ABSTRACT

Metabolism and Quantification of [18F]DPA-714, a New TSPO Positron Emission Tomography Radioligand

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ABSTRACT

[18F]DPA-714 [N,N-diethyl-2-(2-(4-(2-[18F]-fluoroethoxy)phenyl) 5,7-dimethylpyrazolo[1,5a]pyrimidin-3-yl)acetamide] is a new radioligand currently used for imaging the 18-kDa translocator protein in animal models of neuroinflammation and recently in humans. The biodistribution by positron emission tomography (PET) in baboons and the in vitro and in vivo metabolism of [18F]DPA-714 were investigated in rats, baboons, and humans. Whole-body PET experiments showed a high uptake of radioactivity in the kidneys, heart, liver, and gallbladder. The liver was a major route of elimination of [18F]DPA-714, and urine was a route of excretion for radiometabolites. In rat and baboon plasma, high-performance liquid chromatography (HPLC) metabolic profiles showed three major radiometabolites accounting for 85% and 89% of total radioactivity at 120 minutes after injection, respectively. Rat microsomal incubations and analyses by liquid chromatography–mass spectrometry (LC-MS) identified seven metabolites, characterized as O-deethyl, hydroxyl, and N-deethyl derivatives of nonradioactive DPA-714, two of them having the same retention times than those detected in rat and baboon plasma. The third plasma radiometabolite was suggested to be a carboxylic acid compound that accounted for 15% of the rat brain radioactivity. O-deethylation led to a nonradioactive compound and [18F] fluoroacetic acid. Human CyP3A4 and CyP2D6 were shown to be involved in the oxidation of the radioligand. Finally an easy, rapid, and accurate method—indispensable for PET quantitative clinical studies—for quantifying [18F]DPA-714 by solid-phase extraction was developed. In vivo, an extensive metabolism of [18F]DPA-714 was observed in rats and baboons, identified as [18F]deethyl, [18F]hydroxyl, and [18F]carboxylic acid derivatives of [18F]DPA-714. The main route of excretion of the unchanged radioligand in baboons was hepatobiliary while that of radiometabolites was the urinary system.

INTRODUCTION

The 18-kDa translocator protein (TSPO) is a heteropolymeric mitochondrial protein (Scarf, 2011). TSPO is primarily located at outer mitochondrial membranes within transmembrane channels (Lacapère and Papadopoulos, 2003) that have many putative functions (Batarseh and Papadopoulos, 2010; Chelli et al., 2004; Rupprecht et al., 2010). TSPO is now known to have a widespread distribution in the organism (Hirvonen et al., 2010), and low levels of TSPO are found in the normal brain (Papadopoulos et al., 2006a). TSPO is present in activated microglia (Myers et al., 1991) and to a lesser extent in astrocytes; hence, TSPO concentrations are found to be elevated in regions of brain inflammation arising from neurodegenerative disorders or stroke (James et al., 2008; Lang, 2002; Papadopoulos et al., 2006b). Positron emission tomography (PET) imaging of TSPO is therefore recognized as a clinical means to detect and investigate neuroinflammation. Among the PET radioligands that are clinically useful, [11C]PK11195 [N-methyl-N-(1-methylpropyl)-1-(2-chlorophenyl)isoquinoline-3-carboxamide] has been the most widely used (Hirvonen et al., 2010). New candidate PET ligands for TSPO have recently been developed (for review, see Dollé et al., 2009), and these have been shown to have a higher brain uptake and a higher proportion of specific binding than [11C]PK11195 (Lacapère and Papadopoulos, 2003; Chauveau et al., 2008). In a recent study of several animal models of brain inflammation in our institution, [18F]DPA-714 [N,N-diethyl-2-(2-(4-(2-[18F]-fluoroethoxy)phenyl)phenoxy)] 5,7-dimethylpyrazolo[1,5a]pyrimidin-3-yl)acetamide] has been shown to perform better than other tested radioligands such as [11C]PK11195 and [11C]DPA-713 [N,N-diethyl-2-[2-(4-methoxyphenyl)-5,7-dimethylpyrazolo[1,5a]pyrimidin-3-yl]acetamide] (Chauveau et al., 2009). This finding suggested that [18F]DPA-714 could be a promising agent for...
clinical TSPO imaging. [18F]DPA-714 is now undergoing clinical development (Arlicot et al., 2012; Boellaard et al., 2011).

To date, identification of the radiometabolites of [18F]DPA-714 has yet to be published. This is an important step in the development of a new radiotracer since the radiometabolite corrected arterial input function for compartmental modeling is mandatory when no reference region (i.e., a region devoid of the target receptor) can be used. Furthermore, the presence of radiometabolites in the cerebral tissue could interfere with quantification. The quantification of TSPO distribution in the human brain requires the determination of the total plasma radioactivity concentration and the accurate determination of the non-metabolized fraction of [18F]DPA-714 in plasma as a function of time. We have developed and validated a fast, sensitive, and convenient approach using solid-phase extraction (SPE) to quantify [18F]DPA-714 in plasma. In addition, we demonstrate the equivalence of the time-activity curve at early time points of the PET study for the SPE procedure compared with our previous high-performance liquid chromatography (HPLC) method.

Predicting human metabolism before starting clinical studies is also critical. Therefore, the metabolism of nonradioactive DPA-714 was studied in vitro in microsomes (rat, baboon, and human), and the metabolism of [18F]DPA-714 was studied ex vivo in rats and in vivo in rats and baboons.

Materials and Methods

Radiosynthesis

DPA-714 was labeled with fluorine-18 at its 2-fluoroethyl moiety using a tosyloxy-for-fluorine nucleophilic aliphatic substitution according to slight modifications of procedures already reported (Damont et al., 2008) and using a commercially available GE TRACERlab FX F-N synthesizer (GE Healthcare, Waukesha, WI). Ready-to-inject, >99% radiochemically pure [18F]DPA-714 ( formulated in physiologic saline containing less than 10% of ethanol) was obtained with specific radioactivities at the end of the radiosynthesis, ranging from 37 to 111 GBq/μmol.

In Vivo Metabolism of [18F]DPA-714

Animals. All animal use procedures were in strict accordance with the recommendations of the European Community (86/609/CEE) and the French National Committee (décret 87/848) for the care and use of laboratory animals. Four male adult *Pigio anubis* baboons (weight = 12.6 ± 4.3 kg) and male Sprague-Daley rats (weight = 250–300 g) were used.

Stability of [18F]DPA-714 in Plasma and Brain Tissues. The radiochemical stability of [18F]DPA-714 was checked in vitro in spiked rat or baboon plasma and in rat brain homogenate at 37°C. Baboon blood samples were taken immediately before the PET study. Plasma was prepared by centrifugation (5 minutes, 3000g, at room temperature). Six plasma samples (500 μl) were mixed with 20 KBBq/ml [18F]DPA-714. Three of these were extracted with 700 μl of CH3CN and centrifuged at 3000g for 10 minutes. The supernatant was collected in three tubes then was directly analyzed by radio-HPLC.

Radiometabolites Analysis.

Baboons. During the PET experiments performed in isoflurane-anesthetized baboons for the characterization of the kinetics of [18F]DPA-714, 28 blood samples from each of the four baboons (1 ml; six independent PET experiments) were drawn from the femoral artery from 0 to 120 minutes after injection and immediately centrifuged at 4°C for 5 minutes at 3000g to obtain cell-free plasma. The radioactivity in the plasma samples (200 μl) was measured in a cross-calibrated gamma-counter (Cobra Quantum D5003; Perkin-Elmer, Courtabeuf, France) and expressed in becquers. All the values were corrected for fluorine-18 decay (109.7 minutes) until the injection time according to the following equation: 
\[ y = x 	imes \exp(-t/109.7) \]
where x represents the time of counting and y the radioactivity measured at that time.

For the radiometabolite analysis, arterial plasma samples (seven samples of 500 μl) were counted, deproteinized with 700 μl of acetonitrile, and centrifuged at 4°C for 2 minutes (3000g). The radioactivity in the resulting precipitate was counted for calculating the percentage recovery of radioactivity in the acetonitrile. The whole supernatant was injected onto the radio-HPLC system. Baboon urine was collected at the end of the PET experiment (T0 + 180 minutes) using a Foley catheter (NM Medical, Frein, France). Urine sample (1 ml) was directly injected onto the same radio-HPLC system.

The analytical system consisted of a quaternary gradient pump, an AS110T autosampler and a UVD510UV UV-Vis detector (Dionex S.A., Courtabeuf, France) online with a LB-509 radiospectro detector (MX 2500 cell; Berthold, La Garenne Colombes, France). [18F]DPA-714 and its radiometabolites were separated using a 5-μm, 10 × 250 mm, C18 SunFire preparative column (Waters, Saint-Quentin, France). The mobile phases consisted of 0.1% trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in acetonitrile (B). A linear gradient from 20% to 80% of B (and A from 80 to 20%) in 10 minutes was applied to the column at a flow rate of 5 ml/min (UV detection 220 nm). Data acquisition and processing were performed with Chromeleon software (version 1.0; Dionex). The radioactivity due to unchanged [18F]DPA-714 was expressed as a fraction of the total radioembalved peak areas. The percentages of unchanged [18F]DPA-714 in plasma as a function of time was fitted by a nonlinear regression analysis (OriginPro software version 8.5; OriginLab, Northampton, MA) using a standard following biexponential decay equation.

Rats. Two rats were injected via a tail vein with 32.1 ± 2.8 MBq [18F]DPA-714 and were sacrificed by decapitation 120 minutes later. Plasma samples were treated and analyzed as described earlier. The whole brain was excised, weighed, counted, and sonicated in 1 ml of acetonitrile on ice. The homogenate was centrifuged 10 minutes at 3000g. The pellet was resuspended and further sonicated in 1 ml of acetonitrile acetonitrile. After centrifugation of the second homogenate, the two extracts were pooled and concentrated by evaporation before analysis by radio-HPLC. The concentrations of [18F]DPA-714 and its radiometabolites in tissues were expressed as a percentage of injected dose per gram of tissue (% ID/g). The radioactivity was also measured in the rat thighbone and in the bone marrow.

Quantification of [18F]DPA-714 in Baboon Plasma by Solid-Phase Extraction. To develop a more sensitive, rapid, and convenient method to isolate the unchanged radiotracer from its radiometabolites, a method using only SPE was developed and compared with the radio-HPLC method, the latter being considered as the reference method. Five arterial plasma samples from each baboon (n = 3) were counted and analyzed by both radio-HPLC and SPE. A 60-mg HLB Oasis SPE cartridge (Waters) was conditioned with 1 ml of CH3OH and equilibrated with 5 ml of water. For the development of the method, the following procedure was used. Plasma samples (200 μl) were diluted to 400 μl with an aqueous solution containing 4% chlorhydric acid. The radioactivity was measured in the resulting solution, which was directly applied to the HLB cartridge (SPE fraction F1). The cartridge was then sequentially washed with 1 ml of H2O (fraction F2), then with a CH3CN/H2O solution (fraction F3). Several concentrations of CH3CN (15 concentrations between 25% and 39%) were tested for elution of the last fraction of radiometabolites. Then, 1 ml of a CH3CN/H2O 35/65 (v/v) solution was chosen for an optimal separation. [18F]DPA-714 was finally eluted with 1 ml of CH3CN (fraction F4). The radioactivity was measured in all the collected fractions as well as in the cartridge (fraction F5). The last fraction (fraction F6) represents the empty tip and tube containing the small amount of plasma that was not applied on the cartridge. The radioactivity due to unchanged [18F]DPA-714 was expressed as the percentage of the sum of radioactivity found in the eluted fractions or as the fraction of the initial radioactivity measured in each plasma sample.
Major Routes of $^{18}$F[DPA-714 Excretion in Baboons. The main routes of excretion of $^{18}$F[DPA-714 were studied using whole-body PET imaging (HR+ PET tomography; Siemens Molecular Imaging, Knoxville, TN). Transmission scans (3 minutes per step, 5 or 6 steps according to the height of the animal) in two-dimensional mode were used for subsequent correction of attenuation of emission scans. Emission scans (in three-dimensional mode, at increasing durations from 1 to 4 minutes per step) were performed up to 90 minutes after the intravenous injection of 276 ± 60 MBq (4.8 ± 2.1 nmol) of $^{18}$F[DPA-714. An experienced investigator drew volumes of interest (VOIs) over all organs that presented significant radioactivity on the earliest emission image for the liver, gallbladder, spleen, kidneys, and vertebral bodies. For the urinary bladder, a VOI was drawn on the last emission image.

In Vitro Metabolism of DPA-714

Chemicals. A d-glucose 6-phosphate disodium salt (G6P), β-nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-phosphate dehydrogenase (G6PDH) from yeast, formic acid, and bicinchoninic acid (BCA) protein assay kit was purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Solvents used for HPLC and liquid chromatography–tandem mass spectrometry (LC-MS/MS) analyses were from VWR (Val de Fontenay, France).

Liver Microsomes and cDNA-Expressed Human P450s. Microsomes from adult male Sprague-Dawley rats and from baboons were prepared by homogenization of liver samples over ice in a 0.1 M aqueous Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 0.2 M sucrose, followed by differential centrifugations according to Kremer’s method (Kremer et al., 1981). The total cytochrome P450 (P450) content was spectrophotometrically measured (Omura and Sato, 1964). The total microsomal protein content was determined according to the method with serum albumin as the standard. Human liver microsomes (pool of 10 individuals) and insect cell microsomes containing recombinant baculovirus cDNA-expressed human P450 (CYP1A2, CYP2A6, CYP2B6, CYP3A4, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 superexpresses) characterized for their P450 content, protein content, and main specific catalytic activities were purchased from Gentest Corp (BD Biosciences, Le Pont-De-Claix, France). All microsomal fractions were stored at −80°C until used.

Typical Microsomal Incubation Procedure. The incubations contained 1 mg of microsomal proteins (rat, baboon, and human liver microsomes), a NADPH-generating system (5 mM G6P, 1 mM NADP⁺, G6P dehydrogenase 2 U), DPA-714 (100 μM), and 0.1 M aqueous potassium phosphate buffer (potassium dihydrogen phosphate and dipotassium hydrogen phosphate, pH 7.4) in a final volume of 1 ml. The reaction mixtures were preincubated for 2 minutes at 37°C before initiation of the reaction by the NADPH-generating system. The incubations were conducted in a 37°C water bath with gentle shaking for 15, 30, and 60 minutes. The reaction was stopped by the addition of an equal volume of acetonitrile. Samples were centrifuged at 4°C for 5 minutes at 9000 g. The resulting supernatant was filtered (Vetra Spin Micro 0.2 μm; Whatman, Versailles, France), and 20 μl were analyzed by LC-MS/MS as described herein. Controls were incubated either without the NADPH-generating system or without microsomes to ensure the stability of the tracer in the experimental conditions. The percentage of each metabolite was determined as a percentage of the total peak area. Enzymatic catalytic activities are expressed as a formation rate of each metabolite in nanomoles per milligram of microsomal proteins per minute (nmol metabolite/mg per minute).

Oxidation of DPA-714 by cDNA-Expressed Human P450s Isoforms. Microsomes from baculovirus-infected insect cells expressing human P450s and cell microsomes containing control activity were used (Gentest/BD Biosciences, Le Pont-De-Claix, France) to test their ability to oxidize DPA-714. The incubations included DPA-714 (30 μM), 20 pmol of P450, 1.3 mM NADP⁺, 6.3 mM G6P, G6PDH 2 U, and 0.1 M aqueous phosphate buffer, pH 7.4, in a final volume of 500 μL. The reaction proceeded for 30 minutes at 37°C and was stopped by the addition of 500 μL acetonitrile. Samples were centrifuged at 10,000 g for 5 minutes at 9000 g, and 20 μl of the supernatants were analyzed by LC-MS/MS. Results are expressed as a percentage of the total peak areas for each metabolite (% of biotransformation).

Identification of DPA-714 Metabolites by LC-MS/MS. DPA-714 metabolites were analyzed by LC-MS/MS in microsomal extracts on a LCQ Deca XP+ ion trap mass spectrophotometer equipped with an electrospray source (Thermo Electron, Les Ulis, France). The HPLC system interfaced with the LCQ Deca XP+, which consisted of a LC Pump Surveyor (series 54949), PDA Surveyor (series 56470), and a Surveyor autosampler (series 55899, Thermo Electron, Les Ulis, France). Pressurized nitrogen was used as sheath gas with a flow rate of 25 units (arbitrary units for sheath gas pressure as defined by the manufacturer). The source voltage for electrospray ionization was 4.5 kV, and the capillary voltage was 38 V. The capillary temperature was 275°C. The separation of DPA-714 and its metabolites was performed using a reverse-phase Atlantis C18 column 2.1 × 150 mm, 5 μm (Waters). The mobile phase consisted of A) H₂O containing 0.05% formic acid and B) acetonitrile containing 0.05% formic acid. A linear gradient from 20 to 70% of B (80–30% of A) in 30 minutes was applied to the column at a flow rate of 200 μl/min. The whole output of the LC column was introduced into the photodiode array detector (190–600 nm) before the electrospray ionization probe of the LCQ Deca XP+ operated in the positive mode. In the full-scan MS acquisition mode, the instrument method was set up to detect ions in the range of m/z 50–500. The MS was set up in the MS/MS mode for acquiring, isolating with a mass width of 1 Da, and dissociating the ion m/z = 399 and its main metabolites by collision-induced dissociation in the ion trap using helium as the collision gas with an energy of 35%. Data acquisition and processing were performed with Xcalibur software (version 2.0) with the assistance of Mass Frontier software (version 4.0) for proposed fragmentation mechanisms (Thermo Electron).

Results

In Vivo Studies

Stability of $^{18}$F[DPA-714 in Plasma and Brain Tissues. $^{18}$F[DPA-714 proved to be stable in saline and upon incubation of spiked baboon plasma at 37°C for 190 minutes. Indeed, no decomposition products could be observed for $^{18}$F[DPA-714 by radio-HPLC analysis. $^{18}$F[DPA-714 also proved to be stable in rat plasma and brain homogenate for 180 minutes at 37°C.

In Vivo Metabolism in Baboons. Radio-HPLC profiles of plasma extracts showed three main radiometabolite peaks along with $^{18}$F[DPA-714 with retention times at 2.9, 7.2, 8.6, and 10.0 minutes, respectively (Fig. 1). The percentage of non-metabolized $^{18}$F[DPA-714 rapidly and exponentially decreased as a function of time (Fig. 2A) representing 46% at 30 minutes and only 11% at 120 minutes after injection. During the same time, the percentage of the main radiometabolite $^{18}$F[M7 increased and reached 50% of plasma radioactivity (Fig. 2A). The radioactivity measured in the acetonitrile fraction of the baboon plasma was 46% at 30 minutes and only 11% at 120 minutes after injection. During the same time, the percentage of the main radiometabolite $^{18}$F[M7 increased and reached 50% of plasma radioactivity (Fig. 2A). The radioactivity measured in the acetonitrile fraction of the baboon plasma was 46% at 30 minutes and only 11% at 120 minutes after injection. During the same time, the percentage of the main radiometabolite $^{18}$F[M7 increased and reached 50% of plasma radioactivity (Fig. 2A). The radioactivity measured in the acetonitrile fraction of the baboon plasma was 46% at 30 minutes and only 11% at 120 minutes after injection. During the same time, the percentage of the main radiometabolite $^{18}$F[M7 increased and reached 50% of plasma radioactivity (Fig. 2A). The radioactivity measured in the acetonitrile fraction of the baboon plasma was 46% at 30 minutes and only 11% at 120 minutes after injection. During the same time, the percentage of the main radiometabolite $^{18}$F[M7 increased and reached 50% of plasma radioactivity (Fig. 2A).
protein precipitates (acetonitrile pellet) increased as a function of time and accounted for 17% of total plasma radioactivity at 120 minutes (Fig. 2B). This radioactivity may be due to the presence of [18F] fluoroacetate (or [18F]fluoroacetic acid) generated by O-dealkylation of the tracer. This fraction was considered as a radiometabolite for the quantification of [18F]DPA-714 in plasma and the determination of the time-course of the radioligand (Fig. 2A). The plasma concentrations of radioactivity and nonmetabolized [18F]DPA-714 are presented in Fig. 2C (input function and metabolite-corrected input function).

In urine, the same radiometabolites as those detected in plasma were observed along with a very few amount (less than 2%) of [18F]DPA-714 at 180 minutes. The radiometabolites eluted at 2.9, 5.9, and 8.2 minutes represented 51, 30, and 17% of total radioactivity, respectively (data not shown).

**Major Route of [18F]DPA-714 Excretion in Baboons.** The SUV\(_{\text{max}}\) values obtained by whole body PET studies in baboons are summarized in Table 1. High uptake of radioactivity was observed in TSPO-rich regions such as the heart (SUV\(_{\text{max}} = 7.42\)) and parotid gland (SUV\(_{\text{max}} = 1.93\)). The highest radioactivity was observed in the kidneys: SUV\(_{\text{max}} = 12.7\) at 10 minutes after injection, followed by a washout (SUV = 4.6 at 90 minutes after injection). Brain displayed low radioactivity in these normal baboons. Vertebral bodies showed also an uptake of radioactivity (SUV\(_{\text{max}} = 1.99\) at 90 minutes after injection) with a slow increase from the beginning until the end of the PET experiment. One route of excretion was the hepatobiliary system: SUV\(_{\text{max}}\) in the gallbladder was 7.3 at 42 minutes after injection followed by the appearance of a large amount of radioactivity in the duodenal area. The other route of excretion was the urinary bladder with a SUV\(_{\text{max}}\) of 6.5 at 90 minutes after injection.

**Quantification of [18F]DPA-714 and Its Radiometabolites by Solid-Phase Extraction.** With the aim of developing a rapid, sensitive, and accurate quantification method of [18F]DPA-714 and its radiometabolites in plasma for human quantitative imaging studies, we have developed an approach using only SPE. In baboon spiked plasma, the yield of extraction of non-metabolized radiotracer was 95 ± 2% (\(n = 10\)). The limit of detection (LOD) and limit of quantification (LOQ) values were calculated as 1.2 and 1.8 Bq, respectively, compared with 55 and 145 Bq with HPLC. The mean total recovery of plasma-sample radioactivity with this method was 94 ± 4% (\(n = 87\)). The time-activity course of SPE fractions are presented in Fig. 3A. The first fraction corresponded to proteins and radiometabolites that were not retained on the SPE column by hydrophobic and anionic interactions. Polar radiometabolites were

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**TABLE 1**

Biodistribution of [18F]DPA-714 in one baboon

<table>
<thead>
<tr>
<th>Target Organ</th>
<th>SUV(_{\text{max}})</th>
</tr>
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<tbody>
<tr>
<td>Lung</td>
<td>5.67</td>
</tr>
<tr>
<td>Heart</td>
<td>7.42</td>
</tr>
<tr>
<td>Liver</td>
<td>6.28</td>
</tr>
<tr>
<td>Gallbladder</td>
<td>7.31</td>
</tr>
<tr>
<td>Spleen</td>
<td>7.57</td>
</tr>
<tr>
<td>Kidney</td>
<td>12.7</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>6.50</td>
</tr>
<tr>
<td>Parotid</td>
<td>1.93</td>
</tr>
<tr>
<td>Vertebral body</td>
<td>1.99</td>
</tr>
</tbody>
</table>

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**Fig. 2.** (A) time-course of the fraction of unchanged [18F]DPA-714 and radiometabolite [18F]-M7 in baboon plasma (mean ± SD, \(n = 6\) independent PET experiments) determined by radio-HPLC. The results are expressed as a percentage of the total radioactivity (%). Experimental data were fitted as the sum of two exponentials (OriginPro version 8.5). (B) [18F] radioactivity in acetonitrile pellet. The results are expressed as a percentage of the total radioactivity (%). Experimental data were fitted as the sum of two exponentials (OriginPro version 8.5). (C) representative plasma time-activity curve after injection of [18F]DPA-714 in one baboon.
eluted in water (fraction F2). More hydrophilic radiometabolites were eluted with 35% acetonitrile/water (fraction F3). Nonmetabolized \([^{18}F]\)DPA-714 was eluted in the acetonitrile fraction, as verified by radio-HPLC analysis (fraction F4). Figure 3C compares the time-course of unchanged radiotracer measured by SPE and radio-HPLC in the plasma samples of three different baboons. The percentage of unchanged \([^{18}F]\)DPA-714 followed a similar exponential decrease with both techniques (Fig. 3B).

**In Vivo Metabolism in Rats.** Rat plasma sample analyses demonstrated the formation of three radiometabolites that had the same retention times as those detected in baboons. In plasma at 120 minutes after injection, the concentration of non-metabolized \([^{18}F]\)DPA-714 was 15% of the total radioactivity (Fig. 4). The main radiometabolite was suggested to be generated in plasma by further oxidation of hydroxylated radiometabolite(s) into a carboxylic acid derivative (\([^{18}F]\)-M7, see identification of metabolites). This radiometabolite accounted for most of the plasma radioactivity. In the brain, at the same time, the concentration of total radioactivity was 2-fold higher than in plasma while that of non-metabolized \([^{18}F]\)DPA-714 was 11-fold higher than in plasma. This fraction represented 85% of the total brain radioactivity (Fig. 4). The radiometabolite \([^{18}F]\)-M7 represented 15% of the total brain radioactivity, but the concentration of \([^{18}F]\)-M7 was lower than in plasma (ratio of 0.4). The concentrations of the other radiometabolites found in plasma were quantitatively negligible in brain tissue. The radioactivity was also measured in the cortex of the thighbone and in the bone marrow at 120 minutes after injection. The results showed that 65% of the total bone radioactivity was in the bone marrow and 35% was found in the cortex of the thighbone.

**In Vitro Studies**

**Species Comparison.** Representative HPLC chromatograms of rat and human microsomal incubates are shown in Fig. 5A. Seven metabolites more polar than DPA-714 generated from oxidation by cytochromes P450 were detected in rat liver microsomal incubations by LC-MS (M1 to M6b; Fig. 5A). The formation rates for each metabolite expressed in nmol/mg per minute were compared in three species (Fig. 5B). Rat liver microsomes catalyzed the formation of seven metabolites M1 to M6b, with M1 and M3 being the most abundant (0.89 and 0.82 nmol/mg per minute) followed by M2, M4, and M5. In baboon liver microsomes, M4 and M5 were the predominant metabolites (0.46 and 0.64 nmol/mg per minute); in human liver microsomes, M3 and M4 predominated (0.45 and 0.57 nmol/mg per minute). It should be noticed that all the metabolites except M5 would remain radioactive with \([^{18}F]\)DPA-714.

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**Fig. 3.** (A) time-course of the radioactivity in each SPE fractions (F1, F2, F3, F4) in baboon plasma. (B) time course of the fraction of unchanged \([^{18}F]\)DPA-714 in baboon plasma determined by radio-HPLC and SPE (mean ± SD, n = 3 independent experiments). Comparison of radio-HPLC and solid-phase extraction to determine the time-dependent decrease of the fraction of non-metabolized \([^{18}F]\)DPA-714. Data points were fitted as a mono-exponential decrease (OriginPro version 8.5).

**Fig. 4.** Plasma and brain biodistribution of total \([^{18}F]\) radioactivity, unchanged \([^{18}F]\) DPA-714 and \([^{18}F]\)-M7 in rats at 120 minutes after injection of \([^{18}F]\)DPA-714. The concentrations of unchanged \([^{18}F]\)DPA-714 and \([^{18}F]\)-M7 were measured by radio-HPLC and are expressed as a percentage of the injected dose per gram of tissue (% ID/g). Values are the mean ± SD of two independent experiments (with two rats per experiment).
Identification of DPA-714 Metabolites. Positive ions ESI-MS and the major MS-MS fragments obtained for each metabolite (M1 to M6b) detected in microsomal fractions are summarized in Table 2. The mass spectrum of DPA-714 exhibited a molecular ion at \( m/z 399 \) [M+H+]. LC-MS analysis of authentic DPA-714 gave a retention time, mass spectrum, and fragmentation profile identical to those in microsome extracts. The main MS/MS fragments come from the departure of the fluoroethoxy group (\( m/z 353 \)), the \( N \)-diethyl group [\( N(CH_2CH_3)_2, m/z 326 \)], the diethylcarboxamide group [\( (CO)N(CH_2CH_3)_2, m/z 299 \)], and formally a hydroxyl function (\( m/z 382 \), keto-enol equilibrium). The mass spectrum of M1, M2, and M4 exhibited a molecular ion at \( m/z 415 \), 16 Da higher than the molecular ion for DPA-714, which is consistent with the insertion of an oxygen atom and the formation of three different alcohol derivatives by hydroxylation. In comparison with DPA-714, fragmentation of the molecular ion of M1, M2, or M4 generated the same product ions \( m/z 398, 369, 342, 315 \), showing an increment of 16 Da consistent with the presence of a hydroxyl group in the parent molecule and retention of this group in the main fragment ions. These hydroxylations probably occurred at the “upper part of the molecule,” the more likely position being the two methyl substituents of the phenyl ring (as evidenced by a fragment at \( m/z 397 \) corresponding to a loss of water (−18)). M3 exhibited a molecular ion at \( m/z 371 \) (−28), in agreement with the loss of one ethyl group by \( N \)-deethylation and consistent with the preservation of the fragments \( m/z 329 \) and \( m/z 326 \). M5 exhibited a molecular ion at \( m/z 353 \) (−46), in agreement with the loss of the fluoroethyl group by \( O \)-deethylation as attested by the loss of this group in the main fragment ions. M6a exhibited a molecular ion at \( m/z 431 \) (+32), consistent with the formation of a dihydroxylated derivative of DPA-714. M6b exhibited a molecular ion at \( m/z 387 \) consistent with \( N \)-deethylation (−28) and hydroxylation (+16) of DPA-714.

The low mass of \( ^{18}\text{F} \)DPA-714 administered to baboons prevented direct identification of metabolites in plasma by LC-MS. However incubating rat liver microsomes with DPA-714 and analysis by LC-MS identified two compounds having the same retention times as the radiometabolites detected in baboon plasma (Fig. 6). These two in vivo radiometabolites corresponded to \( N \)-dealkyl derivatives of \( ^{18}\text{F} \)DPA-714, \( ^{18}\text{F} \)-M3, and \( ^{18}\text{F} \)-M6b. The peaks detected between 2 and 3 minutes by UV-detection corresponded to the solvent front. The third in vivo radiometabolite was not detected in microsomes. However, based on the metabolism of zolpidem, a closely related analog of DPA-714 (Pichard et al., 1995), this highly polar compound was likely issued from further oxidation in plasma of a hydroxylated metabolite to generate a carboxylic acid derivative (\( ^{18}\text{F} \)-M7). From these findings, metabolic pathways for DPA-714 were proposed (Fig. 7). The main oxidative metabolism of DPA-714 in liver microsomes likely occurred at the methyl of the pyrimidine moiety (hydroxylation), at the nitrogen (\( N \)-deethylation) and oxygen atom (\( O \)-deethylation).

### Table 2: Microsomal metabolites of DPA-714

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Rt</th>
<th>[M+H+]</th>
<th>Main Fragments</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>16.87</td>
<td>415 (+16)</td>
<td>398, 369, 342, 315</td>
<td>r, (h)</td>
</tr>
<tr>
<td>M2</td>
<td>16.43</td>
<td>415 (+16)</td>
<td>398, 369, 342, 315</td>
<td>r, b, h</td>
</tr>
<tr>
<td>M3</td>
<td>16.10</td>
<td>371 (−28)</td>
<td>354, 325, 326, 299</td>
<td>r, b, h</td>
</tr>
<tr>
<td>M4</td>
<td>15.85</td>
<td>415 (+16)</td>
<td>398, 369, 342, 315</td>
<td>r, b, h</td>
</tr>
<tr>
<td>M5</td>
<td>15.05</td>
<td>353 (−46)</td>
<td>336, 307, 280, 253</td>
<td>r, b, h</td>
</tr>
<tr>
<td>M6a</td>
<td>14.28</td>
<td>431 (+32)</td>
<td>414, 385, 358, 331</td>
<td>r</td>
</tr>
<tr>
<td>M6b</td>
<td>13.88</td>
<td>387 (−12)</td>
<td>370, 235, 342, 315</td>
<td>r, (h)</td>
</tr>
</tbody>
</table>

b, baboon; h, human; r, rat.

![Fig. 5](https://example.com/f5.png) (A) analysis of metabolites of DPA-714 in rat and human liver microsomal incubations by HPLC. (B) in vitro metabolism of DPA-714 in different species: comparison of the rates of formation of DPA-714 metabolites in rat, baboon, and human liver microsomes (30-minute incubation) and expressed as nmol metabolite per mg of microsomal proteins per minute. Values are the mean ± SD of \( n = 5 \) independent duplicate experiments.
Oxidation of DPA-714 by cDNA-Expressed Human P450s Isoforms. Microsomes expressing individual human P450s isoforms were used to test their ability to catalyze the oxidation of DPA-714. The fraction of each metabolite was determined using LC-MS (Fig. 8). Human CYP3A4 exhibited the highest catalytic activity for the formation of M4 (9.3%, methyl-hydroxylation), M3 (7%, N-deethylation), and M5 (3.4%, O-deethylation) as in human liver microsomes (Table 2). CYP2D6 was able to convert significantly DPA-714 into M5 (2.32%, O-deethylation). Other human P450s catalyzed the formation of one to four minor metabolites (less than 1%).

Discussion

The major findings of the present study are as follows. First, the main ways of elimination of [18F]DPA-714 in baboons were the hepatobiliary (at least for the parent compound) and urinary (for radiometabolites) systems. Second, [18F]DPA-714 was rapidly metabolized in vivo in rats and baboons into at least three radiometabolites. All of these were more polar than the native radioligand. Third, LC-MS-MS analysis of metabolites produced in vitro identified [M+H+] ions consistent with hydroxyl, N-deethyl, and O-deethyl derivatives of DPA-714. Two of them, deethyl and deethyl-hydroxyl derivatives of DPA-714, have the same retention times than those detected in baboon plasma. Fourth, evidence indicates that a small amount of an acidic [18F]metabolite enters the rat brain at late times but does not appear to display specific binding. Fifth, we have developed a rapid and easy method of quantification of the radioligand and its radiometabolites in plasma by SPE that may be useful in human quantitative PET studies.

The peripheral in vivo biodistribution of [18F]DPA-714 by whole-body PET studies in baboons showed a high uptake of [18F]DPA-714 in TSPO-rich peripheral regions such as the heart, spleen, and kidneys, in accordance with the data observed in rodents (Fookes et al., 2008; James et al., 2008) and recently in humans (Arlicot et al., 2012). The other peripheral organs identified with moderate or high levels of radioactivity were involved in the metabolism or excretion of the tracer such as the liver and gallbladder. This radioactivity uptake has previously been shown to be specific by displacement experiments with nonlabeled DPA-714 (James et al., 2008). Time-activity curves in the gallbladder (data not shown) showed that the liver was a major route of elimination of [18F]DPA-714 in baboons, with a finding also described in humans (Arlicot et al., 2012). Urine was a route of excretion for radiometabolites. Indeed, a very small amount of residual [18F]DPA-714 was found in the urinary samples at 120 minutes after injection. A vertebral uptake of radioactivity was also observed in baboons as well as in humans (Arlicot et al., 2012).

The understanding of the metabolism of a candidate PET radioligand is important in establishing its value for quantitative imaging. Ideally, the candidate radioligand should not give rise to a radiometabolite that can enter the brain and significantly impair the identity of the PET signal. After we administered [18F]DPA-714 in baboons, the radioactivity represented by the nonmetabolized radioligand decreased rapidly in plasma. This radioactivity was also composed of a highly polar radiometabolite and of two radiometabolites with intermediate retention times and lipophilicity. Similar rapid plasma decreases for radiotracers have been observed in baboons with other TSPO ligands: [18F]PBR2102 and [18F]PBR111 (Fookes et al., 2008). In humans, [18F]PBR06 and [11C]PBR28 were shown to be rapidly metabolized in plasma (Dickstein et al., 2011) while [18F]DPA-714 decreased more slowly (Arlicot et al., 2012).

The low mass of radioligand administered to baboons (a few nanomoles) prevented direct identification of metabolites in plasma using LC-MS. However, incubating liver microsomes with DPA-714 and analyses by LC-MS identified at least seven metabolites (M1 to M6b) in rat and human, and four metabolites in baboon, two of them having the same retention times as the radiometabolites in baboon plasma. All were more polar than the native radioligand and in all except one, M5, the fluorine atom was preserved. At least four were the same in all three species, but the relative amount of individual metabolites differed between species. LC-MS data also demonstrated the formation by cytochrome P450 P450 in rat, baboon, and human liver microsomes of seven main metabolites issued from N-deethylation ([18F]-M3, m/z 371), hydroxylations giving three alcohol derivatives ([18F]-M1, [18F]-M2, [18F]-M4, m/z 415), di-hydroxylation ([18F]-M6a m/z 431), N-deethylation and hydroxylation ([18F]-M6b m/z 415), and O-deethylation ([18F]-M5, m/z 353).

Baboon microsomal studies suggested that the radiometabolites detected in plasma arose both from N-deethylation ([18F]-M3) and N-deethylation plus hydroxylation ([18F]-M6b). The third plasma radiometabolite ([18F]-M7) was not found in microsomal incubations and thus could not be identified by this technique. Based on the metabolism of zolpidem (Pichard et al., 1995), a closely structural analogs of DPA-714, this radiometabolite was proposed to be generated by further oxidation of a hydroxylated radiometabolite(s) in plasma leading to the formation of a carboxylic acid derivative ([18F]-M7).

Human recombinant cytochrome P450 was used as a predictive model of human metabolism. CYP3A4 was found to be the major enzyme responsible for one hydroxylation reaction, N-deethylation, and O-deethylation of DPA-714 as shown in cDNA-expressed human microsomes. The third plasma radiometabolite ([18F]-M7) was not found in microsomal incubations and thus could not be identified by this technique. Based on the metabolism of zolpidem (Pichard et al., 1995, a closely structural analog of DPA-714, this radiometabolite was proposed to be generated by further oxidation of a hydroxylated radiometabolite(s) in plasma leading to the formation of a carboxylic acid derivative ([18F]-M7).

In vivo, O-deethylation led to the formation of two metabolites: a nonlabeled metabolite (M5) undetectable by radio-HPLC or LC-MS due to the low mass administered and [18F]fluoroacetaldehyde, which is further converted to [18F]fluoroacetate (or fluoroacetic acid) and defluorinated into free [18F]fluoride. This transformation should not be quantitatively important, as shown by the low uptake of radioactivity in the bones (skull and vertebra). It should be noticed that red marrow is present in these bones and red marrow also contains TSPO (Kam...
et al., 2012). Indeed, PET cannot distinguish between free [$^{18}$F] trapped in the hydroxyapatite of bones and [$^{18}$F]DPA-714 binding to red marrow cells. The radioactivity found in the acetonitrile pellet increased with time, consistent with the formation of a radiometabolite such as [$^{18}$F]fluoroacetic acid during the biotransformation process of $O$-deethylation. [$^{18}$F]fluoroacetate has been proposed as a radiotracer for prostate tumors and for imaging of glial metabolism. This compound is known to be further converted to [$^{18}$F]fluoride (Tecle and Casida, 1989; Marik et al., 2009; Ponde et al., 2007; Nishii et al., 2012). In previous imaging studies in rodents, the free [$^{18}$F]fluoride contributed to a significant level of bone uptake in rodents after [$^{18}$F]DPA-714 administration (Fookes et al., 2008). The in vitro findings described in this report demonstrate the formation by rat, baboon, and human cytochrome P450 of $O$-dealkylated DPA-714 and thus [$^{18}$F]fluoroacetate. Our present results confirmed this hypothesis. In our study, the measurement of the radioactivity in the rat thighbone and in the bone marrow showed a higher level of radioactivity (almost 2-fold) in the bone marrow than in the cortical zone of the thighbone. This result, in accordance with previously published data (Fookes et al., 2008) may reflect TSPO expression in the bone marrow. The radioactivity in the cortical zone of the thighbone may result from the fixation of [$^{18}$F]fluoride, provided from [$^{18}$F]fluoroacetate generated by $O$-deethylation of the radioligand. In baboons, the radioactivity uptake observed in vertebra was probably due both to specific uptake of radioactivity in the bone marrow and to the uptake of [$^{18}$F]fluoride in the bones. Different metabolic pathways and species differences have been described for [$^{18}$F]fluoroacetate (Nishii et al., 2012). The differences in the extent of defluorination of [$^{18}$F]-DPA-714 between species (Fookes et al., 2008; James et al., 2008; Arlicot et al., 2012) may be explained by a difference in the metabolism of the radioligand by $O$-deethylation and a difference in the metabolic pathways of fluoroacetate (Tecle and Casida, 1989).

Experiments were performed in rats to gain greater insight into the metabolism of [$^{18}$F]DPA-714 and the potential contribution of radiometabolites to the brain radioactivity. Radiometabolites appeared quite rapidly in rat plasma. In the brain, no radiometabolite had been detected at earlier times, 10 minutes and 30 minutes after injection.

![Fig. 7. Proposed metabolic pathways for DPA-714 and its main metabolites in liver microsomes based on LC-MS and MS/MS experiments.](image-url)
$[^{18}F]$-M7 was detected in the brain at 120 minutes after injection. Ratios of ex vivo measurements of brain to plasma of $[^{18}F]$DPA-714 and $[^{18}F]$-M7 suggested the lack of specific accumulation of this radiometabolite.

Finally, a simplified method of quantification of $[^{18}F]$DPA-714 in plasma was developed using direct SPE to improve the estimation of a metabolite-corrected input function for future quantitative human PET studies. A similar exponential decrease of $[^{18}F]$DPA-714 in plasma was obtained with SPE and radio-HPLC, the latter considered as the reference method. Compared with radio-HPLC, the main advantages of the SPE method were the higher sensitivity (increased by a factor 80), faster processing (less than 30 minutes for more than 20 samples for SPE against 30 minutes for one time point by HPLC), the possibility of processing simultaneously multiple low radioactivity samples even at late times in the PET scan, and the low volume of plasma required. These advantages are particularly important in light of the decay of $[^{18}F]$-fluorine and the low activity injected to humans. Furthermore, this highly sensitive and specific procedure can be easily automated and used on a routine clinical basis. Another study by Katsifis et al. (2011) has also described a SPE method for measurement of intact TSPO ligands belonging to a closely related chemical family (PBR102 and PBR111), and they also found a good agreement between the SPE and HPLC methods.

In conclusion, $[^{18}F]$DPA-714 is rapidly and extensively converted in vivo into several radiometabolites in rats and baboons. In vitro rat, baboon, and human microsomal studies demonstrate the formation by cytochrome P450 of hydroxylated and deethylated metabolites, some of which are detected in rat and baboon plasma. Secondary oxidation in plasma of a $[^{18}F]$-hydroxylated metabolite is likely to produce the main radiometabolite, which crosses the rat brain-blood barrier. In vivo $O$-deethylation generates an unlabeled compound concomitantly with $[^{18}F]$fluoracetalddehyde, leading to formation of a small uptake of $[^{18}F]$ fluoride in the bones. The SPE developed for the quantification of the non-metabolized fraction of $[^{18}F]$DPA-714 in plasma is the method of choice for the quantification in humans with low-activity samples.

Authorship contributions:

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Contributed new reagents or analytic tools: Peyronneau, Damont, Kassiou.
Performed data analysis: Peyronneau, Saba, Goutal, Valette.
Wrote or contributed to the writing of the manuscript: Peyronneau, Bottlaender, Valette.

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