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Cytochrome P450 and radiopharmaceutical metabolism

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Positron emission tomography (PET) is a powerful non-invasive probe to investigate human physiology. A large number of radiotracers have been studied as imaging agents, but only a few have found clinical applications in pharmacology. A potential radiopharmaceutical is designed with very specific physiochemical characteristics, but, generally, less attention is paid to its adsorption, distribution, metabolism, and excretion properties, especially metabolism. Understanding the metabolic fate of radiopharmaceutical probes is essential for an accurate analysis and interpretation of PET measurements. The inherent inability of PET to differentiate between a parent compound and its metabolites confounds the interpretation of images and may impact the identification of the pathologically induced biochemical changes under investigation. Cytochrome P450 plays a major role in mammalian xenobiotic biotransformation and many *in vitro* methods are available to study and predict drug metabolism. The purpose of this review is to highlight the existing *in vitro* techniques available to investigate the biotransformation of xenobiotics in a fashion analogous to small molecule drug discovery. The aim is to facilitate the development and validation phases of PET tracers during preclinical evaluation. Emphasis is placed also on describing how cross species comparisons are essential in establishing appropriate translational pharmacology. Procedures of analysis (tandem liquid chromatography-mass spectrometry), typically used for studying the metabolism of drugs, are proposed as quick and accurate tools for the determination of a radiopharmaceutical's metabolic stability at the tracer level.

KEY WORDS: Radiopharmaceuticals - Tomography, emission computed - Cytochrome P-450 enzyme system - Metabolism - Biotransformation - Metabolic detoxification, drug - Species specificity.

Positron emission tomography (PET) is a rapidly evolving imaging tool that provides insight into physiological, biochemical and pharmacological functions at the molecular level. This technique has been used for over three decades in research and has had a clinical role for more than 15 years. Major clinical applications of PET to date have been in the areas of cardiology and neurology, but over 90% of its workload is in tumor diagnosis and targeting in oncology.[1]

PET studies involve the administration of compounds labeled with positron-emitting isotopes that have been formulated for intravenous injection. It is generally believed that successful drugs can be excellent PET radiopharmaceuticals if susceptible to labeling with PET isotopes, but unfortunately this does

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not always hold true for several reasons. Fluoxetine (*i.e.* Prozac) is an example of a successful, heavily prescribed antidepressant failing to be effectively used as a PET tracer for imaging the serotonin reuptake sites due to its non-specific binding in humans.[2] Fluoxetine's non specific binding, reflected in a high volume of distribution of the drug, and its extensive metabolic conversion to an active metabolite (norfluoxetine) with pharmacological activity similar to that of the parent, renders the drug less than ideal for PET studies.[2, 3]

In the last 30 years, several PET radiotracers have been developed for imaging cancerous lesions, brain receptors, transporters and enzyme systems, but currently only a few of them are used as imaging agents in clinical practice. The process involved in developing new PET radiopharmaceuticals begins with compound selection, through *in vitro* and *in vivo* efficacy evaluation, leading ultimately to clinical investigation, not unlike drug discovery.

Pharmacokinetics comprises the study of adsorption, distribution, metabolism and excretion (ADME) of a xenobiotic. Although the potential new radiotracer has the desired *in vitro* characteristics (*i.e.* high affinity, selectivity, appropriate pharmacological properties) to investigate the desired target, it could fail upon *in vivo* administration due to non specific affinity (as seen in the case of fluoxetine), low metabolic stability and unfavorable pharmacokinetics. These are all factors contributing to the attrition in the PET radiopharmaceutical development process.

The major issue with PET is the fact that *in vivo* measurements only reflect the amount of radioactivity in tissue, but do not provide any information about the chemical disposition of the pharmacophore itself. PET scans thus reflect only the sum of the parent radiotracer and its radiolabeled metabolic products formed during biotransfomation reactions. In the preclinical evaluation of a new PET radiotracer, it is important to take into account that the metabolic products of the radiotracer may show substantially different activity and distribution patterns in the body compared to the parent compound. Knowledge of these biotransformations is essential for the full understanding of the radioactivity distribution throughout the body. As an example, significant inter-tissue differences in the further metabolism of 2-fluoro-2-deoxyglucose (FDG) beyond FDG-6-phosphate have been demonstrated *ex vivo* in different rat organs. Moreover, a relative tissue-dependent accumulation of each metabolite reflecting the physiological characteristics of each organ has been demonstrated.[4] Therefore, by knowing the biochemical nature and occurrence of diverse metabolic products, it is possible to gain deeper insight into the physiological and pathological processes by means of PET.

To calculate physiological parameters such as receptor density or metabolic rates, it is necessary to know the concentration of the tracer and its metabolites in the blood and tissues over time. For this purpose, mathematical models have been developed to describe the relationship between the measured data and the physiological parameters affecting tracer uptake and biotransformation.[5] Moreover, taking into account that tracer metabolism markedly affects the arterial input function, a parameter used for quantitative analyses of PET images, metabolite analysis in the plasma becomes an essential prerequisite for the correct evaluation and interpretation of PET imaging as well as for the identification of pathological changes in metabolic pathways.[6] This review focuses on key concepts and techniques used to understand the role of cytochrome P450 in metabolism of xenobiotics, PET tracers or new chemical entities (NCEs) alike.

Metabolism (biotransformation)

Metabolism, or biotransformation, is the enzyme-catalyzed conversion of drugs and other foreign compounds (xenobiotics) to their metabolites. Most of these biotransformations take place in the liver, but there are metabolizing enzymes in many other tissues, including gut, kidney, brain, lung and skin. The primary biologic purpose of biotransformation is to inactivate and detoxify xenobiotics that may cause harm to the body. The metabolites are usually rendered more polar and less lipid soluble than the parent molecule *via* oxidation, dealkylation and conjugation, thereby aiding their excretion. However, there is no *a priori* relationship between biotransformation and pharmacological activity; some drug metabolites are active while others are inactive.

Drug biotransformation is achieved by a series of reactions classified as phase I and phase II which often, although not invariably, occur sequentially. While phase I metabolites may have pharmacological activity, most phase II metabolites rarely do.

Phase I metabolic reactions usually convert the parent drug to a more polar metabolite by introducing or

unmasking a functional group (-OH, -NH_2, -SH, -COOH) and are mediated by enzymes such as cytochrome P450, flavin-containing monooxygenase (FMO), esterases and amidases. These reactions are catabolic (*e.g.* oxidation, reduction or hydrolysis) and often the products can be more chemically reactive and paradoxically more toxic, carcinogenic, or immunogenic than the parent drug.

Phase II reactions (non-cytochrome P450 mediated and herein briefly mentioned) are anabolic and involve conjugation with an endogenous substrate such as acetate, glucoronate, glutathione, sulfate, or an amino acid and usually form inactive products. Just as with phase I reactions, phase II conjugation is intended for, but does not always result in, more rapid elimination and detoxification. Enzymes of the cytochrome P450 (CYP) superfamily represent the major catalysts for most phase I biotransformations of xenobiotics and endobiotics (steroids, fatty acids, hormones, etc.) with a wide range of chemical structures. CYP enzymes are bound to membranes of the endoplasmic reticulum and are associated with other cytochromes (b5) as well as with the NADPH-cytochrome P450 reductase. The most common chemical reactions catalyzed by CYPs are aliphatic hydroxylation, aromatic hydroxylation, N-dealkylation, and O-dealkylation, or oxidation, but ring-opening and reduction can also take place. Phase I biotransformation of any specific NCE is not entirely predictable: a specific site of metabolism may be favored for one compound and a completely different site for another compound, although very structurally related.

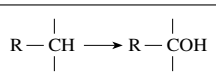
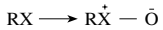
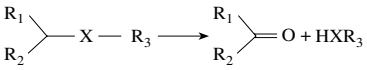
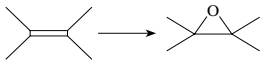
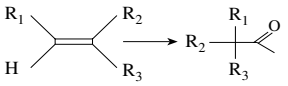
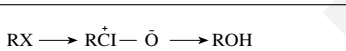
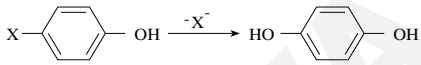
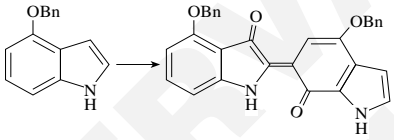
Substrate specificity is very low for the CYP enzyme complex: high lipid solubility is the only common property that renders CYP substrates a wide variety of structurally unrelated drugs, ranging from a molecular weight of 28 Da (ethylene) to 1 203 Da (cyclosporine). In Figure 1 examples of CYP-mediated chemical transformations are reported to illustrate some of the dominant pathways.

Larger molecules, such as proteins that are either currently marketed or under investigation, are not CYP substrates and little is known regarding their catabolism. It is widely believed that therapeutic proteins are metabolized by the same catabolic pathways as endogenous proteins, and can be broken down into amino acid fragments. Generally, the metabolic products of proteins are not considered a safety risk and classical biotransformation studies as performed for small molecules are not needed. Compared with small molecules or conventional drugs, characterizing the metabolites of potential therapeutic proteins is a much more difficult task. These difficulties arise because of the lack of suitable analytical method(s) and the abundance of potential sites of metabolism due to the complex structure of therapeutic proteins. The metabolism of proteins is highly dependent on structure (including sugars), charge (density and distribution), size, and hydrophilicity/lipophilicity. Most proteins are catabolized by proteolytic enzymes which are distributed throughout the body.[7-10]

Molecules of relatively small size and with highly hydrophobic characteristics permeate the hepatocyte membrane by simple non-ionic passive diffusion. An example of peptides of this nature are the cyclosporins (cyclic peptides). Other cyclic and linear peptides of small size (<1.4 kDa) and hydrophobic nature (containing aromatic amino acids), such as cholecystokinin-8 (CCK-8), are cleared by the hepatocytes by carrier-mediated transport. After internalization into the cytosol, these peptides are usually metabolized by microsomal enzymes (*i.e.* CYP mediates hydroxylation for cyclosporine) or cytosolic peptidases for CCK-8.[7-10]

Species differences between animal and human drug biotransformation

Animal models are commonly used in the preclinical development of NCEs to predict their metabolic behavior in humans. It is, however, important to realize that drug-metabolizing enzymes vary across species (homolog speciation) and within a species (phenotype and expressional levels) as a result of age, gender, disease, hormonal status and even diet.[11, 12] Even though the validity of animal testing to predict efficacy and safety of a NCE in humans has been questioned, it is generally believed that the pharmacokinetics can be extrapolated reasonably well to humans by using appropriate pharmacokinetic models.[13] In general, the use of rodents is preferable because of low cost, genetic homology, ease of handling and ethical concerns. However, because of dissimilarities between rodentia and humans in many physiological functions, the pharmaceutical industry is required to include a non-rodent species in preclinical metabolism studies to validate toxicity and human dose models extrapolated from rodents. In addition, large animals, being more similar in size to humans and having a

Reaction class	Structural change
C-hydroxylation	
Heteroatom oxygenation	
Heteroatom release	
Epoxide formation	
Group migration	
Chlorine oxygenation	
Aromatic dehalogenation	
Dimer formation	

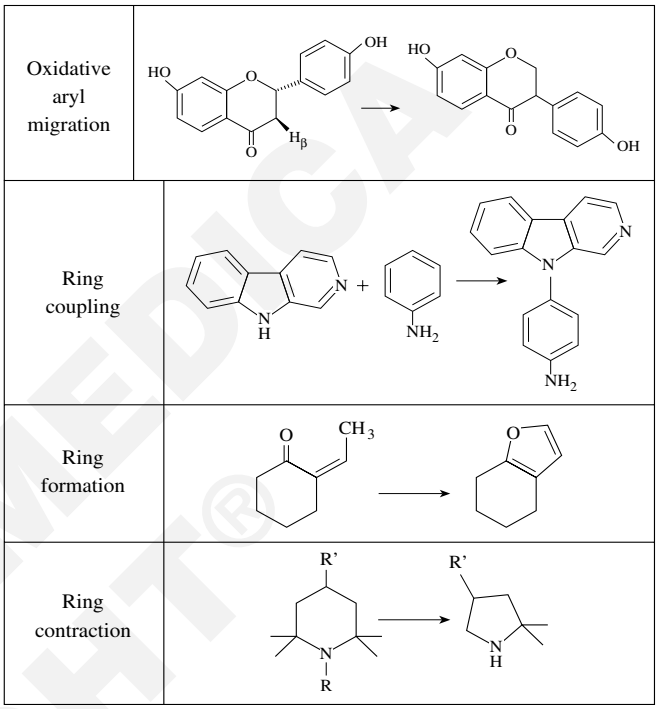


Figure 1.—Examples of chemical reactions catalyzed by cytochromes P450 (CYPs). Many are formal oxidations, but reductions and rearrangements have been also described.

longer life, are preferred for addressing issues related to scaling up to human therapy and for performing longitudinal studies. Body size, weight and average heart rate have always been considered important covariables for allometric scaling in order to determine the major pharmacokinetic parameters of NCEs.[14, 15] As an empirical approach, allometric scaling is widely used in predicting how anatomical, physiological and biochemical variables in mammals (*e.g.* tissue volumes, blood flow and process rates) can be scaled across species as a function of anatomical and physiological properties such as body weight or liver weight as a percentage of body weight.[14, 15] As a consequence of the relative amount of hepatic enzymes, such as CYP/gram body weight, higher in small animals compared to humans, in general, humans will tend to eliminate xenobiotics less rapidly than small animals when compared on a weight-normalized basis. Other physiological parameters including hematocrit, serum albumin concentration and body temperature, are relatively well conserved among animals and independent of animal size.[16] It is worth mentioning that key differences across species include plasma protein binding, transporters, clearance mechanisms and metabolism. This article is focusing on the latter with emphasis on the role of CYPs.

Although the CYP system is highly conserved among species, small differences in the primary sequences of CYPs are present and can give rise to profound diversity in substrate specificity and catalytic activities across species. Thus, differences in CYP isoforms are a major cause of diversity in drug metabolism between animal species and humans. Various CYP isoforms have been identified. Some drugs are specific substrates for these isoforms, but different CYPs often catalyze an overlapping spectrum of metabolic reactions.

The CYP superfamily is divided into families (*e.g.* CYP1, CYP2, CYP3), in which the primary structure is >40% identical, and into subfamilies (labeled with letters A, B, C, etc.) the members of which have >55% identical protein sequences, and finally by an Arabic

TABLE I.—*Major human drug-metabolizing cytochrome P450s.*

Gene subfamily	CYP	Expression	Tissue localization	Relative protein abundance (%) [1]
1A	1A1	Inducible	Lung, placenta	<1
	1A2	Inducible	Liver	8-15
1B	1B1	Constitutive?	Kidney?, tumors	
2A	2A6	Constitutive	Liver, nasal mucosa?	5-12
	2A7	Constitutive	Liver,	
	2A13	Constitutive	Liver, nasal mucosa?	
2B	2B6	Inducible?	Liver	1-5
	2B7	?	Lung	Absent
2C	2C8	Constitutive	Liver, kidney	10
	2C9	Constitutive	Liver	15-20
	2C18	Constitutive	Liver	
	2C19	Constitutive	Liver	<5
2D	2D6	Constitutive	Liver, duodenum	2
2E	2E1	Constitutive/inducible	Liver, lung, placenta, skin, brain	7-11
2F	2F1		Lung	Absent
2J	2J2			
3A	3A4	Constitutive/inducible	Liver, placenta, duodenum	30-40
	3A5	Constitutive	Liver?, stomach, placenta	<1
	3A7	Constitutive	Liver (fetal)	

CYP: cytochrome P450; ?: unknown or uncertain. [From Donato *et al.*[17] with permission from Wolters Kluwer Health].

number, representing the individual enzyme. In humans, 16 gene families and 29 subfamilies have been identified to date.[17] More than 50 isoforms have been isolated in man and about 35 CYP isoenzymes are of clinical relevance. Three CYP families, called CYP1, CYP2, CYP3, are responsible for the majority of drug biotransformations, but they also catalyze the metabolic conversion of endogenous compounds (*e.g.* vitamins, bile acids and hormones). In all animal species and humans, CYPs are located in virtually all organs.[17, 18] However, the liver (300 pmol of total CYPs/mg microsomal protein) and the intestinal epithelia (about 20 pmol of total CYPs/mg microsomal protein) are the predominant sites for CYP-mediated drug elimination, whereas CYPs in other tissues contribute to drug elimination to a much smaller extent. In Table I major human drug-metabolizing CYPs are listed together with their expression, tissue localization, relative protein abundance in the liver and their potential inducibility. Inducibility by xenobiotics is also one of the major characteristics of many CYP isoenzymes (Table I). Most human drug-metabolizing CYPs are polymorphic and/or inducible, accounting for the large interindividual variability in pharmacokinetics, efficacy and potential toxicity.[19]

CYP3A4 and its related 3A5 are the most abundantly expressed isoforms and represent approximately 30-40% of the total in human adult liver. They are involved in the biotransformation of ~50% of therapeutic drugs on the market at present.[20]

Based on the highly conserved regions found in CYPs from different animal species, it has been possible to classify the genes and to establish equivalence of CYP isoenzymes across species (Table II). The CYP1A subfamily is present in humans and in all considered animals (*i.e.* mouse, rat, rabbit, pig, dog and monkey) where, in particular CYP1A2 seems to be the predominant isoform. This is not a common pattern: the predominant isoform within the same CYP family is usually different among animal species (for example CYP3A4 in humans, CYP3A1 in rats and CYP3A6 in rabbits, CYP2C9 in humans, CYP2C11 in rats and several minor isoforms in the dog, rabbit, pig and mouse). Many animals express CYP genes with no known equivalent in humans or other species, an example is the CYP2 family (Table II).[11, 21]

In vitro models for drug metabolism studies

During the last decade, several *in vitro* and *in silico* approaches for CYP screening purposes have been developed and taken into routine use within industry and academia. Most of the interest has

TABLE II.—*Cytochrome P450 isoenzymes in humans and other animal species.*

CYP subfamily	Human	Monkey	Dog	Pig	Rabbit	Rat	Mouse
1A	1, 2	1, 2	1, 2	1, 2	1, 2	1, 2	1,2
1B	1	1	1			1	1
2A	6, 7, 13	2, 24	13, 25	19	10, 11	1-3	4, 5, 12, 22
2B	6, 7	17	11	22	4, 5	1-3, 8, 12, 15, 22-24	9, 10, 13, 19, 20
2C	8, 9, 18, 19	20, 43	21, 41, 42	32-36	1-5, 14-16, 30	6, 7, 11-13, 22, 23, 24	9, 10, 29, 37-40, 44, 50, 54, 55
2D	6, 7, 8	17, 19, 29, 30	15	15, 21	24	1-5, 18	9-13, 22, 26, 34, 40
2E	1	1	1	1	1, 2	1	1
3A	3, 4, 5, 7, 43	8	12, 26	12, 29	6	1, 2, 9, 18, 23, 62	11, 13, 16, 25, 41, 44

CYP: cytochrome P450. [Modified from Martignoni *et al.* and Donato *et al.*[11, 177] with permission from Wolters Kluwer Health].

focused on developing assays suitable for high-throughput screening (HTS), but recently, increased scrutiny has been placed on *in vivo* relevance.[22-29] All *in vitro* models used for metabolite analysis have strengths and weaknesses, hence, an integrated approach of several different techniques is usually preferable. Enzyme sources for most *in vitro* models are human-derived. These systems consist mainly of liver slices, liver hepatocytes, microsomes, and individual recombinant CYPs expressed in host cell systems. Table III shows a comparison of different human-derived enzyme sources used *in vitro*.[30]

During the past few years, genetically modified mice lines expressing various human CYP isoforms [31-33] and chimeric mice with humanized livers [34, 35] have been developed with the aim of creating more predictive models of human response. The availability of these strains is still very limited and requires further validation and characterization. Tissue sections and perfused organs can also be extremely useful in metabolism studies as they are inherent surrogates of whole body metabolism but handling complexity and poor availability hinders widespread use.

Primary human hepatocytes

Hepatocytes are known to contain the full ensemble of both phase I and phase II drug metabolizing enzymes (DMEs). Therefore, they are considered a valuable and reliable tool for metabolite identification, induction and mechanistic toxicity studies. Good *in vitro-in vivo* correlations in the metabolic activity of a number of drugs have been demonstrated and consequently, cultured human hepatocytes are the most recommended tools to study CYP-mediated metabolism and induction.[36-41]

The restricted availability of liver tissue limits the widespread use of primary human hepatocytes. Hepatocyte cultures can be prepared from surgical wedge biopsies or whole liver. The maintenance of normal cellular physiology and intercellular contacts in hepatocytes requires special matrix configurations and demanding technical abilities. Satisfactory cryopreservation of adult hepatocytes has been achieved and frozen cells are now sold entrapped in a polysaccharide matrix (*e.g.* alginate). Although several cryopreservation applications have been developed, the time frame for efficient use of a single hepatocyte batch is still quite short.[26, 42-44] Tissue banking has further increased the use of rarely obtainable tissues, *e.g.* those of human origin, by making the material more available.

Human liver microsomes

Microsomes from different animal species are commercially available and can be stored at -80 °C for years with little or no loss of enzyme activities. Due to their ease of use, minimal cost, and speed, most companies are using human liver microsomes to measure the disappearance of a compound over time and determine half-life or intrinsic clearance. In a first approach, the metabolic stability of a NCE and its metabolic profile can easily be investigated by incubation with hepatic microsomes followed by chromatographic analysis (*e.g.* tandem mass spectrometry coupled to high-pressure liquid chromatography [HPLC-MS/MS]), greatly simplifying the identification of the metabolites.

Microsomes are derived from smooth endoplasmic reticulum during tissue homogenization and are fractionated from subcellular organelles by differential ultracentrifugation.[45] A microsomal fraction from

TABLE III.—*Comparison of in vitro enzyme sources used in preclinical research.*

Model	Advantages	Disadvantages
Isolated perfused liver	Closest to *in vivo* Three dimensional cytoarchitecture *In vivo* like expression of DMEs and transporters Functional bile canaliculi	Not high throughput system Complicated to use Hepatic function is preserved for only a few hours Human organs are difficult to obtain
Liver slices	*In vivo* cytoarchitecture is preserved Reasonably high throughput Human tissue slices are more easily available than whole organs The use of cryopreserved liver slices can reduce the use of experimental animals Functional DMEs, transporters and bile canaliculi Cell-cell connections are preserved	Hepatic function is not preserved for >10 h Difficult to obtain, relatively healthy tissue needed Necrotic cells/scar tissue at edges of the slice Bile cannot be collected and analyzed Presence of necrotic cells might affect active transport of drug through the outer cells Limited viability
Freshly isolated hepatocytes in suspension	Reasonably high throughput Most DMEs well-preserved at *in vivo* levels Easy to use Zone specific metabolism and toxicity may be studied depending upon the method of isolation	Lack of cell polarity limits use for drug transporters Lacks functional bile canaliculi Limited survival (2-4 h) Lack of cell-cell and cell-matrix contacts Viability of isolated human hepatocytes may be variable
Primary hepatocyte cultures	Throughput depends on the technology used to preserve the tissue function Relatively easy to use Differentiated function maintained in many short-term and some long-term culture Potential for use in chronic toxicity studies and drug-drug interaction studies	Loss in DMEs activities in long term culture May or may not have functional bile canaliculi No single system has been able to preserve all the different liver specific functions *in vitro* Culture may need special supplements in media Difficult to obtain Relatively healthy tissue needed Commercially available
Microsomes	High throughput system Maintain expression of Phase I enzymes Can be recovered from frozen tissues Can be used in evaluating intrinsic clearance, covalent binding and drug inhibition studies Relatively simple and inexpensive technique Commercial sources available Easy storage	The only functionally expressed DMEs are Phase I enzymes and UGTs Can be used only for limited studies Cofactor addition necessary Inadequate representation of the diversity of hepatic functions
cDNA-expressed individual CYP enzymes	Commercial sources available Useful in high throughput single enzyme studies Metabolic profiles due to specific CYPs	Only one DME at a time can be studied Used to address very specific questions Studies may lack *in vivo* relevance

DMEs: drug metabolizing enzymes; UGTs: UDP-glucuronosyltransferases; CYPs: cytochromes P450. [From Sivaraman *et al.*[307] with permission from Bentham Science Publishers Ltd.].

human liver contains a full complement of the transmembrane CYPs, which makes it a suitable tool for studying inhibitory interactions and CYP-catalyzed metabolite formation.[46] By employing relevant cofactors and other reaction components, one can readily investigate and distinguish between CYPs, FMO and glucuronosyl transferases. In addition, microsomes are relatively easy to prepare, and enzymatic activities are stable during prolonged storage,[47] if the original tissue is correctly handled and frozen immediately

after excision.[46] The validation and harmonization of the handling of liver tissue between different laboratories would lead to less inter-laboratory variation between assays employing subcellular fractions.[45, 46] The experimental setup is simple and amenable to HTS, making this method widely utilized. In this model system, the incubation can be performed only for a limited amount of time (usually less than 1 h) before enzyme activity loss occurs. These systems give no insight into phase II metabolism.

Recombinant (cDNA-expressed) CYPs

DMEs are available commercially as heterologously expressed enzyme systems. In these preparations, an individual enzyme is produced in the endoplasmic reticulum of an eukaryote host cell. The expression of human liver CYPs in different artificial systems has become easier due to the rapid development of recombinant DNA techniques. The systems employed for the production of cDNA-expressed CYPs include bacteria, yeast, mammalian cell lines and baculovirus systems.[48-51] cDNA-expressed enzymes can be studied in isolation and thus are a valuable tool and, because they lack the whole complement of other hepatic enzymes, the *in vivo* predictive value of the data obtained is under debate.[52] However, cDNA-expressed enzymes are suitable for HTS and are a valuable tool in understanding substrate specificity (phenotyping) and metabolic pathways in early drug development. Moreover, recombinant enzymes can be used as small-scale bioreactors to generate usable amounts of metabolic product.[53]

Liver slices

Precision-cut liver slices retain a wide range of enzymatic activities and most closely mimics true organ function. Since the transport systems are present in liver slices, they are suitable for studying the transport of an NCE through cell membranes. If whole cell metabolism needs to be studied for short periods, liver slices represent a valuable tool.[43]

Maintenance of liver slices is demanding. For proper oxygen and nutrient transportation the thickness of the slice needs to be as thin as possible within the limits of the optimal number of cell layers and oxygen and nutrient transportation. Therefore, special tissue slicers are required.[54] The time available for preparation and use of a single slice is limited by the survival of the cells; however, cryopreservation methods have overcome some of these problems.[43, 55, 56] Many studies have shown lower cellular uptake, clearance, and metabolic capacity in liver slices compared to hepatocytes,[39, 43, 57] which has probably limited their popularity. Despite these limitations, tissue slices can be used for metabolism studies for about 24 h, which is considerably longer than by microsome incubations.

Methods for analysis of metabolites

Important prerequisites for effective drug metabolism studies are the ease and availability of methods to identify and quantify both the parent compound and its principal metabolites.[58] Metabolite identification can be a challenging task and is often a major investment in preclinical ADME assessment.

In a PET study, extensive metabolism combined with the short half-life of the positron emitting radionuclides can yield samples containing very low amounts of radioactivity at late time points. Since extensive metabolism may interfere with PET quantification and interpretation, it is essential to obtain accurate metabolic information early in the development process. This may yield plasma data of poor precision and accuracy, which in turn may affect the quality of PET data, such as receptor binding potential. Additionally, radiolabeled metabolites can potentially enter the target area of the radiotracer, thus complicating PET quantification. It is thus important to investigate the metabolism at an early stage in the development of a new PET tracer. During the time course of the study, the metabolic stability can be evaluated by performing simultaneous assays of the radiotracer in different body fluids. This has been accomplished by using different chromatographic techniques, with the most common being HPLC and sensitive on-line or off-line γ-ray detection.[59, 60] One approach is to spike samples with the co-eluting unlabeled tracer compound followed by injection onto a preparative liquid chromatography (LC) system. The spiking must be performed to obtain high enough concentrations for detection with UV absorption.[60] When the radiotracer is separated from its labeled metabolites, the radioactive content of the analyte fraction is determined in a well counter. The main advantage as well as the main limitation with this analytical method is that it relies on radioactivity from rapidly decaying samples for detection. The advantage is that detection

can be performed with high sensitivity. The disadvantage is that, in working with substances with a short half-life, the measurements can only be performed during a very limited time before the signal-to-noise level becomes too low for reliable analysis due to decay of radioactivity.[60] The time available for analysis and also the accuracy of the last determinations in the metabolic decay curve could be increased if some other detection technique was used for the analysis.

The total quantity of a labeled compound administered during a PET examination is usually in the picomolar to nanomolar range, which is several orders of magnitude below pharmacological levels.[61] Therefore, the amounts of metabolites that can be isolated from the samples obtained are for most applications not sufficiently high to be identified.

Due to isotopic dilution from the stable non-radioactive analogue present during nuclide production and synthesis, the radiotracer product always contains a mixture of labeled and unlabeled compound.[62] The ratio between them is determined as the specific radioactivity in Becquerels per mole (Bq/mol). It should thus be possible to determine the unlabeled fraction of the tracer substance by other detection techniques than radiodetection, providing the tracer is not endogenous and that the required sensitivity can be reached.[62]

Mass spectrometry coupled to HPLC (HPLC/LC-MS) is known to be more sensitive and selective than UV absorbance detection and has proven to be a valuable tool for the analysis of compounds being developed as radiopharmaceuticals, allowing detection of trace-level impurities,[63] determination of specific activity[64] and identification of metabolites.[65, 66] The main advantage of LC-MS analysis of the stable isotope compound lies in the non time dependent sensitivity. Theoretically, this leads to a constant precision and accuracy with time. LC-MS analysis also holds the potential for highly efficient metabolic screening of radiotracer candidates. If high sensitivity is reached, the substrate can be collected from the labeling batch used for biodistribution and autoradiography assays. The labeling batch can be stored and used to repeat metabolic stability assays, which is not possible with radiodetection of short-lived radionuclides. Also, MS/MS analysis provides both structural information and high selectivity. When electrospray ionization is used, this detection technique behaves as a concentration sensitive device.[67] Therefore, increased mass sensitivity will be obtained if columns with small inner diameters are used. This is important to consider when working with limited sample amounts, such as plasma samples. Recently, a number of methods utilizing quadrupole mass spectrometers have been developed for determining the *in vitro* metabolic stability of radiotracers.[62] Lately this radio-LC-MS approach has been used to confirm the identity of radiopharmaceuticals labeled with radiometals, such as ^{99m}Tc and ^{64}Cu where the incorporation of the metal radioisotope into the desired ligand was confirmed by comparing the retention times of radiolabeled species with the fully characterized natural metal isotope complexes detected by LC-MS.[68-70]

Cytochrome P450-mediated metabolism of radiopharmaceuticals: some examples

As stated earlier, the metabolic fate of a NCE is influenced by numerous factors, such as enzyme expression, interspecies differences, inter-animal and inter-individual variability, age, hormonal status, health status, coadministration of anesthetics or interaction with other drugs or xenobiotics.

An illustrative example of the implications of CYP-mediated metabolism during bolus/infusion PET studies can be found in the pharmacokinetics of the radiotracer [^{18}F]CPFPX, a novel PET ligand used for *in vivo* quantification of cerebral A1 adenosine receptors. In humans, this radioligand is supposedly metabolized to one major metabolite by CYP1A2, as depicted in Figure 2, and at least 6 less abundant Phase I metabolites. Its metabolism is, therefore, expected to have a strict dependence on disease related or xenobiotic-induced changes of CYP1A2 activity. In patients with liver disease (*i.e.* cirrhosis, hepatitis, etc.) or exposure to CYP1A2 inducers (*i.e.* tobacco smoke, carbamazepine, omeprazole, etc.) or inhibitors (*i.e.* fluvoxamine, ciprofloxacin, phenacetine, etc.) it will result in an increase or decrease, respectively, in the metabolism of [^{18}F]CPFPX and thus alter its plasma clearance and quantitation of cerebral A1 adenosine receptors.[71]

In the development of a central nervous system PET radiopharmaceutical, it is essential to evaluate and determine if radiolabeled metabolites enter the brain and, thus, confound the PET signal.

Non-invasive quantitation of brain dopamine transporter (DAT) density and affinity has been successfully

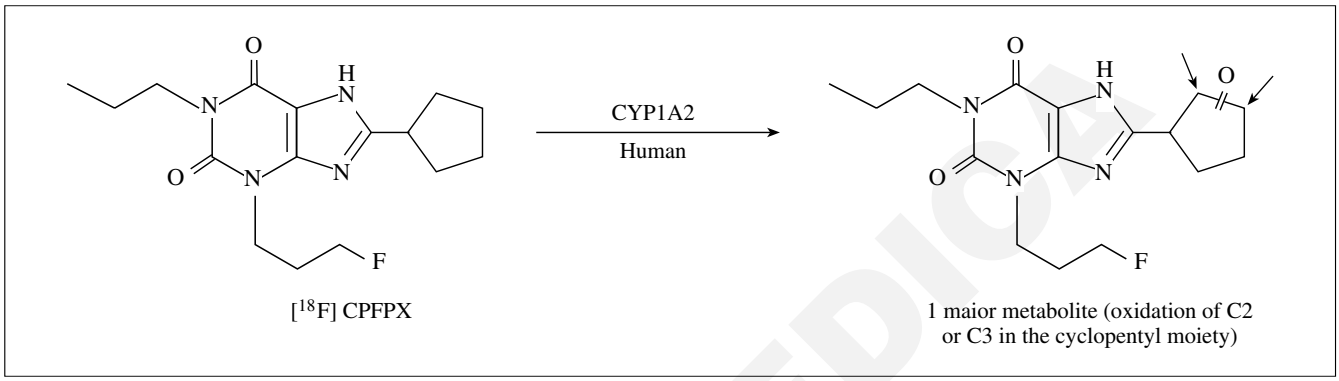


Figure 2.—CYP1A2-mediated preferential oxidation of [18F]CPFPX in humans.[71]

used to determine pathophysiology, disease progression, and effects of therapy in Parkinson's disease and cocaine abuse. Fluorine-18-FECNT is an established PET tracer for DAT quantitation that has successfully been used in humans and monkeys. However, in rats, primates, and humans a polar [18F]fluoralkyl metabolite of [18F]FECNT, was identified to originate in the periphery and enter the brain, confounding brain radioligand measurements. In Figure 3, the possible route for *in vivo* oxidative metabolism of [18F]FECNT, mediated by liver CYPs, is depicted. Three 18F-radiolabeled metabolites, identified as ethanol, acetaldehyde and carboxylate were characterized by radio-HPLC and LC-MS analyses. The radiometabolite found in the brain was likely [18F]2-fluoroacetaldehyde, the product expected to originate from a CYP-mediated N-dealkylation, or its oxidation product, [18F]2-fluoroacetic acid. In addition, the rate of metabolism of [18F]FECNT was higher in monkeys than in humans. Thus, analysis with a measured arterial input function will be prone to more reliable data of DAT quantification than reference tissue modeling.[72]

Another example is the antagonist des-cyclohexanecarbonyl derivative [*O*-methyl-11C]WAY100635, recognized as the first highly effective radioligand for visualizing brain serotonin 5-HT_{1A} receptors in rodents. In rats radio-metabolites, more polar than the parent compound were formed and did not pass the blood brain barrier.[73] However, in humans and primates [*O*-methyl-11C]-WAY100634 (Figure 4), a different radiolabeled metabolite was identified to form and enter into the brain, contributing to specific and non-specific binding.[74] This drawback has led to the development of more metabolically stable compounds which are currently under evaluation in animals and humans.

Conclusions

Metabolism is a major determinant of both pharmacokinetics and disposition and hence needs to be taken into account when developing effective PET tracers. Understanding the metabolic fate of radiopharmaceutical probes is essential for an accurate, high quality analysis and correct interpretation of PET measurements. The inherent inability of PET to differentiate between parent and metabolites confounds interpretation of images and may impact the identification of the pathological biochemical changes under investigation.

A great deal of effort is now directed at assessing key metabolic parameters at early stages of radiopharmaceutical drug development. Several *in vitro* methods are available to gain insight into the metabolic fate of a radiotracer that correlate well with *in vivo* behavior. Human hepatocytes remain the best simple model to gain information on both phase I and phase II metabolism owing to their relative ease-of-use and relevance to *in vivo* systems. Human liver microsomes are widely utilized as a first line *in vitro* system due to their availability, relatively low cost and simplicity of use, all characteristics that make them suitable for HTS. Other *in vitro* models, such as recombinant CYPs, remain more remote surrogates of *in vivo* systems, but are suitable for academic mechanistic research. Animal models are commonly used in the

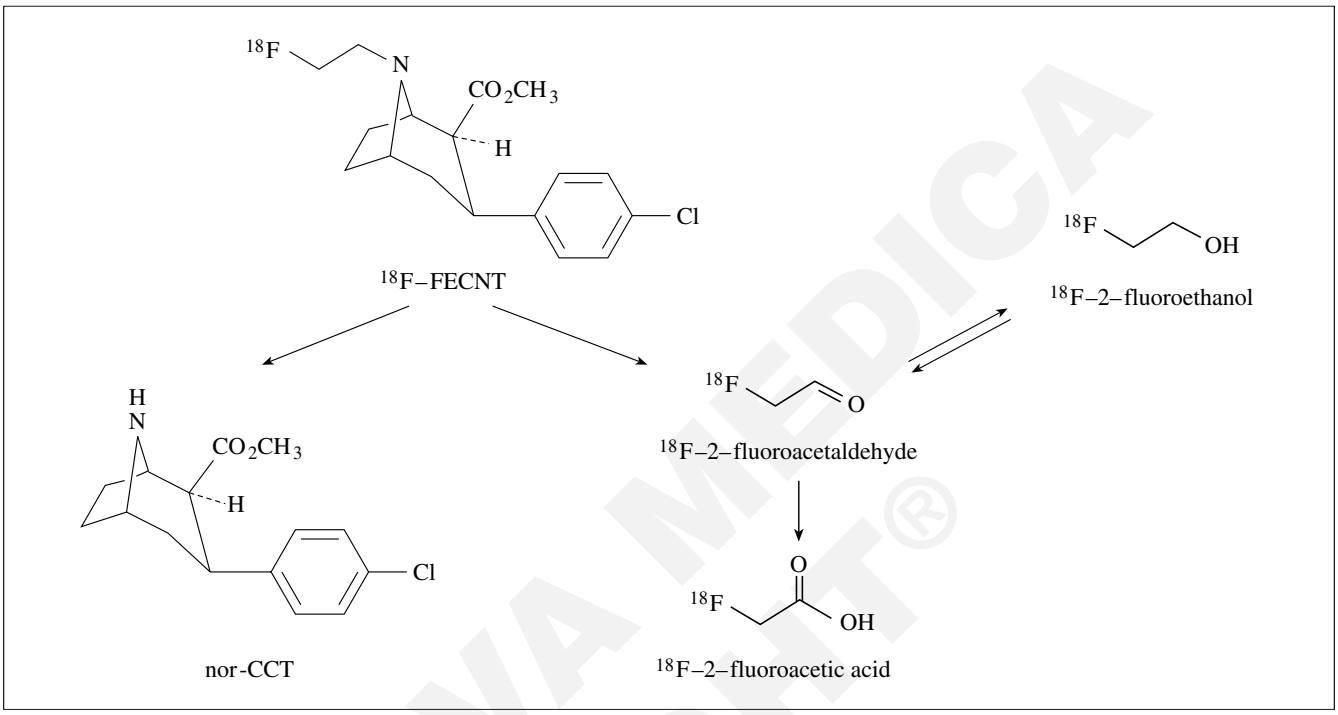


Figure 3.—Possible route for oxidative metabolism of [18F]FECNT mediated by liver cytochromes P450 (CYPs) in rodents and humans.[72]

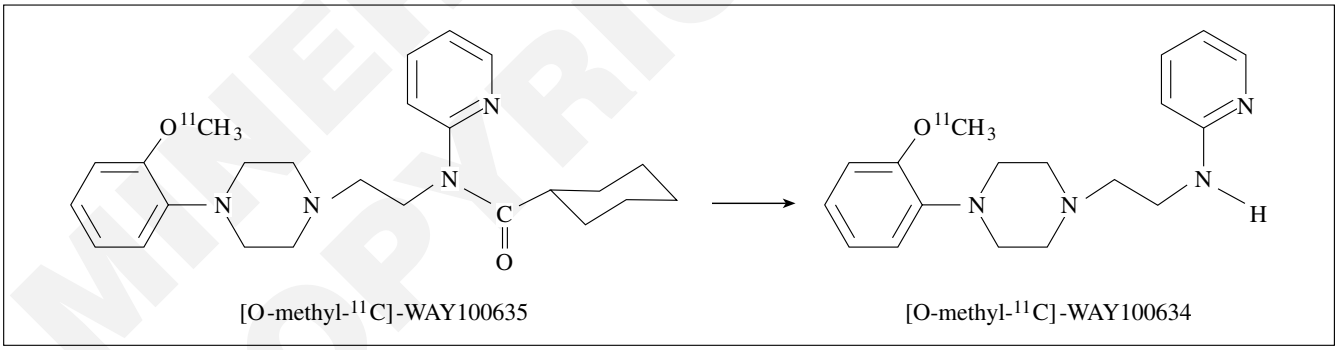


Figure 4.—Metabolism of [O-methyl-11C]-WAY100635 in primates and humans.[74]

preclinical evaluation of NCEs to predict *in vivo* specificity of the tracer to the target gene product in humans. However, it is important to realize that humans differ from other species in regard to isoform composition, expression and catalytic activities of drug-metabolizing enzymes.[75] When developing a new radiopharmaceutical, it is essential to understand its metabolic fate in the target species in order to enable the fullest interpretation of PET imaging. Interspecies differences in radiotracer metabolism must be taken into consideration when predicting the impact of metabolism in humans from preclinical animal studies.[76]

Knowledge of the intrinsic pharmacophore metabolism should also be considered when determining the locus of the label. LC/MS can afford a full metabolic profile using cold compounds before radiolabeling is commenced.

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