scans to be analyzed) (Hastie et al., 2009). Here, this training set was obtained from a tracer-specific template comprising an average of 36 healthy controls (Fink et al., 2009; Stein et al., 2008). Tissue classes (gray matter, white matter, cerebrospinal fluid) for the training set were defined from SPM5 probability atlases (Wellcome Trust Centre for Neuroimaging, London, UK; <http://www.fil.ion.ucl.ac.uk/spm/>). In addition, regions of interest (ROIs) for the carotid arteries were drawn manually within the PET template summed over the first minute. The individual PET scans were then scaled to the maximum intensity of the template, since classification was carried out using the voxel values rather than spatial location. Here, the maximum of the individual scans were scaled to the maximum of the template and all remaining voxels were adjusted proportionally. Classification into different tissue classes was carried out with the standard Matlab algorithm for linear discriminant analysis 'classify'. The input for LDA were voxel intensities for the training set (4 tissue groups: GM, WM, CSF, carotid arteries) and the test set (i.e., the scan to be analyzed). 'Classify' uses the training data to obtain a discriminant function, which in turn defines boundaries in multidimensional space of classification features. The features are given by the time points of the PET data. Since only the first minute of the PET scan was used, this approach may generally be considered as an adaptive thresholding technique. The blood vessel ROI resulting from LDA (Fig. S1) was then further refined. First, the peak activity and the corresponding frame when this maximum occurs were identified. The final blood vessel ROI was then restricted in order that each voxel exhibits 0.66 times the peak intensity within the identified time frame. This yields a blood pool with virtually homogenous kinetic characteristics.

To correct for spill-in from and spill-out to surrounding regions, the obtained vessel area was subjected to a ROI-based partial volume effects correction (PVC) as implemented in PMOD 3.3 (Rousset et al., 1998). The algorithm describes the spill-over between ROIs in a geometric transfer matrix (a system of linear equations) after convolution with the point spread function. The true activity for each ROI is then

estimated by matrix inversion. The point spread function was defined according to the resolution of the reconstructed image by a 3D Gaussian function (4.36mm FWHM). The region surrounding the blood pool was defined as the ROI within a 4-voxel radius around the vessels (approximately 2.8 times the FWHM of the PET camera (Izquierdo-Garcia et al., 2009)). For the final input function, the peak was taken from the partial volume corrected IDIF, whereas the tail was defined by manual venous blood samples from 10min onwards (IDIF+VIF). This is feasible since it may be difficult to obtain an accurate representation for both peak and tail from a single IDIF (Zanotti-Fregonara et al., 2011a) and venous samples showed only negligible differences to arterial ones after 10min (see results and figure 2). In 5 scans late venous samples (35 and 50min) were not available due to logistical problems (only one chemist available) and hence were replaced with arterial ones (see results and discussion). No scaling was applied to the IDIF (see discussion). Similar to the arterial input functions, IDIF+VIF were corrected for plasma to whole-blood ratio, radioactive metabolites and delay (mean $\pm$ SD = 6.5 $\pm$ 4.8s) but using only venous samples. Although in theory no delay correction is required for IDIF (Liptrot et al., 2004) this additional correction improved the model fits, most probably since IDIF were obtained from venous blood vessels. Again, the final input function was fitted with a sum of 3 exponentials.

Regions of interest and kinetic modeling

PET scans were spatially normalized to stereotactic space as defined by the Montreal Neurological Institute (MNI) with SPM8 (standard algorithm and parameters) using a tracer-specific template (Fink et al., 2009; Stein et al., 2008). For 5-HT_{1A} receptor quantification regions of interest were taken from an atlas (Stein et al., 2008): frontal, orbitofrontal, parietal, temporal, occipital and cingulate cortices, insula, amygdala-hippocampus complex, midbrain (including the dorsal raphe nucleus) as well as cerebellar gray (excluding vermis) and cerebellar white matter. These ROIs cover a great range of 5-HT_{1A} binding and include areas typically reported in psychiatric disorders (Drevets et al., 2007; Hahn et al., 2010a; Hirvonen et al., 2008; Lanzenberger et al., 2007; Parsey et al., 2010; Spindelegger et al., 2009). To improve model fits, the time activity curves of each ROI were resampled to 10x1min, 5x2min and 14x5min.

Quantification of the 5-HT_{1A} receptor binding potential (BP_p (Innis et al., 2007)) was carried out separately for AIF and IDIF+VIF with PMOD 3.3. A two-tissue compartment model was used, with K_1/k_2 fixed to that of cerebellar white matter (Hirvonen et al., 2007; Parsey et al., 2010). A cerebral blood volume component of 5% was assumed for all kinetic modeling procedures, using CBV for the whole-blood curve and 1-CBV for the tissue component (Gunn et al., 1998; Hirvonen et al., 2007; Parsey et al., 2010). In addition to ROI-based quantification, we computed voxel-wise binding potential maps to facilitate visualization of the results. A two-tissue compartment model with ridge regression was used to provide stable fits (Byrtek et al., 2005) with K_1/k_2 fixed to that of cerebellar white matter as obtained from the ROI analysis. Standard values of PMOD 3.3 (=1 = penalty of factor 10) were chosen for ridge factors, which provided more stable parametric modeling estimates than the standard two-tissue compartment model. Taking into account potential uncertainties associated with variations in the cerebral blood volume component (CBV) (Gunn et al., 1998; Slifstein et al., 2000), this was included as fit parameter in an additional voxel-wise analysis.

To evaluate the performance in relation to a reference region model, 5-HT_{1A} receptor binding potentials were compared between the two-tissue compartment model with $BP_{ND}=(V_T-V_{ND})/V_{ND}$ and the multilinear reference tissue model 2 (MRTM2 (Ichise et al., 2003)). For the latter one, k_2' was estimated from time activity curves of a receptor rich and poor region (insula and cerebellar white matter, respectively) (Hahn et al., 2010a; Ichise et al., 2003) using the simplified reference tissue model (Lammertsma and Hume, 1996).

Statistical analysis

Comparisons between manual arterial (A) and venous (V) blood samples and input function parameters were carried out with paired- or two-sample t-tests where appropriate. Also, we calculated the variability (reproducibility) as $(A-V)/\text{mean}(A,V)*100$ (Hirvonen et al., 2007) and computed Bland-Altman plots. Direct comparison of 5-HT_{1A} BP_p obtained from AIF and IDIF+VIF was evaluated by linear

regression analysis. Furthermore, to assess bias in 5-HT_{1A} binding obtained with the two different input functions, a repeated measures ANOVA was computed with method and ROI as fixed effects and subject as random effect (Ogden et al., 2010). All statistical tests were carried out two-tailed and calculated with MatlabR2010a or SPSS 18 (IBM, New York, USA).

RESULTS

For manual samples there was no significant difference in whole-blood activity between arterial and venous samples from 10min onwards, variability was below 10% and absolute difference was less than 0.5kBq/ml ($p>0.4$). Similarly, no significant difference was found for plasma to whole-blood ratio between arterial and venous samples with a variability of 1.9% ($p>0.4$, Fig. S2). For radioactive metabolites, the first three venous samples (2, 5, 10min) showed significantly higher parent fractions but with absolute differences below 6% ($p<0.05$, Fig. 1). Also, maximum variability (24% at 5min) was in line with reported reproducibility values (Hirvonen et al., 2007; Parsey et al., 2000). Venous parent fractions obtained at later time points (20, 35, 50min) were not significantly different from arterial samples (variability < 13%, $p>0.12$). Bland-Altman plots generally confirm these results between arterial and venous manual blood samples (Fig. S3).

Automatically extracted regions of interest for IDIFs comprised 29.4 ± 18.3 voxels (mean $\pm$ sd), of which only 0.9 ± 1.9 were located within the carotid arteries. Signal to noise ratio of uncorrected IDIF peaks to surrounding activity was 5.2 ± 0.8 . The magnitude of partial volume correction was consistent across subjects with a ratio of IDIF peaks after/before PVC of 1.75 ± 0.2 . Compared to image-derived input functions after PVC, the arterial input functions showed slightly but non-significantly lower peak values (mean difference $\Delta=-7.6\pm16.9$ kBq/ml, $p>0.1$). However, the AIF peaks emerged significantly later ($\Delta=4\pm6.4$ s, $p<0.05$) and exhibited a higher full-width at half-maximum ($\Delta=15.9\pm5.2$ s, $p<0.001$, Fig. 2).

Direct comparisons of 5-HT_{1A} binding obtained with AIF and IDIF+VIF are given in Figures 3, Figure 5 and Table 1. For ROI-analysis, linear regression showed strong correlations between the two methods at baseline ($R^2=0.95$), after treatment ($R^2=0.93$) and when pooling all scans ($R^2=0.93$). Similarly, intercepts were close to zero (-0.05 to 0.16) and slopes close to unity (0.97 to 1.07, Table 1). The differences between baseline and treatment regression parameters were not significant for slopes ($p=0.87$), intercepts ($p=0.73$) nor correlation coefficients ($p=0.28$). Including only those 10 subjects where late venous sampling (35min and 50min) was fully available did not change the results ($R^2=0.94$, intercept=0.03, slope=1.03, table 1). Furthermore, there was no significant bias in 5-HT_{1A} BP_p between AIF and IDIF+VIF as assessed by repeated measures ANOVA ($F_{1,43} = 2.4$, $p>0.1$). Although individual model fits of the time activity curves were similar (Fig. 4), AIF showed slightly but significantly lower sum of squared differences ($F_{1,21} = 10.8$, $p<0.01$). Bland-Altman plots show no absolute or proportional errors, but a slightly increased variation with increasing binding potentials between the two methods (Fig. S4). Similarly, voxel-wise analysis showed excellent agreement between the two methods, where differences appeared only in areas with lowest receptor binding (Fig. 5A and B). These differences almost vanished after inclusion of CBV as additional fit parameter (Fig. 5C and D).

The comparison of BP_{ND} values between the two-tissue compartment model and the MRTM2 was similar for AIF and IDIF+VIF. Although intercepts were close to zero (0.09 and 0.008), the agreement was weaker than within the two input function models when pooling all subjects ($R^2_{AIF}=0.823$ and $R^2_{IDIF+VIF}=0.818$). Slopes indicate a considerable underestimation by the reference tissue model (0.42 and 0.33), which is in line with previous results (Gunn et al., 1998). See table 1 and figure S5 for details.

DISCUSSION

This work demonstrates the applicability of image-derived input functions combined with venous blood samples to quantify serotonin-1A receptor binding using [*carbonyl*-¹¹C]WAY-100635. Moreover, we were able to resolve essential issues for the use of IDIFs. Direct comparison showed that arterial samples can be substituted by venous

ones for plasma to whole-blood ratios and parent fractions despite the fast metabolism of the radioligand. Hence, the combination of image-derived and venous input functions represents a promising quantification method which is entirely independent of arterial samples. Importantly, the approach yielded robust estimates of 5-HT_{1A} receptor binding at baseline and after 8 weeks of hormone treatment, which holds true for both ROI-based and voxel-wise quantification.

Although image-derived input functions have been evaluated for a variety of radioligands (see introduction), recent work demonstrated the limited applicability of IDIFs (Zanotti-Fregonara et al., 2011a). Potential problems include poor identification of cerebral blood vessels within the PET images, unreliable estimation of the input function shape, fast metabolism of the radioligand and differences between arterial and venous manual blood samples. Despite these issues, our results indicate that IDIFs combined with venous blood samples represent a promising approach for modeling of [*carbonyl*-¹¹C]WAY-100635. The validation of IDIFs in clinical research protocols is a major step towards reduced invasiveness, however, to date only a few studies have used IDIFs as a routine tool (Zanotti-Fregonara et al., 2011a). Here, we investigate the application of IDIFs for both baseline and treatment measurements yielding robust 5-HT_{1A} binding estimates independent of hormone therapy. This combination of image-derived and venous input functions may provide a method that can be widely used in clinical studies, thereby offering a less invasive procedure for the patient. Still, the extrapolation to other radioligands requires further investigation especially for tracers with different plasma kinetics.

Manual blood samples, metabolites and kinetic modeling

Direct comparison of manual blood samples showed excellent agreement between arterial and venous data. For whole-blood samples it seems that arterio-venous equilibrium is reached at 10min post injection for [*carbonyl*-¹¹C]WAY-100635, which is a prerequisite for the complete avoidance of arterial cannulation (Zanotti-Fregonara et al., 2011a) unless a consistent relationship can be obtained to scale venous samples. This lack of equilibrium prior to 10min may at least in part explain the higher parent

fraction observed in early venous samples (2, 5, 10min). Though, these differences are still within reported test-retest values of 26% (Hirvonen et al., 2007; Parsey et al., 2000).

Despite the aforementioned difference in the metabolite profile, linear regression analysis indicates a high degree of agreement of 5-HT_{1A} binding between IDIF+VIF and AIF based modelling ($R^2=0.93-0.95$). These values are comparable to previous studies using IDIFs for tracer kinetic modeling (Mourik et al., 2009; Sanabria-Bohorquez et al., 2003; Zanotti-Fregonara et al., 2011b). Notably, in the majority of these studies only the arterial whole blood time-activity curve was replaced by the image-derived input functions, but arterial blood samples were used for plasma to whole-blood ratios and metabolite correction. A residual variability of 5-7% between AIF and IDIF+VIF is well below previous test-retest values of 14% (Parsey et al., 2000) and 12% (Hirvonen et al., 2007) for 5-HT_{1A} binding.

Although the Logan plot (Logan et al., 1990) appears to be the preferred approach for assessing IDIFs (Mourik et al., 2009; Zanotti-Fregonara et al., 2011a; Zanotti-Fregonara et al., 2011b), we did not evaluate this method for two reasons. The fast metabolism of [*carbonyl*-¹¹C]WAY-100635 considerably decreases the area under the tail of the input function, which may complicate a robust estimation of 5-HT_{1A} binding due to potentially high variations in IDIF peaks (Zanotti-Fregonara et al., 2011a). Second, the two-tissue compartment model is considered as the model of choice for this radioligand when using input function modeling (Gunn et al., 1998; Hirvonen et al., 2007; Parsey et al., 2010). Individual rate constants may be difficult to estimate with IDIFs (Zanotti-Fregonara et al., 2011a), though, our results indicate that macroparameters such as binding potentials can be robustly identified with the combination of IDIFs and venous blood samples. This is also confirmed by the agreement between the two methods in 5-HT_{1A} binding obtained from both ROI-based and voxel-wise modeling. Moreover, the direct comparison between arterial and venous samples enables the application of venous blood for other non-invasive quantification techniques (Ogden et al., 2010).

Extraction of image-derived input functions

The radioligand [*carbonyl*- ^{11}C]WAY-100635 showed a high signal contrast of cerebral vessel activity as compared to the surrounding tissue, which is another requirement when extracting IDIFs using blood vessel ROIs directly delineated from PET data (Zanotti-Fregonara et al., 2011a). The high signal to noise ratio also makes the algorithm robust against scaling errors induced by noise. Alternatively, the algorithm could be further improved by scaling individual scans not simply to the maximum but e.g., to the average of several maximum intensity voxels. Several techniques have been introduced for the definition of blood vessel ROIs, each comprising certain advantages. This includes simple manual delineation (Chen et al., 1998) as well as automatic methods such as clustering (Liptrot et al., 2004), fixed volumes of high intensity voxels (Mourik et al., 2008) and independent component analysis (Chen et al., 2007). We used linear discriminant analysis for a first definition of the cerebral blood vessels (Hahn et al., 2010b) followed by two additional restrictions (percentage of maximum in certain time frame). More generally, the algorithm automatically determines individually adaptive thresholds in several steps. Although it might be more intuitive to extract image-derived input functions from arteries (Zanotti-Fregonara et al., 2011a), the vast majority of our final ROIs comprised venous blood vessels. However, this freedom to let the algorithm choose may yield a higher signal-to-noise ratio for several reasons. Venous vessels showed higher tracer concentrations and the typical field of view of PET brain scans covers more voxels within venous vessels than arterial ones. The latter also indicates that venous cerebral vessels are larger than the carotid arteries, which in turn causes less pronounced partial volume effects. This may explain the similarly high IDIF peaks as compared to AIF, although manual venous blood samples showed lower activities than arterial ones. Alternatively, the slightly lower AIF peaks may result from dispersion caused by the automated blood sampling system or uncertainties in partial volume correction algorithm. Therefore, a thorough evaluation of issues such as dispersion correction as well as the definition of the point spread function and ROI size should be subject to future studies. Furthermore, it is theoretically possible that (depending on the transport across the blood brain barrier) the tracer concentration is more similar between arterial and venous blood in cerebral

capillaries than at distal sites such as the hand. Nevertheless, it might be more important to focus on a homogenous blood pool for IDIF extraction rather than on its location (Naganawa et al., 2005; Turkheimer et al., 2007). Still, further evaluation is required for the extrapolation of the method to other radioligands and for the assessment of other modeling parameters such as individual rate constants.

Concerning peak recovery, recent work suggested that scaling of IDIFs with blood samples may provide better estimates than PVC solely based on image data (Zanotti-Fregonara et al., 2011a). However, the authors also acknowledged that IDIF methods are tracer specific and may not work equally well for other radioligands. Using an established scaling technique (Chen et al., 1998), we observed a high variability in IDIF peak values when compared to arterial input functions (data not shown), hence this approach was not further evaluated. Instead of scaling, partial volume corrected IDIFs were used for peaks only, whereas manual venous blood samples represented the tail of the input function. This is reasonable since a single IDIF may not give an accurate representation for both peak and tail of the input function (Zanotti-Fregonara et al., 2011a).

CONCLUSIONS

This study validates the use of image-derived input functions combined with venous blood data for the modeling of [*carbonyl*-¹¹C]WAY-100635. The direct comparison of manual arterial samples with venous ones enables the complete substitution of arterial cannulation, yielding a less-invasive procedure for the patient. Moreover, the evaluation of the method before and after treatment demonstrates its potential for routine use in clinical research protocols and encourages further investigation for other radioligands with different kinetics characteristics.

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CONFLICT OF INTEREST

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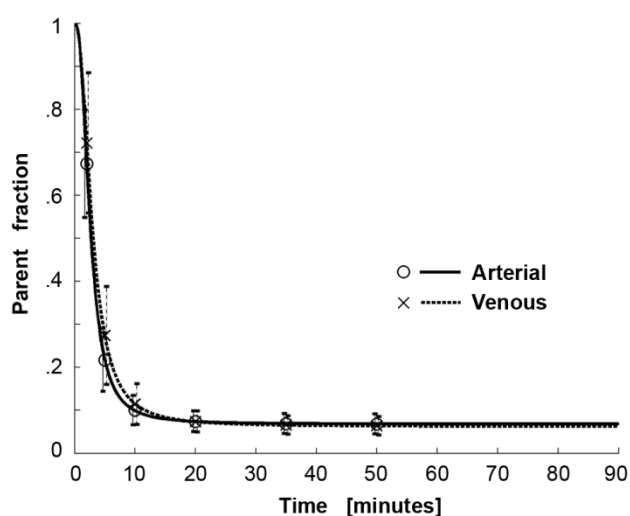
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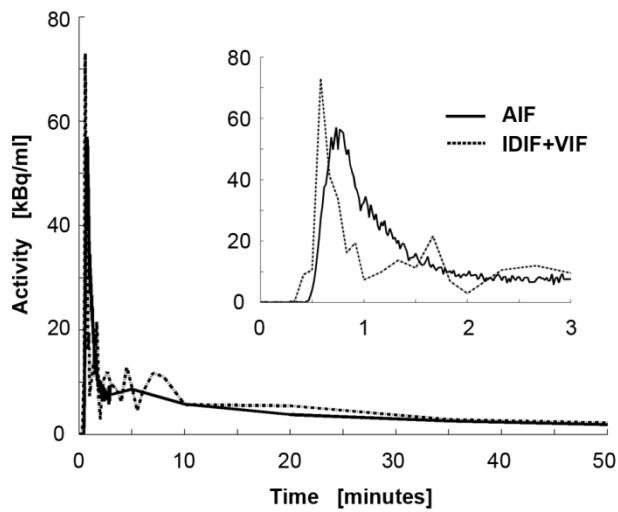
FIGURE LEGENDS

Figure 1



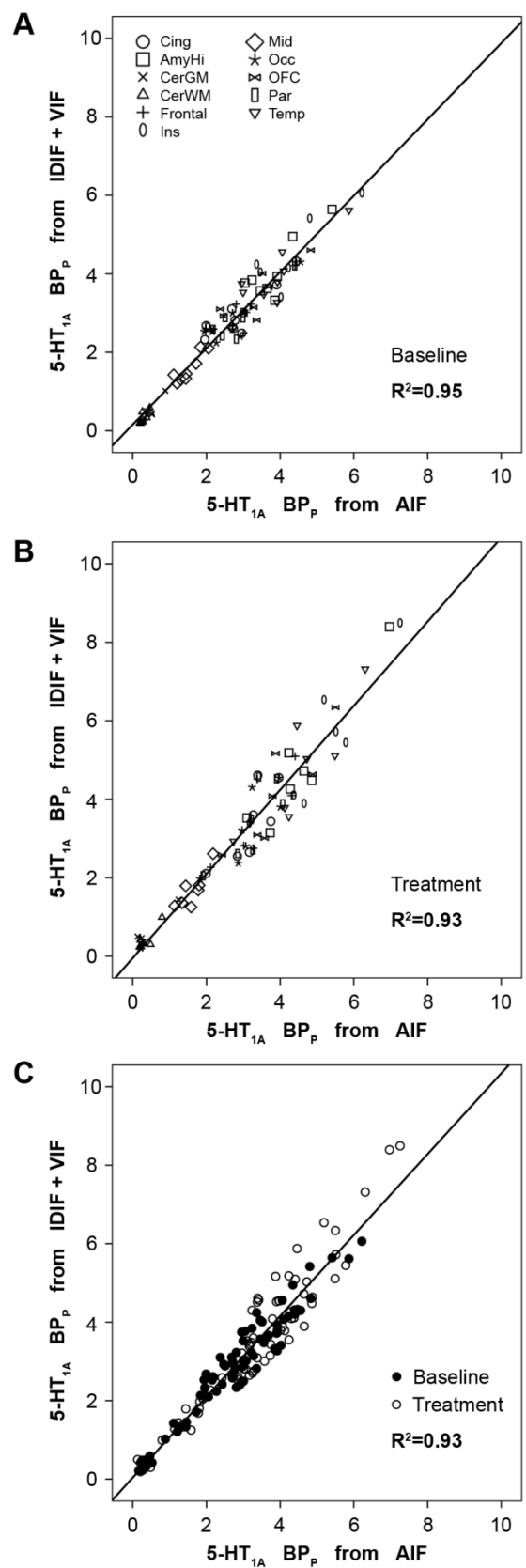
Parent fraction of [carbonyl-¹¹C]WAY-100635 (mean±SD of all 15 PET scans). The 6 arterial and 6 venous manual blood samples were taken at 2, 5, 10, 20, 35 and 50min post injection. Model fits were calculated with a Hill-type function (Gunn et al., 1998) for arterial (solid line, circles) and venous samples (dotted line, crosses) separately. Parent fraction was similar for both samples series with maximum variability of 24% at 5min. Bars denoting the standard deviation are shifted on abscissa to distinguish arterial and venous samples.

Figure 2



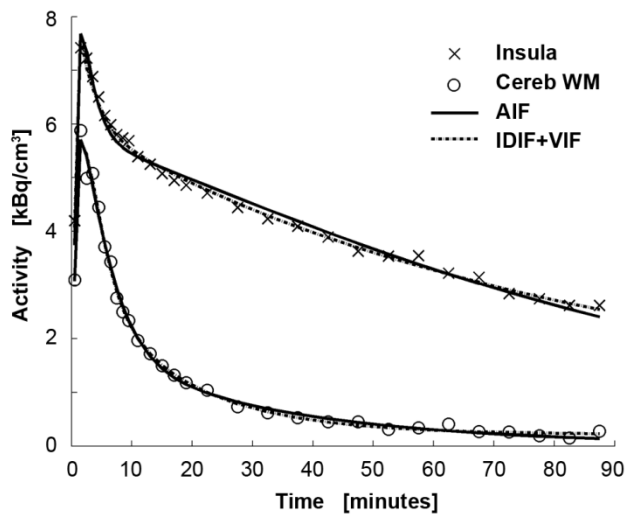
Whole-blood radioactivity concentrations of the two input functions used for kinetic modeling for a representative subject. Compared to the arterial input function (AIF, solid line), the image-derived input function (IDIF) shows good recovery of the initial peak after partial volume correction. The IDIF was combined with manual venous blood samples from 10min onwards (IDIF+VIF, dotted line), resulting in similar tails for the two input functions. Zoom shows the first 3 minutes of the scan.

Figure 3



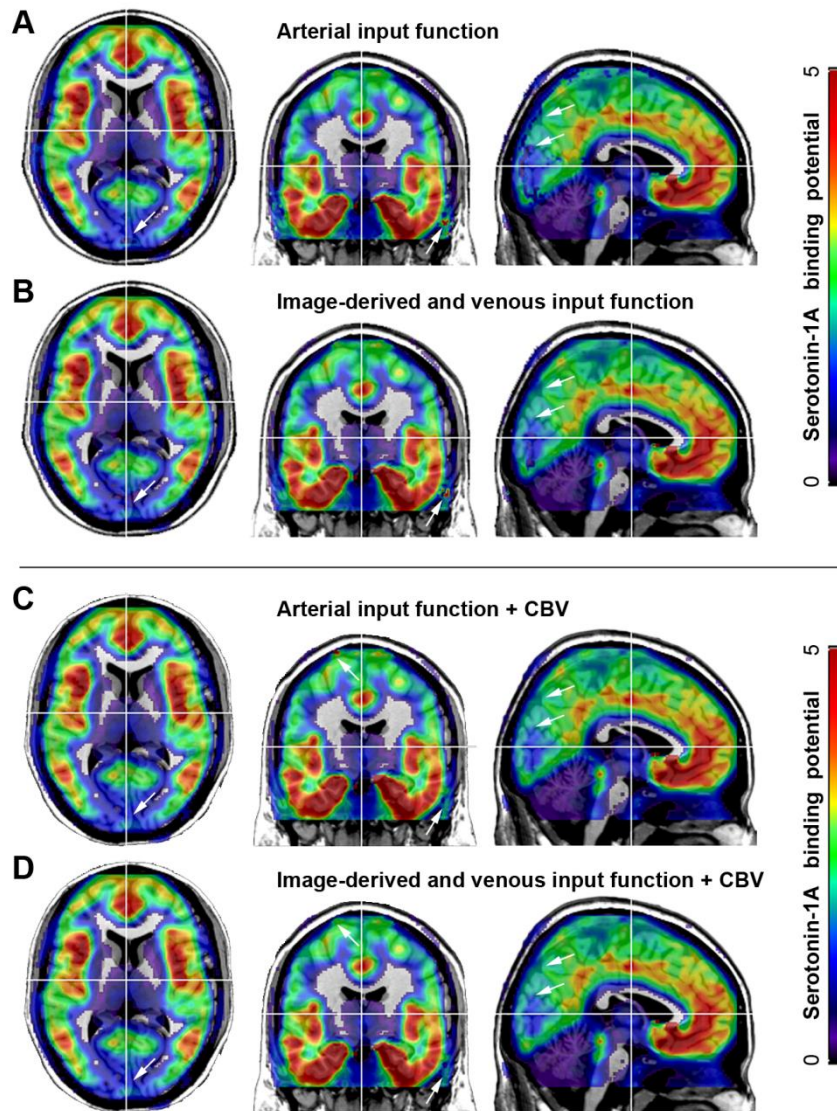
Direct comparison of serotonin-1A receptor (5-HT_{1A}) binding potential (BP_p). Receptor binding was obtained from arterial input functions (AIF) or the combination of image-derived and venous input functions (IDIF+VIF). Linear regression analysis shows strong agreement between these two independent approaches for PET measurements A) at baseline, B) after treatment and C) when pooling all scans (see table 1 for details). Symbols in A and B represent different regions of interest. Cing: cingulate cortex, AmyHip: amygdala-hippocampus complex, CerGM/CerWM: cerebellar gray/white matter, Ins: insula, Mid: midbrain, Frontal/OFC/Occ/Par/Temp: frontal, orbitofrontal, occipital parietal and temporal cortices.

Figure 4



Kinetic modeling of [carbonyl-¹¹C]WAY-100635. Time activity curves are shown for the insula (crosses) and cerebellar white matter (Cereb WM, circles) for a representative subject. Model fits calculated with a two-tissue compartment model resulted in similar fits when using the arterial input function (AIF, solid line) or the combination of the image-derived and venous input functions (IDIF+VIF, dotted line).

Figure 5



Voxel-wise quantification of [*carbonyl*-¹¹C]WAY-100635 for a representative subject. Maps were calculated with a two-tissue compartment model with ridge-regression fitting (Byrtek et al., 2005), with K_1/k_2 fixed to that of cerebellar white matter from ROI-based modeling. The model was applied with the cerebral blood volume (CBV) component fixed to 5% (A, B) and included as fit parameter (C, D). Serotonin-1A receptor binding potentials (BP_p) were computed with the arterial input function (AIF; A, C) and the combination of image-derived and venous input functions (IDIF+VIF; B, D) yielding virtually identical results. Of note, differences in binding potentials between the two methods were observed in some areas with lowest receptor binding (A, B;

medial occipital cortex, extra-cerebral areas as indicated by white arrows). These differences almost vanished after inclusion of CBV as additional fit parameter (**C, D**).

TABLES

Table 1

Condition	n	R^2	Slope	Intercept
5-HT _{1A} BP _P AIF vs IDIF+VIF				
Baseline	8	0.95	0.97	0.16
Treatment	7	0.93	1.07	-0.05
Pooled	15	0.93	1.03	0.04
Late Venous	10	0.94	1.03	0.03
5-HT _{1A} BP _{ND} AIF vs MRTM2				
Pooled	15	0.82	0.42	0.09
5-HT _{1A} BP _{ND} IDIF+VIF vs MRTM2				
Pooled	15	0.82	0.33	0.01

Linear regression analysis was carried out for the direct comparison of serotonin-1A (5-HT_{1A}) receptor binding (BP_P) as quantified by arterial input functions (AIF) or the combination of image-derived and venous input functions (IDIF+VIF). Results showed good agreement between the two methods for PET measurements at baseline, after treatment and when pooling all scans (see also figure 3). Similar results were obtained when including only those 10 subjects where late venous sampling (35min and 50min) was fully available. Comparing two-tissue compartment models (AIF and IDIF+VIF) with the multilinear reference tissue model 2 (MRTM2, (Ichise et al., 2003)) gives weaker agreement and indicates an underestimation of receptor binding (BP_{ND}) with the latter (Gunn et al., 1998) (see also figure S5).

14. Results and Discussion

14.1 GENERAL

The development of PET-radiotracers is a particular challenge since there are a lot of critical points which are important to be followed. If a new PET-tracer is planned to being introduced for clinical application, particular attention should be paid to its behaviour during the preclinical process.

- The tracer should display high affinity to the target region (e.g. receptor, transporter, etc.) of choice.
- A high selectivity to the target region is mandatory.
- The tracer should not have a tendency to non-specific binding.
- If the degradation of the tracer results in radio-metabolites, they should not interfere with the signal at the target tissue

The last point requires a high metabolic stability against enzymatic degradation. The evaluation of the metabolic stability of the selected tracers is a complex process of sequential evaluation steps. These steps include (1) the enzymatic *in-vitro* stability of the tracer against single enzymes regarding to phase I metabolism, (2) the enzymatic *in-vitro* stability against a group of phase I catalytic enzymes such as the superfamily cytochrom P-450, and (3) the enzymatic *in-vitro* stability against the metabolizing enzymes in pooled plasma.

Further *in-vitro* tests with the focus on BBB penetration via passive diffusion have to be carried out. After the assessment of the lipophilicity, which can be evaluated using various log P methods, models which mimic the BBB such as immobilized artificial membrane (IAM) HPLC are nowadays methods of choice.

The use of biological tissues such as fractioned enzymes or body fluids of both animal and human origin provides the advantage of additional information of possible species differences. This knowledge, then, limits the future choice of suitable animal species regarding the determination of metabolic stability.

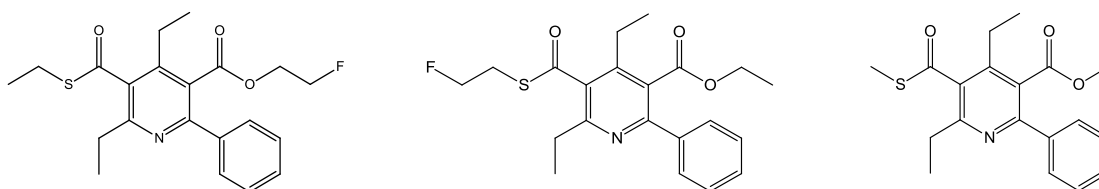
In-vivo experiments are the consecutive step to verify the results from *in-vitro* testing. Animal experiments have to be performed before a possible application in men.

Additionally, comparison of *in-vitro* data (animal and human) with *in-vivo* data of animal experiments can provide valuable information for the following human studies.

14.2 STABILITY AGAINST THE SINGLE ENZYME CARBOXYLESTERASE

Porcine liver esterase (PLE) was the enzyme of choice to conduct our enzymatic studies. The use of PLE as common alternative enzyme to human carboxylesterase (hCE) is appropriate since it is inexpensive, commercially available and became a biochemical model for enzymatic ester hydrolysis [Jones, 1993] but also for organic syntheses [Bornscheuer, 2005]. PLE has been widely used as a model for *in-vitro* studies of drug metabolism in recent years [Kawaguchi, 1985a,b]. The PLE has a reasonable sequence homology (about 75%) to hCE which crystal structure is known [Hasenpusch, 2011].

FE@SUPPY, FE@SUPPY:2 and (Me)²@SUPPY



Structure 1: FE@SUPPY, FE@SUPPY:2 and (Me)²@SUPPY

FE@SUPPY (2-Fluoroethyl 2,4-diethyl-3-ethylsulfanylcarbonyl-6-phenylpyridine-5-carboxylate) and its derivatives FE@SUPPY:2 (5-ethyl-2,4-diethyl-3-((2-[¹⁸F]fluoroethyl)sulfanylcarbonyl)-6-phenylpyridine-5-carboxylate) and (Me)²@SUPPY (methyl 2,4-diethyl-3-methylsulfanylcarbonyl-6-phenylpyridine-5-carboxylate) are antagonists for the adenosin A₃ receptor (A₃R) and belong to the class of dialkylpyridines. These substances comprise two ester functions within their molecular structure and are therefore predestined to be metabolically tested using carboxylesterases (CES).

The Michaelis-Menten constants (K_m) and limiting velocities (V_{max}) which show their stability against CES were as follows:

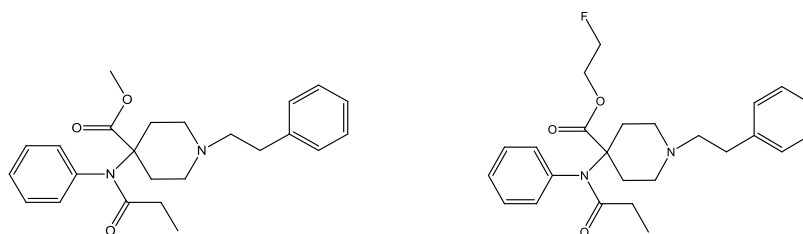
FE@SUPPY	K_m 20.15 μ M; V_{max} 0.035 μ M/min
FE@SUPPY:2	K_m 13.11 μ M; V_{max} 0.015 μ M/min
(Me) ² @SUPPY	K_m 24.63 μ M; V_{max} 0.021 μ M/min

These findings show that the metabolic stability is comparable in all three cases. Additionally they led us to further preclinical experiments and animal studies regarding FE@SUPPY and FE@SUPPY:2 [Mitterhauser, 2009, Nics, 2009, 2011].

Referring to paper 1, the stability of the radioactive labelled derivative [¹⁸F]FE@SUPPY:2 did not increase in rat studies as would have been expected. In both *in-vitro* and *in-vivo* studies, [¹⁸F]FE@SUPPY was more stable against enzymatic degradation. 50% of [¹⁸F]FE@SUPPY was intact after 15 min p.i. and 30% after 30 min, but only 30% of [¹⁸F]FE@SUPPY:2 after 15 min and 15% after 30 min. Regarding rat brain experiments, we observed an early metabolite for [¹⁸F]FE@SUPPY:2 but none for [¹⁸F]FE@SUPPY before 30 min p.i.

The observation of the same degradation pathway in all cases (cleavage only at the carboxylester moiety) could be explained by the structural similarity of the presented tracers.

CFN and FE@CFN



Structure 2: CFN and FE@CFN

Carfentanyl (CFN) (2-fluoroethyl 1-phenylethyl-4-(N-propanoylanilino)piperidine-4-carboxylate) and fluoroethylcarfentanyl (FE@CFN) (ethyl 8-fluoro-5-methyl-6-oxo-5,6-

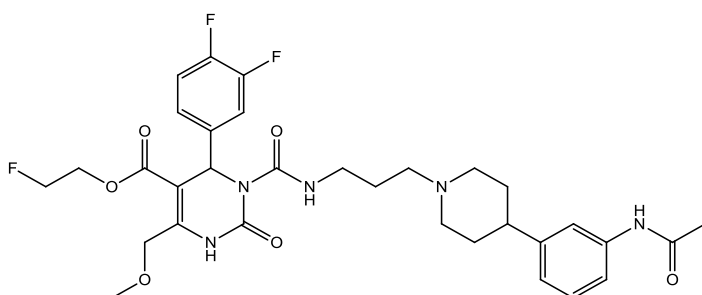
dihydro-4H-benzo-[f]imidazo[1,5-a]-[1,4]diazepine-3-carboxylate) which are agonists for the μ -opioid receptor showed high stability against enzymatic degradation.

CFN: K_m 2503 μ M; V_{max} 1.47 μ M/min

FE@CFN: K_m 2439 μ M; V_{max} 4.13 μ M/min

These findings support - from a metabolic point of view - the promise of a high stability in further preclinical *in-vitro* and *in-vivo* applications such as cytochrome P-450, plasma stability and animal studies.

FE@SNAP



Structure 3: FE@SNAP

FE@SNAP (3-fluoroethyl 3-((3-(4-(3-(acetamidophenyl)piperidin-1-yl)propyl)carbamoyl)-4-(3,4-difluorophenyl)-6-(methoxymethyl)-2-oxo-1,2,3,4-tetrahydro pyrimidine-5-carboxylate) which is an antagonist for the melanin concentrating hormone receptor 1 (MCHR1) showed a relatively high stability against CES catalyzed degradation.

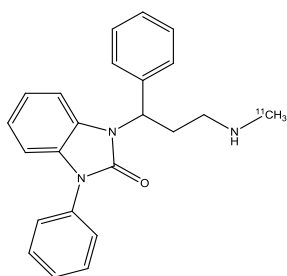
K_m 347.3 μ M; V_{max} 0.874 μ M/min

These findings substantiate, that from a metabolic point of view further metabolic experiments regarding stability testing against cytochrome P-450 but also plasma metabolite trials can be proceeded.

14.3 STABILITY AGAINST CYTOCHROME P-450 AND PLASMA

A pooled protein solution of rat and human origin containing the cytochrome P-450 superfamily and the regenerating substrates were used to evaluate the stability of selected tracers. Plasma metabolic experiments were conducted using pooled heparinized plasma of rat and human origin. Both sets of experiments took place using the radiolabelled analogues [^{11}C]Me@APPI and [^{18}F]FE@SNAP.

[^{11}C]Me@APPI



Structure 4: [^{18}F]FE@APPI

[^{11}C]Me@APPI (1-(3-([^{11}C]methylamino)-1-phenylpropyl)-3-phenyl-1H-benzodimidazol-2(3H)-one) showed no significant metabolism in human liver microsome experiments so far. After 1 hour, 99.5% of the tracer was found to be still intact. Recent studies with CES and plasma from human and rat origins showed also no metabolism after 1 hour of incubation. These findings led us to hypothesize that from a metabolic point of view Me@APPI could be a very promising PET-tracer for the norepinephrine transporter.

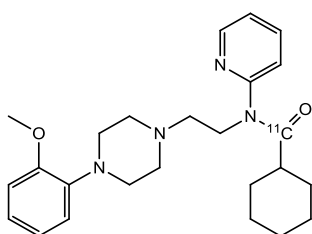
[^{18}F]FE@SNAP

[^{18}F]FE@SNAP (3-[^{18}F]fluoroethyl-3-((3-(4-(3-acetamidophenyl)piperidin-1-yl)propyl)carbamoyl)-4-(3,4-difluorophenyl)-6-(methoxymethyl)-2-oxo-1,2,3,4-tetrahydro pyrimidine-5-carboxylate) showed an enzymatic degradation of 5.4% using human microsomes and 2.6% using rat microsomes after an incubation time of 1 hour in liver microsomes experiments. The degradation of [^{18}F]FE@SNAP in human plasma showed

3.9% after 1 hour. In rat plasma experiments, we observed 32% metabolites in the initial phase of incubation and 100% metabolites after 1 hour.

These findings showed a high stability of the tracer using human and rat liver microsomes and human plasma but very poor stability in rat plasma. We assume, from a metabolic point of view, that rat plasma contains degradation enzymes which make it impossible to choose rats as animal species to predict human behaviour in that context.

14.4 *IN-VIVO* METABOLITE MEASUREMENTS IN A CLINICAL STUDY



Structure 5: [carbonyl- ^{11}C]WAY-100635

The 5-HT_{1A} receptor antagonist [carbonyl- ^{11}C]WAY-100635 (N-(2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)cyclohexane[^{11}C]carbox-amide) was the tracer of choice in a human study. Its radioactive metabolites [carbonyl- ^{11}C]desmethyl-WAY-100635 (N-(2-(4-(2-hydroxyphenyl)piperazin-1-yl)ethyl)-N-(pyridin-2-yl)cyclohexane[^{11}C]carbox-amide) and [^{11}C]cyclohexyl-carboxylic acid should be quantified using radio-HPLC analysis. Our demand to this study was to shorten the complete set-up of the method to be able to analyse twice as many samples in the same time-period. The reason for this research question was a parallel measurement of arterial and venous blood samples to figure out any statistical differences between both approaches.

The main improvement of the set-up was the implementation of a new HPLC-column with an adopted solvent. To analyse late blood samples with a focus to a high counting statistic we had to use a fraction collector connected to an offline gamma-detector.

The successful implementation of this new *in-vivo* HPLC-method using [carbonyl-¹¹C]-WAY-100635 enabled the conduction of an interesting clinical trial [Hahn, 2013] .

14.5 BLOOD-BRAIN BARRIER PENETRATION

It is a prerequisite for a newly developed PET-tracer for the brain to be able to pass the blood-brain barrier (BBB) effectively. Passive diffusion is the main pathway to penetrate the BBB. In addition to the common lipophilicity parameters such as log P, a new HPLC method using a modified column with immobilized artificial membranes (IAM) as stationary phase showed more effectiveness to predict a statement for a passive diffusion into the brain.

Comparing the log P HPLC method with the IAM HPLC method, log P shows a weak prediction of brain penetration. The described association between log P and peak % injected dose (ID) in brain had an r^2 value of 0.47 [Tavares, 2012]. This value is low since log P provides a measure of hydrophobicity and polarity interactions (i.e. lipophilicity), whereas it does not reflect ionic bonding that is also involved in compound-membrane interactions [Ong, 1996, Yang, 1996, Giaginis, 2008a, 2008b].

Me@APPI, FE@SNAP

IAM chromatography experiments prooved membrane partition coefficient (K_m) and permeability (P_m) values:

Me@APPI $K_m = 539.55$; $P_m = 1.51$

FE@SNAP $K_m = 327.38$; $P_m = 0.51$

BBB-penetration experiments showed that Me@APPI (1-(3-(methylamino)-1-phenylpropyl)-3-phenyl-1H-benzo[d]imid-azol-2(3H)-one) and FE@SNAP showed values which are in a permeability (P_m)-range of β -CIT (0.22), PIB (1.00), DASB (1.20) and ADAM (1.41) [Tavares, 2012]. These tracers are known to be able to penetrate BBB sufficiently for *in-vivo* imaging. These findings are a strong indication for the possibility of Me@APPI and FE@SNAP to pass the BBB via passive diffusion.

15. Conclusion and outlook

In this thesis, various preclinical *in-vitro* methods regarding the determination of the metabolic stability against catalytic enzymes of newly developed PET-tracers were evaluated. In addition, an improved method for the determination of tracers to pass the BBB was carried out. Besides, *in-vivo* methods concerning a PET-tracer which is already in clinical application were presented.

Step-by-step applications of the different presented metabolic methods lead to reliable statements, whether a tracer is stable enough for clinical application. The knowledge of the stability of a tracer and information of a possible BBB penetration represents a significant part in the complex drug development process of a PET probe.

The following list summarizes the experiments that were conducted during this PhD-thesis (in chronological order):

- stability studies using single enzymes such as carboxylesterase which has a very broad and overlapping catalytic activity as phase I enzyme and which is distributed in various tissues of mammals;
- stability studies using a group of catalyzing enzymes such as the cytochrome P-450 family which is mainly available in the liver tissue and responsible for metabolic reactions of approximately 75% of all phase I enzymes;
- stability studies using pooled plasma which has a high concentration of catalytic (e.g. liver) enzymes which are permanently released into blood; and
- immobilized artificial membrane HPLC to receive detailed information about BBB penetration via passive diffusion.

The use of tissue or body fluids of human origin should be implemented in the preclinical process to get “human” *in-vitro* and *in-vivo* information as early as possible. Comparison with material of animal origin can lead to the choice of suitable animal species.

The implementation of HPLC-MS would be important in the future. It is of utmost importance to determine structures of discovered metabolites and receive information

about possible toxic metabolites or those which increase or decrease enzyme capacities (drug-interaction).

Metabolite studies should serve as preclinical methods in any evaluation of newly developed tracers, but its limitations should always be taken into consideration.

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Curriculum Vitae

Name

Mag.^{rer.nat.} Lukas Nics



Current Position

Research fellow at the Department of Nuclear Medicine at the Medical University of Vienna

Education

1976-1980	Primary school in Vienna and Kaltenleutgeben
1980-1984	AHS lower cycle
1984-1985	Polytechnical school
1985-1989	Apprenticeship as watchmaker and graduation
1989	Military service in Eisenstadt and Villach (military position: non-commissioned officer; rank sergeant)
1989-1996	Employee as watchmaker
1993	Master craftsman's examination for watchmaker
1996-2002	Self-employed watchmaker
2002	General qualification for university entrance
2002-2008	Education in Nutritional sciences, University of Vienna, Faculty of Life Sciences,
2008	Master's Degree in Nutritional sciences; Title of master thesis: "Veränderungen des oxidativen Stresses und nutritiver Antioxidantien durch einen Iron-Man Triathlon" (Supervisor: Prof. Mag. Dr. Karl-Heinz Wagner)

2008-now Doctoral studies at the Department of Nutritional sciences, University of Vienna, Faculty of Life Sciences, Title of the doctoral thesis: “Metabolite studies *in-vitro* and *in-vivo*: From preclinical evaluation to clinical trials ” (Supervisor: Prof. Mag. Dr. Karl-Heinz Wagner)

Languages

German (mother tongue), English

Scientific and academical experiences

2005-2008	Tutor at the Department of Nutritional sciences, University of Vienna, Faculty of Life Sciences “Übungen zur Ernährungslehre”
2008-now	Teaching assistant at the Department of Nutritional sciences, University of Vienna, Faculty of Life Sciences “Ernährung des Menschen I”
2008-2012	Employed at the Medical University of Vienna, Department of Nuclear Medicine, Radiochemistry and biomarker development unit
2011-now	Lecturer at the Department of Nutritional sciences, University of Vienna, Faculty of Life Sciences “Chromatographie in der qualitativen und quantitativen Bioanalytik - Schwerpunkt HPLC”
2013-now	Employed at the Medical University of Vienna, Department of Psychiatry and Psychotherapy
since 2013	Reviewer at the Journal of Nutrition Research (IP 1.974)

Diploma Students

finished	Hoda El-Samahi, Sabine Hartmann, Chrysoula Vraka
ongoing	Matthias Hendl, Zarko Solajic

List of publications (peer reviewed research articles)

1. König D., Neubauer O., Nics L., Kern N., Berg A., Bisse E., Wagner KH. (2007): Biomarkers of exercise-induced myocardial stress in relation to inflammatory and oxidative stress. *Exerc Immunol Rev.*, 13: 15-36.
2. Neubauer O., König D., Kern N., Nics L., Wagner KH. (2008): No indications of persistent oxidative stress in response to an ironman triathlon. *Med Sci Sports Exerc.*, Dec, 40(12): 2119-28.
3. Mitterhauser M., Haeusler D., Mien LK., Ungersboeck J., Nics L., Lanzenberger R., Sindelar K., Viernstein H., Dudczak R., Kletter K., Spreitzer H., Wadsak W. (2009): Automatisierung und erste Evaluation von [¹⁸F]FE@SUPPY:2, ein alternativer PET-Tracer für den Adenosin A3 Rezeptor: Ein Vergleich mit [¹⁸F]FE@SUPPY. *The Open Nuclear Medicine Journal.*, 1: 15-23.

4. Haeusler D., Mien LK., Nics L., Ungersboeck J., Phillipe C., Lanzenberger R., Kletter K., Dudczak R., Mitterhauser M., Wadsak W. (2009): Simple and rapid preparation of [^{11}C]DASB with high quality and reliability for routine applications. *Appl Radiat Isot.*, 67(9): 1654-60.
5. Haeusler D., Mitterhauser M., Mien L.K., Shanab K., Lanzenberger R., Schirmer E., Ungersboeck J., Nics L., Spreitzer H., Viernstein H., Dudczak R., Kletter K., Wadsak W. (2009): Radiosynthesis of a novel potential adenosine A3 receptor ligand, 5-ethyl 2,4-diethyl-3-((2-[^{18}F]fluoroethyl)sulfanylcarbonyl)-6-phenylpyridine-5-carboxylate ([^{18}F]FE@SUPPY:2). *Radiochim Acta.*, 97: 753-58.
6. Wagner KH., Reichhold S., Holz C., Knasmüller S., Nics L., Meisel M., Neubauer O. (2010): Well-trained, healthy triathletes experience no adverse health risks regarding oxidative stress and DNA damage by participating in an ultra-endurance event. *Toxicology.*, 278(2): 211-6.
7. Haeusler D., Nics L., Ungersboeck J., Mien LK., Lanzenberger R., Shanab K., Sindelar KM., Viernstein H., Wagner KH., Dudczak R., Kletter K., Wadsak W., Mitterhauser M. (2010): [^{18}F]FE@SUPPY and [^{18}F]FE@SUPPY:2 - metabolic considerations. *Nucl Med Biol.*, 37(4): 421-6. (the authors Haeusler and Nics contributed equally).
8. Nics L., Haeusler D., Wadsak W., Wagner KH., Dudczak R., Kletter K., Mitterhauser M. (2010): The stability of methyl-, ethyl- and fluoroethylesters against carboxylesterases in vitro: there is no difference. *Nucl Med Biol.*, 38(1): 13-7.
9. Neubauer O., Reichhold S., Nics L., Hoelzl C., Valentini J., Stadlmayr B., Knasmüller S., Wagner KH. (2010): Antioxidant responses to an acute ultra-endurance exercise: impact on DNA stability and indications for an increased need for nutritive antioxidants in the early recovery phase. *Br J Nutr.*, 104(8): 1129-38.
10. Hahn A., Wadsak W., Windischberger C., Baldinger P., Höflich A., Losak J., Nics L., Philippe C., Kranz G., Kraus C., Mitterhauser M., Karanikas G., Kasper S., Lanzenberger R. (2012): Differential modulation of the default mode network via serotonin-1A receptors. *Proc Natl Acad Sci U S A.*, 109(7): 2619-24.
11. Hahn A., Nics L., Baldinger P., Ungersboeck J., Dolliner P., Frey R., Birkfellner W., Mitterhauser M., Wadsak W., Karanikas G., Kasper S., Lanzenberger R. (2012): Combining image-derived and venous input functions enables quantification of serotonin-1A receptors with [carbonyl- ^{11}C]WAY-100635 independent of arterial sampling. *Neuroimage.*, 62(1): 199–206 (the authors Hahn and Nics contributed equally).
12. Philippe C., Ungersboeck J., Schirmer E., Zdravkovic M., Nics L., Zeilinger M., Shanab K., Lanzenberger R., Karanikas G., Spreitzer H., Viernstein H., Mitterhauser M., Wadsak W. (2012): [^{18}F]FE@SNAP - a new PET tracer for the melanin concentrating hormone receptor 1 (MCHR1): microfluidic and vessel-based approaches. *Bioorg Med Chem.*, 20(19):5936-40.

13. Nics L., Hahn A., Zeilinger M., Vraka C., Ungersboeck J., Haeusler D., Hartmann S., Wagner KH., Dudczak R., Lanzenberger R., Wadsak W., Mitterhauser M. (2012): Quantification of the radio-metabolites of the serotonin-1A receptor radioligand [carbonyl- ^{11}C]WAY-100635 in human plasma: An HPLC-assay which enables measurement of two patients in parallel. *Appl Radiat Isot.*, 70(12):2730-6.
14. Mark C., Bornatowicz B., Mitterhauser M., Hendl M., Nics L., Haeusler D., Lanzenberger R., Berger ML., Spreitzer H., Wadsak W. (2013): Development and automation of a novel NET-PET tracer: [^{11}C]Me@APPI. *Nucl Med Biol.*, 40(2):295-303.
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16. Ungersboeck J., Mark C., Haeusler D., Nics L., Philippe C., Mitterhauser M., Willeit M., Lanzenberger R., Karanikas G., Wadsak W. (2012): Reliable set-up for in-loop ^{11}C -carboxylations using Grignard reactions for the preparation of [carbonyl- ^{11}C]WAY-100635 and [^{11}C]-(+)-PHNO. *Appl Radiat Isot.*, (submitted)
17. Philippe C., Nics L., Zeilinger M., Schirmer E., Spreitzer H., Karanikas G., Lanzenberger R., Viernstein H., Wadsak W., Mitterhauser M. (2013): Preparation and first preclinical evaluation of [^{18}F]FE@SNAP: a new PET tracer for the melanin concentrating hormone receptor 1 (MCHR1). *Bioorg Med Chem.*, (submitted)

Invited lectures

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| March 2011 | Winterseminar der Biologischen Psychiatrie Oberlech, Austria Title: Metabolitenanalysen als Grundlage für quantitative PET |
| March 2012 | Imaging Biomarker für das dopaminerge System – Biochemische Aspekte |
| March 2013 | Winterseminar der Biologischen Psychiatrie Oberlech, Austria Title: [^{11}C]Harmin oder Wo die Limitationen einer Metabolitenanalyse liegen |

Congress presentations

oral

1. Nics L., Hartmann S., Haeusler D., Wadsak W., Wagner KH., Kletter K., Dudczak R., Mitterhauser M. (2009): Stabilität der Adenosin A3 Rezeptor Antagonisten [^{18}F]FE@SUPPY / [^{18}F]FE@SUPPY:2 gegenüber humanen Lebermikrosomen. 18.

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