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Identification of metabolites of fluorine-18-labeled M2 muscarinic receptor agonist, 3-(3-[(3-fluoropropyl)thio]-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine, produced by human and rat hepatocytes

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Abstract

An accurate, rapid method for the determination of unmetabolized 3-(3-{(3-[^{18}F]fluoropropyl)thio}-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (FP-TZTP), a selective M2 muscarinic agonist, is necessary in order to obtain quantitative information from positron emission tomography (PET) imaging. Using LC–MS–MS to analyze products from cultured human and rat hepatocytes, we identified metabolites resulting from oxidation of the nitrogen in the tetrahydropyridine ring, sulfur-oxidation, demethylation of the tertiary amine, and oxidation of the tetrahydropyridine ring. From the knowledge of the structure of the metabolites, we have developed a two-step extraction sequence that allows rapid determination of the parent fraction in plasma without time-consuming chromatographic analysis. Published by Elsevier Science B.V.

Keywords: 3-(3-{(3-[^{18}F]Fluoropropyl)thio}-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine; FP-TZTP; Muscarinic receptor

1. Introduction

We have synthesized 3-(3-{(3-[^{18}F]fluoropropyl)thio} - 1,2,5 - thiadiazol - 4 - yl)- 1,2,5,6 - tetrahydro-1-methylpyridine ([^{18}F]FP-TZTP), and are evaluating its potential as an imaging agent of the muscarinic system for use in positron emission tomography (PET) [1–3]. Post-mortem evaluation of brain tissue from Alzheimer's subjects revealed a decrease of M2 receptor subtype in cortical regions [4]. We believe this ligand has the potential for determining if the decrease of M2 subtype occurs prior to the final stages of the disease and to evaluate treatment protocols.

[^{18}F]FP-TZTP displayed M2 selectivity in vitro [1] and its in vivo distribution in rats and rhesus monkeys was also consistent with M2 selectivity [2]. A single tissue compartment model can be used to convert PET imaging data into the biologically relevant K_1, the tissue uptake rate, and V, the volume

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of distribution [3]. *V* is linearly proportional to the receptor concentration. By using this model, we observed that the in vivo uptake in monkeys was sensitive to manipulation of endogenous acetylcholine through pre-infusion of the acetylcholine esterase inhibitor, physostigmine [3]. Infusion of physostigmine caused an increase in endogenous acetylcholine and, thus, higher receptor occupancy resulting in a lower *V* for [^{18}F]FP-TZTP. M2 receptors are widespread in the brain, so there is no clear receptor-free region that can be used as a reference region. In order to apply this model to experimental data, we need to know the time activity function of the unmetabolized [^{18}F]FP-TZTP in plasma and in brain tissue during the course of each imaging study.

We have previously conducted in vivo metabolism studies of [^{18}F]FP-TZTP in rat and monkey plasma by radio-thin-layer chromatography (TLC). All of the metabolites observed by radio-TLC in the monkey plasma have corresponding metabolite peaks in rat plasma although apparent differences in metabolic rate and proportions of metabolites were observed. We have shown in vivo that the radioactivity in rat brain is greater than 90% unmetabolized [^{18}F]FP-TZTP up to 45 min post-injection. Knowledge of the structure of the metabolites allows determination of their potential for brain uptake. In the present study, we identified the metabolites of [^{18}F]FP-TZTP produced by rat and human hepatocytes using high-performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometry (ESI-MS). We then synthesized the main proposed metabolites and compared them with HPLC–MS analyses of metabolites produced by hepatocytes. In addition, with knowledge of the structure of plasma metabolites, a simple extraction procedure was developed to facilitate the determination of the concentration of unmetabolized radiotracer in plasma as a function of time during the course of a PET study.

2. Experimental

2.1. Materials

3-(3-{(3-Fluoropropyl)thio}-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methylpyridine (**1**, FP-TZTP) and its radiolabeled analog [^{18}F]FP-TZTP were prepared as previously described [1]. 3-(3-{(3-Fluoropropyl)thio}-1,2,5-thiadiazol-4-yl)-pyridine (**4**) was prepared as previously described [1]. All commercially available reagents were purchased from Aldrich (Milwaukee, WI, USA). Water (HPLC grade) and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA, USA). HPLC purity information for each of the metabolites was determined by UV absorbance at 254 nm, expressed as area percent of all peaks, utilizing the chromatographic conditions described below for HPLC–MS.

2.2. Chemical syntheses

2.2.1. 5-(4-[(3-Fluoropropyl)sulfinyl]-1,2,5-thiadiazol-3-yl)-1-methyl-1,2,3,6-tetrahydropyridine (2)

1 (108 mg, 0.395 mmol) was dissolved in 2 ml 0.6 *M* HCl and cooled in ice. Oxone (123 mg, 0.2 mmol), was dissolved in 1 ml water and added to the acidic solution of substrate. After 15 min, TLC indicated complete conversion of starting material to the product. The reaction solution was treated with 1.5 ml 1 *M* NaOH and extracted with 2×5 ml CH_2Cl_2. The combined organic layers were dried over Na_2SO_4 and evaporated. The residue was subjected to flash chromatography (silica gel, 90% $CHCl_3$, 9% methanol, 1% NH_4OH) to give 108 mg of product.

^{1}H-Nuclear magnetic resonance (NMR) δ 2.05–2.4 (m, 2H), 2.48 (s, 3H), 2.48–2.72 (m, 3H), 3.25–3.60 (m, 4H), 4.60 (dm, J=46 Hz, 2H), 6.60–6.65 (m, 1H).

^{13}C-NMR 23.65 (d, J=21 Hz), 26.72, 45.71, 48.91 (d, J=3 Hz), 50.60, 82.09 (d, J=168 Hz), 129.34, 132.63, 160.16, 160.53.

^{19}F-NMR (external $CFCl_3$) −220.651 (tt, J=47, 24 Hz).

LC–MS 290 [M+H]. Purity by UV (254 nm)= 95.3%.

2.2.2. 5-(4-[3-(3-Fluoropropyl)sulfanyl]-1,2,5-thiadiazol-3-yl)-1,2,5,6-tetrahydropyridine (3)

1 (90 mg, 0.32 mmol) was dissolved in 3 ml dichloroethane, treated with 1-chloroethyl chloroformate (50 μl, 0.32 mmol), and heated at 80°C for 2 h. No reaction was detected, so Proton Sponge (6 mg, 0.03 mmol) was added. After an additional 3 h,

TLC indicated that a reaction was occurring. An additional 50 μl 1-chloroethyl chloroformate was added and heated for 17 h. A third equivalent of 1-chloroethyl chloroformate was added and after an additional 6 h the reaction was deemed complete by TLC. The reaction was acidified with 30 μl 4 *M* HCl in dioxane and filtered through a short plug of silica gel to remove proton sponge. The solvent was evaporated and the residue dissolved in methanol and heated to reflux overnight. The methanol was evaporated and the residue dissolved in CH_2Cl_2 and washed with water containing a few drops of 1 *M* NaOH. Evaporation of the CH_2Cl_2 gave 84 mg of oil.

^{1}H-NMR 2.06–2.50 (m, 4H), 2.40 (brs, 1H), 3.05 (t, *J*=5.9 Hz, 2H), 3.40 (t, *J*=7.1 Hz, 2H), 3.81–3.84 (m, 2H), 4.27 (dt, *J*=47, 5.5 Hz, 2H), 6.77 (quin, *J*=2 Hz, 2H).

^{13}C-NMR 25.80, 28.83 (d, *J*=5 Hz), 29.95 (d, *J*=20 Hz), 42.21, 46.28, 82.30 (d, *J*=166 Hz), 130.49, 131.33, 155.43, 157.16.

Gas chromatography (GC)–MS retention time: 8.65 min *m*/*z* (relative abundance) 259 (100), 198 (50), 169, (70). Purity by GC >90.5%.

LC–MS 260 [M+H]. Purity by UV (254 nm)= 96.7%.

*2.2.3. 3-(4-[(3-Fluoropropyl)sulfinyl]-1,2,5-thiadiazol-3-yl)-pyridine (**5**)*

4 (100 mg, 0.392 mmol) was dissolved in 2 ml water. HCl (100 μl of 12 *M*) was added followed by a solution of Oxone (123 mg, 0.2 mmol) in 1 ml water. The solution was allowed to stand for 2 h. The solution was poured into 1.5 ml 1 *M* NaOH and extracted with CH_2Cl_2 (3×2 ml). The combined organic layers were dried (Na_2SO_4) and evaporated. The residue was subjected to flash chromatography (100% ethyl acetate) to give 74 mg of the product.

^{1}H-NMR 2.10–2.38 (m, 2H), 3.30–3.60 (m, 2H), 4.60 (dm, *J*=47 Hz, 2H), 7.49 (dd, *J*=7, 5 Hz, 1H), 8.26 (dt, *J*=8, 2 Hz, 1H), 8.76 (dd, *J*=5, 2 Hz, 1H), 9.10 (d, *J*=2 Hz, 1H).

^{13}C-NMR 23.78 (d, *J*=20 Hz), 49.34 (d, *J*=3 Hz), 82.08 (d, *J*=158 Hz), 123.73, 127.04, 136.51, 149.83, 151.48, 159.05, 160.74.

LC–MS 272 [M+H]. Purity by UV (254 nm)= 96.5%.

*2.2.4. 3-(4-[(3-Fluoropropyl)sulfanyl]-1,2,5-thiadiazol-3-yl)-1-methylpyridinium iodide (**6**)*

4 (300 mg, 1.17 mmol) was dissolved in 2.5 ml acetone, treated with CH_3I (366 μl, 5.88 mmol), and allowed to stand overnight. The solution was scratched to cause precipitation of a yellow solid. The solid was filtered to give the product (380 mg).

^{1}H-NMR (DMSO-d6) δ 2.14 (dquin, *J*=25, 7 Hz, 2H), 3.45 (t, *J*=7 Hz, 2H), 4.46 (s, 3H), 4.58 (dt, *J*=47, 5 Hz, 2H), 8.35 (dd, *J*=8, 6 Hz, 1H), 9.00 (d, *J*=8 Hz, 1H), 9.13 (d, *J*=6 Hz, 1H), 9.46 (s, 1H).

^{13}C-NMR (DMSO-d6) 28.86 (d, *J*=5 Hz), 29.30 (d, *J*=20 Hz), 48.47, 82.30 (d, *J*=163 Hz), 127.81, 130.77, 143.14, 145.19, 146.18, 151.59, 156.50.

ESI-MS 270 [M+H]. Purity by UV (254 nm)= 94.8%.

*2.2.5. 3-(4-[(3-Fluoropropyl)sulfinyl]-1,2,5-thiadiazol-3-yl)-1-methylpyridinium iodide (**7**)*

5 (59 mg, 0.217 mmol) was dissolved in acetone (0.5 ml), treated with CH_3I (154 mg, 1.088 mmol), and allowed to stand at room temperature overnight. An oily second layer formed. The acetone was decanted from the oil. The oil was triturated with ether and then dissolved in CH_2Cl_2 and the solvent evaporated to give 75 mg of yellow foam.

^{1}H-NMR (CD_3CN) 1.94–2.26 (m, 2H), 3.42 (t, *J*=7 Hz, 2H), 4.47 (s, 3H), 4.68 (dt, *J*=46, 5 Hz, 2H), 8.21 (dd, *J*=7, 7 Hz), 8.96 (d, *J*=7 Hz), 9.37 (brs, 1H).

^{13}C-NMR (CD_3CN) 24.20 (d, *J*=20 Hz), 49.93, 51.17 (d, *J*=3 Hz), 82.54 (d, *J*=164 Hz), 128.66, 132.42, 146.42, 146.86, 155.41, 163.00.

LC–MS 286 [M+H]. Purity by UV (254 nm)= 97.3%.

*2.2.6. 5-(4-[(3-Fluoropropyl)sulfanyl]-[1,2,5]thiadiazol-3-yl)-1-methyl-1,2,3,6-tetrahydropyridine 1-oxide (**8**)*

1 (101 mg, 0.370 mmol) was dissolved in 0.5 ml methanol. The solution was treated with 30% H_2O_2 (42 μl) and allowed to stir at room temperature. After 4 h, an additional 42 μl portion of H_2O_2 was added and the solution allowed to continue overnight. TLC analysis indicated that conversion was incomplete. A third portion of H_2O_2 (42 μl) was added and the reaction stirred for another day. The reaction was concentrated and subjected to flash

chromatography (silica gel, 15 mm×6 in. column, chloroform–methanol–ammonium hydroxide) (1 in.=2.54 cm). The elution consisted of 100 ml of: chloroform–methanol–ammonium hydroxide (90:9:1) followed by 100 ml of chloroform–methanol–ammonium hydroxide (85:13.5:1.5). The product was collected and evaporated to give 112 mg (115%) of product.

^{1}H-NMR δ 2.197 (dp, J=27, 7 Hz, 2H), 2.5–2.7 (m, 1H), 3.0–3.33 (m, 1H), 3.35 (s, 3H), 3.4–3.5 m, 4H), 4.41 (brs, 2H), 4.59 (dt, J=47, 5.3 Hz, 2H), 6.95 (brt, J=5 Hz, 1H).

^{13}C-NMR 23.78, 28.89 (d, J=4.9 Hz), 29.79 (d, J=20.0 Hz), 58.53, 62.70, 67.75, 82.10 (d, J=166 Hz), 125.63, 127.48, 154.33, 155.43.

Chemical ionization (CI)-MS (NH_3) 307 (M+18), 290 (M+1), 275 (M−15).

LC–MS 290 [M+H]. Purity by UV (254 nm)= 95.2%.

2.3. HPLC–MS

All experiments were performed on a Finnigan LCQ mass spectrometer (Finnigan, San Jose, CA, USA) equipped with an ESI interface and coupled to a Hewlett-Packard 1100 HPLC system (Hewlett-Packard, San Fernando, CA, USA). The separation was accomplished on YMC-CN column (150×4.6 mm, YMC, Waters, Milford, MA, USA) with gradient elution at 0.5 ml/min. The solvent system was composed of 50 m*M* ammonium acetate (A) and acetonitrile (B). A gradient of 30–85% B over 20 min was used. The entire column effluent was passed through a UV (254 nm) and a flow-count radioactivity detector (Bioscan, Washington DC, USA) and introduced into the ESI interface. Mass spectral data were acquired in the positive ion mode and using a high flow tune method. Ion detection was achieved with ESI using a spray voltage of +4200 V, capillary heater temperature of 200°C, sheath gas flow of 80 ml/min (N_2), and an auxiliary gas flow of 20 ml/min (N_2), and relative collision energy of 25–40%.

2.4. Analysis of N-oxide *8*

The peak corresponding to structure **8** was isolated by HPLC and infused into a Finnigan TSQ700 operated in the electrospray mode [5] with a 3.3 kV spray voltage.

2.5. Hepatocyte incubation

In vitro metabolism studies of **1**, **2**, and **3** were conducted using cryopreserved hepatocytes from male Sprague–Dawley rats and male human liver tissue (In vitro Technologies, Baltimore, MD, USA). The cells, stored in liquid nitrogen, were thawed rapidly in a 37°C water bath and gradually diluted with cell culture medium (RPMI Medium 1640 media, Life Technologies, Rockville, MD, USA). After washing the cells with the medium, the viable cell concentration was adjusted to 1.0 million per ml and the resulting cell suspension was incubated at 37°C in CO_2–air (5:95) for 15 min prior to the introduction of test compound. To 1 ml of cell suspension, 10 μl of the stock solution of FP-TZTP (2.0 mg/ml in 10% aqueous EtOH) was added to give a final concentration of 20 μg/ml. The suspension was maintained at 37°C, 100 μl of cell suspension was removed and added to 100 μl acetonitrile at 0.5, 2, and 4 h. Each suspension was centrifuged at 3310 *g* for 5 min. The metabolites in 20 μl supernatant were analyzed by LC–MS.

For the analysis of radiolabeled **1**, the above procedure was followed with the following modifications. To 1.0 ml suspension of cells and unlabeled **1**, 0.5 mCi of [^{18}F]FP-TZTP in 20 μl EtOH was added. Metabolite analysis was conducted at 0.5 and 2 h.

2.6. In vivo rat metabolism study

All animal studies are conducted under a protocol approved by the NIH Animal Care and Use Committee. A metabolism study was performed in normal male Sprague–Dawley rats weighing about 250 g. The rats were administered 0.5 mCi [^{18}F]FP-TZTP along with 2 mg of FP-TZTP in 0.1 ml of 10% EtOH saline solution through the tail vein. At 10 and 30 min after injection, the rat was sacrificed and 2 ml blood was removed. After centrifugation at 17 000 *g* for 4 min, 0.5 ml serum was taken. An equal volume of acetonitrile was added and centrifuged at 17 000 *g* for 4 min. Supernatant (20 μl) was injected into the

HPLC system with a radioactivity detector and analyzed by LC–MS.

2.7. Extraction procedure

Blood containing [^{18}F]FP-TZTP metabolites was centrifuged at 17 000 *g* for 4 min and the plasma was separated. To 150 μl plasma, 450 μl buffer (125 m*M* KCl adjusted to pH 12.5 with NaOH) and 1.2 ml organic solvent (hexane–EtOAc, 4:1) were added. After mixing, the layers were separated by centrifugation at 2500 *g* for 5 min. The aqueous phase was frozen in dry ice; the organic phase was collected and treated with 50 μl of acetic anhydride, and then extracted with 1.2 ml of 0.1 *M* HCl. The aqueous phase contained unmetabolized parent compound. All phases were counted in a gamma counter to determine extraction efficiency.

3. Results

3.1. Chemical synthesis of proposed metabolites

Syntheses of proposed metabolites identified by LC–MS were conducted, when practical, as shown in Fig. 1. All products were characterized by MS and ^{1}H-NMR. Sulfur oxidation products, **2** and **5**, were obtained by oxidation of the appropriate precursor with one equivalent of Oxone. Metabolite **3** was prepared by demethylation of FP-TZTP (**1**) using 1-chloroethyl chloroformate. Pyridinium metabolites, **6** and **7**, were obtained by methylation of the corresponding pyridine analogue with methyl iodide. The major metabolite **8**, the *N*-oxide of 1-methyltetrahydropyridine, was obtained synthetically by preferentially oxidizing the nitrogen without oxidizing the alkyl sulfur atom.

3.2. Identification of metabolites produced by rat and human hepatocytes

Rat hepatocytes were cultured with [^{18}F]FP-TZTP with (carrier-added) or without (no-carrier-added) the addition of 20 μg unlabeled FP-TZTP in 1 ml incubation solution. The time course of metabolites from the sequential analysis of the no-carrier-added experiment revealed that the parent compound was nearly consumed within 2 h concomitant with the formation of seven radiolabeled metabolites. The carrier-added experiment showed slower metabolism. The 30 min, carrier-added sample was selected for identification of the metabolites by LC–MS–MS (Table 1 and Fig. 2). An *N*-oxide (**8**, Fig. 4) is the major radioactive metabolite. Structure confirmation was obtained for all compounds, except oxidation product (**9**), by comparison of retention time and MS–MS fragmentation with the synthesized standards.

FP-TZTP was identified by parent ion [M+H]

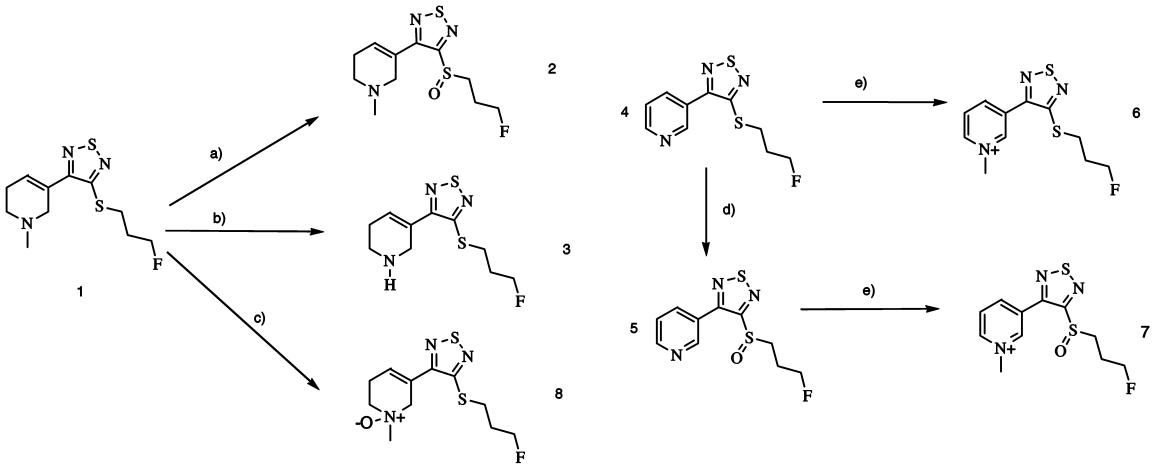

Fig. 1. Synthesis of FP-TZTP metabolites. (a) 1 equiv. Oxone; (b) $CH_3ClCHCOCl$; (c) H_2O_2; (d) 1 equiv. Oxone; (e) CH_3I.

Table 1
Radioactive metabolites formed by rat and human hepatocytes

Retention (min)	Identity	Parent $[M+H]^+$ m/z (MS–MS)
15.5	(1)	274 (214)
13.5	(3)	260 (231)
13.1	(6)	270 $[M]^+$ (210)
12.0	(9)	286 (226)
10.4	(8)	290 (273, 230)
8.0	(2)	290 (230)
4.9	Unknown	
3.6	Fluoride	

(m/z 274) and MS–MS fragment (m/z 214) resulting from cleavage of the 3-fluoropropyl side chain. The major metabolite (**8**) exhibited a molecular ion $[M+H]^+$ of m/z 290, which is 16 more than the parent FP-TZTP, suggesting an oxidation product. Other MS–MS fragments include m/z 273 (loss of OH) and m/z 230 (loss of 3-fluoropropyl side chain). Confirmation of **8** as an *N*-oxide came from literature precedent on the metabolism of a structurally related compound, xanomeline, by the group at Eli Lilly [6–8]. In their work, the position of oxidation was determined by observation of a MS–MS fragment of m/z 60 and rationalized as a fragmentation of the tetrahydropyridine ring. The Finnigan TSQ mass spectrometer is required to see this mass peak because the LCQ uses a different secondary ionization technique. Our analysis on the Finnigan TSQ of the major metabolite peak also showed a MS–MS ion of m/z 60 compared to a fragment m/z 44 for parent FP-TZTP. The structure **8** was also confirmed by comparison to the independently synthesized standard.

Metabolite peaks at 8.0, 13.5, and 13.1 min were identified as **2**, **3**, and **6**, respectively, by co-elution with the synthesized standard and comparing MS–MS fragments. The metabolite **2, 3** and **6** resulted from sulfur-oxidation, demethylation of the tertiary amine, and dehydrogenation of the tetrahydropyridine ring, respectively. The metabolite eluting at 12.0 min exhibited $[M+H]^+$ m/z 286 suggesting oxidation of **6**. N-Oxidation is precluded by the

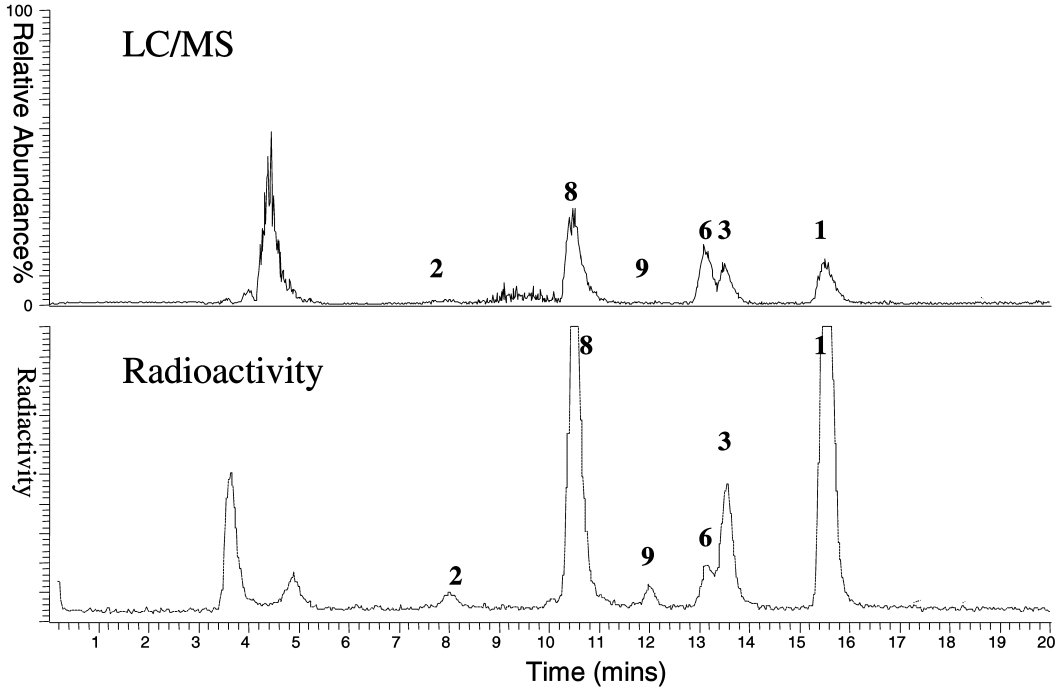

Fig. 2. Chromatogram of carrier-added [^{18}F]FP-TZTP metabolites produced by 0.5 h incubation of rat hepatocytes. The HPLC elution was monitored by a radioactivity detector (lower chromatogram) and then MS (upper chromatogram). The peaks are labeled with the assigned structure.

positive charge of the methylpyridinum and sulfur oxidation is ruled out because the synthesized standard **7** elutes at 7.30 min. We believe the structure may be a pyridine amide **9** analogous to that reported by Christensen et al. [9].

In addition to the major metabolites of FP-TZTP identified above, two more polar radioactivity peaks were found in the radiochromatograms (Figs. 2 and 3). The peak eluting at the solvent front is identified as [^{18}F]fluoride based on co-elution with authentic [^{18}F]fluoride. The remaining peak is unidentified as we could not arrive at a structural assignment based on the MS–MS data.

Compared with rat hepatocytes, human hepatocytes produced a similar metabolite profile (Table 1 and Fig. 3). An *N*-oxide **8** (Fig. 4) was also the major metabolite identified by LC–MS and radio chromatography. However, **8** was not obtained with the same dominance as observed in rat hepatocytes. Demethylation **3** and *S*-oxidation **2** metabolites were also produced by human hepatocytes. The ring oxidation metabolite **6** and its hydroxylated analogue **9** were also found from human hepatocytes.

3.3. Comparison of metabolites in vivo and in vitro

The 10 and 30 min rat plasma were analyzed after intravenous injection of carrier added [^{18}F]FP-TZTP. HPLC with radioactivity and mass spectrometry detection indicated that the metabolism profile in vivo is similar with that of in vitro. At 30 min, the parent **1** represents 91% ($n=2$) of extracted brain radioactivity and less than 5% ($n=2$) of the plasma radioactivity. The major metabolite **8** was unable to pass the blood–brain barrier.

3.4. Development of a rapid extraction method for determination of percent parent [^{18}F]FP-TZTP from metabolites

With knowledge of the structural identity of the

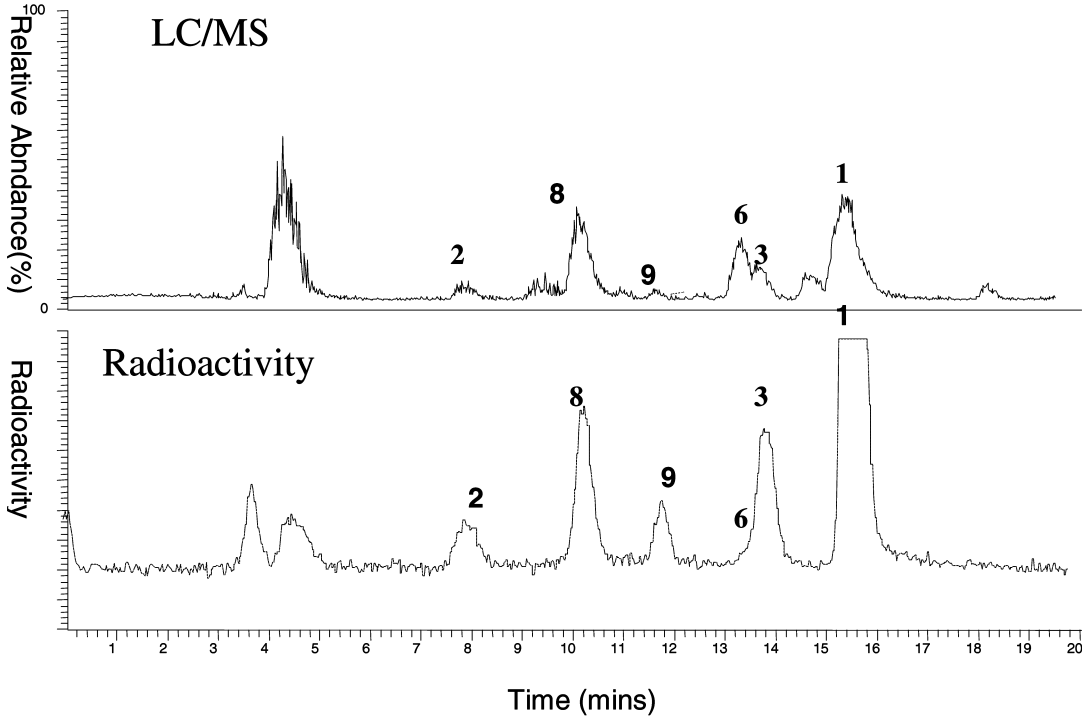

Fig. 3. Chromatogram of carrier-added [^{18}F]FP-TZTP metabolites produced by 2 h incubation of human hepatocytes. The HPLC elution was monitored by a radioactivity detector (lower chromatogram) and then MS (upper chromatogram). The peaks are labeled with the assigned structure.

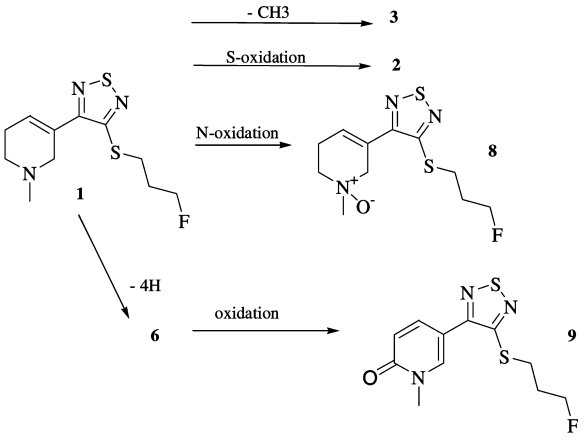

Fig. 4. Proposed metabolic pathway.

plasma metabolites, a simple extraction method for quantification of unmetabolized [^{18}F]FP-TZTP in plasma samples was developed. Extraction of metabolites from a pH 12.5 buffer phase (125 m*M* KCl, adjusted with NaOH) with ethyl acetate–hexane (1:4) results in the efficient extraction of parent FP-TZTP, its desmethyl metabolite **3** and pyridine amide metabolite **9** but leave all zwitterionic, cationic, and the sulfur oxidation metabolites in the aqueous phase (Fig. 5A). Separation of metabolite **3** and **9** from parent compound was achieved by addition of Ac_2O to the organic phase to convert **3** to the corresponding acetamide. Then 0.1 *M* HCl solution was used to extract the basic parent [^{18}F]FP-TZTP from the organic phase (Fig. 5B). LC–MS identification indicated that the HCl aqueous phase contains only parent compound **1**. We previously established that in the first extraction pure [^{18}F]FP-TZTP is extracted with 95±4% (n=5). The extraction efficiency for pure [^{18}F]FP-TZTP for the complete two step procedure is 88±3% (n=5). After applying corrections for extraction efficiency, the percent parent in the original plasma sample can be calculated.

4. Discussion

Determining the metabolic profiles of new radiopharmaceuticals is an important aspect of their design, development, and validation. Identification of metabolites allows rational synthetic design to minimize their presence and also facilitates the development of simple systems to determine the concentrations in plasma for those compounds capable of entering the target tissue. [^{18}F]FP-TZTP (**1**) is being evaluated as a muscarinic receptor imaging agent [1]. Since M2 muscarinic receptors are widely distributed throughout the human brain, no gray matter region devoid of receptors has been found which could serve as a reference region for quantitation [10]. Consequently, we must determine the delivery of radioligand to the brain by construction of an input function that is the integral of the plasma concentration of unmetabolized [^{18}F]FP-TZTP [3]. The determination of the plasma concentration requires correction for metabolites present in the plasma.

From our previous studies of in vivo metabolism of [^{18}F]FP-TZTP using radio-TLC in rat and monkey, we detected three major radiolabeled metabolites. Only one metabolite slowly accumulated in the brain of rats [1]. In order to assess the delivery of parent and metabolite to the brain, we needed more information on the structural identity of metabolites. In vitro metabolism studies with both human and rat hepatocytes provide a method of rapidly generating metabolic profiles expected from liver metabolism and some insight into expected species differences [11].

We employed LC–ESI-MS–MS, a powerful technique that reliably allows structural identification of metabolites from complex biological matrices, to identify metabolites generated in vitro by rat and human hepatocytes. We have optimized the chromatographic method for separation of metabolites from in vitro hepatocyte incubation. This method can reliably achieve limits of quantitation in the 1–10 pg/ml of incubation solution for FP-TZTP and its major metabolites. The sensitivity level was based on the MS ion peak with S/N greater than 4.

Our proposed metabolic pathway based on metabolite identification is shown in Fig. 4. We tested **2** and **3** in rat and human hepatocytes and observed no metabolism within the same time course. This suggests that these two are metabolic end products. There remains one polar radioactive metabolite that

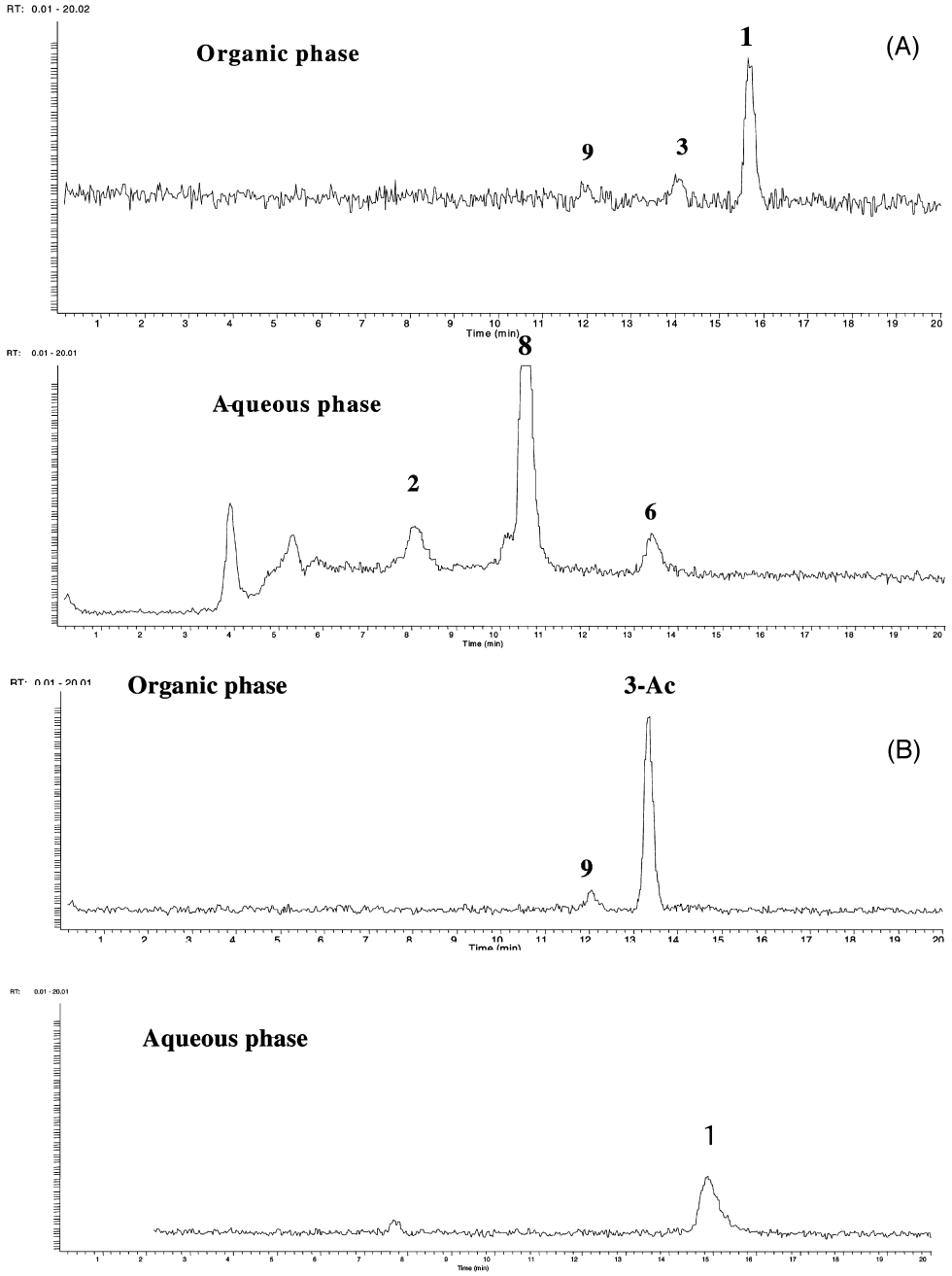

Fig. 5. (A) Radio-HPLC of the layers from the first extraction. The extraction was according to the following procedure: to 150 μl human plasma and 100 μl 2 h [^{18}F]FP-TZTP with (carrier-added) human hepatocytes incubation solution, 450 μl buffer (125 m*M* KCl adjusted to pH 12.5 with NaOH) and 1.2 ml organic solvent (hexane–EtOAc, 4:1) were added. After mixing, the layers were separated by centrifugation at 2500 *g* for 5 min. A 100-μl volume of organic phase and 50 μl aqueous phase was loaded onto the HPLC system. (B) Radio-HPLC of the layers from the second extraction. The extraction was according to the following procedure: the organic phase from the first extraction was collected and treated with 50 μl of acetic anhydride, and then extracted with 1.2 ml of 0.1 *M* HCl. After mixing, the layers were separated by centrifugation at 2500 *g* for 5 min. The organic solvent in 600 μl organic phase was evaporated and dissolved in 100 μl acetontrile which was loaded onto the HPLC system. A 200-μl volume of aqueous phase was loaded onto the HPLC system.

is unidentified, as we could not rationalize any structure from the mass spectral data in the area of the observed radioactivity peak. Previous studies on related compounds by the Eli Lilly group [6–8] showed metabolites arising from oxidation of the alkyl side chain. While we cannot rule out some metabolism of the alkyl chain, the fluorine atom at the end of the chain would be expected to change the metabolism. Any terminal oxidation to generate an acid would result in loss of fluoride and, therefore, the observed radioactive metabolite would be fluoride. If a terminal acid were generated we would expect to observe the mass spectral signals. Our gradient chromatography method would certainly elute this type of metabolite. We surmise that the terminal fluorine atom inhibits side chain oxidation.

In order to provide quantitative results from a PET study, the concentration of unmetabolized radioligand in blood plasma and in the target tissue must be determined. The plasma assay traditionally utilizes extraction followed by HPLC or TLC. Since HPLC or TLC are more labor intensive, time consuming, and generally yield low count rates per peak, we developed a simplified extraction procedure based on the knowledge of metabolite structures. This procedure could be applied to multiple samples in order to improve the reliability of radioactive counting of low count rate samples. The initial partitioning of basic (pH 12.5) plasma separates the metabolites based on polarity. The parent compound (**1**), the desmethyl compound (**3**), and the trace pyridine amide product (**9**) are extracted into the organic phase while the remaining metabolites are retained in the basic aqueous phase. In studies of extraction efficiency, parent compound (**1**) is extracted into the organic phase with high efficiency ($95\pm4\%$, $n=5$). Treatment of the organic phase with acetic anhydride converts **3** into its neutral acetamide. The organic phase is extracted with 1 *M* HCl. The resulting aqueous phase contains **1**, the only remaining basic component, while the acetamide and metabolite **9** remain in the organic layer. When using pure parent **1**, the total extraction efficiency of **1** for the two extraction steps is $88\pm3\%$ ($n=5$). Radioactivity assay of the acid phase and correcting for extraction efficiencies will provide the proportion of **1** in the original plasma sample.

5. Conclusion

We have determined the metabolic profile of [^{18}F]FP-TZTP (**1**) generated by rat and human hepatocytes. Structures were determined by LC–MS–MS and, where possible, comparison with synthesized standards. With the knowledge of the structures, we have developed an extraction procedure that quantitatively separates parent ligand from its metabolites. This allows us to determine the plasma concentration of the unmetabolized radiotracer by a simple technique that allows multiple sampling with high statistical accuracy. We can provide an accurate plasma input function of [^{18}F]FP-TZTP for determination of changes in M2 receptor concentration as a function of Alzheimer's disease

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References

[1] D.O. Kiesewetter, J.T. Lee, L. Lang, S.H. Park, C.H. Paik, W.C. Eckelman, J. Med. Chem. 38 (1995) 5.
[2] D.O. Kiesewetter, R.E. Carson, E.M. Jagoda, P. Herscovitch, W.C. Eckelman, Synapse 31 (1999) 29.
[3] R.E. Carson, D.O. Kiesewetter, E.M. Jagoda, M.G. Der, P. Herscovitch, W.C. Eckelman, J. Cereb. Blood Flow Metab. 18 (1998) 1130.
[4] R. Rodriguez-Puertas, J. Pascual, T. Vilaro, A. Pazos, Synapse 26 (1997) 341.
[5] S. Konig, H.M. Fales, K.D. Haegele, Anal. Chem. 70 (1998) 4453.
[6] T.A. Gillespie, T.J. Lindsay, D. Cornpropst, P.L. Bonate, T.G. Skaggs, A.F. DeLong, L.A. Shipley, in: Biological and Biotechnological Applications of ESI-MS, ACS Symposium Series, Vol. 619, American Chemical Society, 1996, p. 315.
[7] A.T. Murphy, P.L. Bonate, S.C. Kasper, T.A. Gillespie, A.F. DeLong, Biol. Mass Spectrom. 23 (1994) 621.
[8] A.T. Murphy, S.C. Kasper, T.A. Gillespie, A.F. DeLong, J. Chromatogr. B 668 (1995) 273.

[9] E.B. Christensen, J.B. Andersen, H. Pedersen, K.G. Jensen, L. Dalgaard, Drug Metab. Dispos. 27 (1999) 1341.
[10] D.D. Flynn, G. Ferrari-DiLeo, D.C. Mash, A.I. Levey, J. Neurochem. 64 (1995) 1888.
[11] D.W. Straughan, J.H. Fentem, M. Balls, in: J.V. Castell, M.J. Gomez-Lechon (Eds.), In Vitro Method in Pharmaceutical Research, Academic Press, 1997, p. 1.