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Liquid chromatography–tandem mass spectrometry identification of metabolites of two 5-HT_{1A} antagonists, *N*-{2-[4-(2-methoxylphenyl)piperazino]ethyl}-*N*-(2-pyridyl) *trans*- and *cis*-4-fluorocyclohexanecarboxamide, produced by human and rat hepatocytes

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Abstract

Two 5-HT_{1A} antagonists, t-FCWAY and c-FCWAY, were developed as imaging agents for positron emission tomography (PET). In order to evaluate these compounds, hepatocytes from both human and rat were utilized to produce metabolites and LC–MS–MS was used to identify metabolites. These in vitro metabolism studies indicate that hydrolysis of the amide linkage is the major metabolism pathway for humans, whereas aromatic ring-oxidation is the major metabolism pathway for rat. The rat hepatocyte results correlate well with in vivo rat metabolism studies. Based on the structures of the metabolites, we have developed an extraction procedure to determine the concentration of the parent compound in plasma. Published by Elsevier Science B.V.

Keywords: 5-HT_{1A} antagonists

1. Introduction

We have developed two fluorinated analogues of Wyeth-Ayerst's selective 5-HT_{1A} antagonists WAY100635 (**1**, Table 1). *N*-{2-[4-(2-methoxyphenyl)piperazino]ethyl}-*N*-(2-pyridinyl) *trans*-4-fluorocyclohexanecarboxamide (**2a**, t-FCWAY) and *N*-{2-[4-(2-methoxylphenyl)piperazino]-ethyl}-*N*-(2-pyridinyl) *cis*-4-fluorocyclohexanecarboxamide (**2b**, c-FCWAY) [1] were synthesized in both unlabeled and radiolabeled form [1,2]. The *trans* isomer (**2a**) has higher affinity than the *cis* isomer (**2b**). When labeled with the short-lived positron emitting fluorine-18 ($t_{1/2}$=109.8 min), t-FCWAY is a potential radioligand for static measurement of 5-HT_{1A} receptor distribution in the human brain utilizing positron emission tomography (PET) in patients with psychiatric and neurological disorders, such as anxiety, depression and Alzheimer' disease [1,3].

The determination of 5-HT_{1A} receptor concen-

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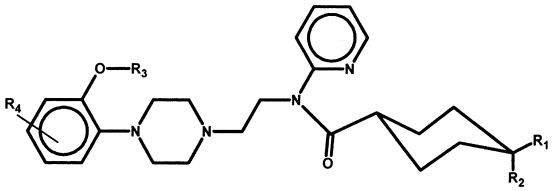
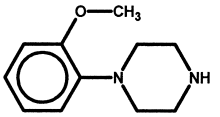
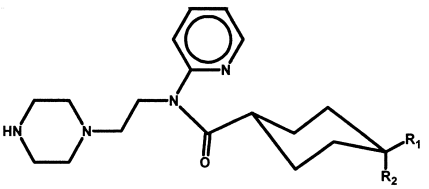
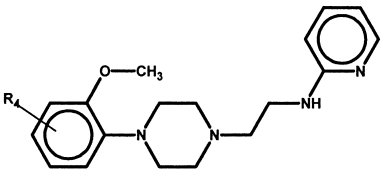
Table 1
The structures of FCWAY and metabolites



Structure	Name	R_1	R_2	R_3	R_4
1	WAY100635	H	H	CH_3	H
2a	t-FCWAY	F	H	CH_3	H
2b	c-FCWAY	H	F	CH_3	H
3a	t-OFCWAY	F	H	CH_3	OH
3b	c-OFCWAY	H	F	CH_3	OH
4a	Glu-t-OFCWAY	F	H	CH_3	OGlu
4b	Glu-c-OFCWAY	H	F	CH_3	OGlu
5a	t-OHCWAY	OH	H	CH_3	H
5b	c-OHCWAY	H	OH	CH_3	H
5c	DOHWAY	Keto		CH_3	H
6a	t-desMeFCWAY	F	H	H	H
6b	c-desMeFCWAY	H	F	H	H
7					
8a (R_1 = F, R_2 = H)					
8b (R_1 = H, R_2 = F)					
9 (R_4 = H)	WAY100634				
10 (R_4 = OH)	OWAY100634				

tration in human brain using PET is a major theme of current research in psychiatric and neurological disorders [1]. In order to provide quantitative results from a PET study, one must be able to determine the concentration of the unmetabolized radioligand in blood plasma and target tissue. The concentration of radiolabeled metabolites in target tissue is determined by knowledge of metabolite profile of the parent compound and studies of tissue extraction in rats. The concentration determination of parent in blood plasma has traditionally relied on extraction followed by high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC). Of course, the procedure can be simplified if the extraction only extracts parent compound.

The defluorination of FCWAY (**2a**, **2b**) was found in previous metabolic studies of in vivo (extraction of rat plasma [1]) and in vitro (incubation with rat hepatocytes [4]). Defluorination was observed to a greater extent from fluorocyclohexanecarboxylic acid

[4]. The study [4] also showed a linear correlation between in vitro defluorination and in vivo femur uptake of [^{18}F]fluoride in rats. Those studies, however, provide limited information about the structural identity of the radiolabeled metabolites.

In addition to our previous metabolism studies of FCWAY [1,4], the lead compound (*O*-methyl-^{11}C or carbonyl-^{11}C) WAY100635 has also been studied [5–8]. *N*-{2-[4-(2-Methoxyphenyl)-piperazino]ethyl}-2-pyridinamine (**9**) and cyclohexanecarboxylic acid were the major metabolites in both monkey and human blood [6,7]. The studies in rat showed a different pattern of metabolism with no significant formation of **9** and cyclohexanecarboxylic acid [5,8]. It also showed the appearance of more polar radioactive metabolites compared with the parent [5]. No structural information for these polar metabolites has been published.

In this study we identified the metabolites of FCWAY produced by human and rat hepatocytes using HPLC coupled with electrospray ionization tandem mass spectrometry (ESI-MS–MS). In addition, with knowledge of the structural identity of plasma metabolites, a simple extraction procedure was developed to determine the concentration of parent radiotracer that can be used as a function of time during the course of a PET study. The extraction procedure has advantages over a targeted LC–MS–MS or HPLC assay for the parent compound concentration because of its simplicity and speed.

2. Experimental

2.1. Chemical syntheses

Compounds **2a**, **2b**, WAY100635 [8], WAY100634 [8] were prepared according to the published procedure [1]. Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). All other reagents for synthesis and analysis were purchased from Aldrich (Milwaukee, WI, USA) unless otherwise indicated in the text.

N-{2-[4-(2-Methoxyphenyl) piperazino]ethyl} - *N* - (2 - pyridyl)*trans*- and *cis* - 4 - hydroxycyclohexanecarboxamide (**5a** and **5b**) were synthesized as follows: to a mixture of *cis*- and *trans*-4-hydroxycyclohexanecarboxylic acid [1] (0.52 g, 3.6 mmol) in 10 ml 1,2-dichloroethane was added 0.26 ml of acetyl chloride and 0.29 ml of pyridine. The mixture was refluxed for 2 h, then cooled to the room temperature, and passed through a small silica gel column to remove the insoluble material. The solvent was evaporated under reduced pressure to give a mixture of *cis*- and *trans*-4-acetyloxycyclohexanecarboxylic acid. The solid mixture was used without further purification.

To a mixture of *cis* and *trans* isomers (above) was added 0.8 ml α,α-dichloromethyl methylether in 10 ml methylene chloride. The mixture was refluxed for 2 h until all acid was converted to acid chloride. The solvent and unreacted α,α-dichloromethyl methylether were evaporated under reduced pressure. The residue was redissolved in 10 ml methylene chloride and added to a solution of 1.2 g of *N*-{2-[4-(2-methoxyphenyl)piperazino]ethyl}-2-pyridinamine and 0.54 ml of triethylamine in 10 ml methylene chloride. This was refluxed for 2 h, then the solvent was evaporated and the residue redissolved in 10 ml of methanol. To the solution, 0.4 g of potassium carbonate was added and the mixture stirred at room temperature overnight to remove the O-acetyl group. After filtration, solvent was removed in vacuo and the residue purified by flash chromatography on silica gel eluting with ethyl acetate (EtOAc) containing 0.5% triethylamine. The fractions containing the product were pooled and the solvent evaporated to give 0.8 g of *cis* and *trans* mixture as an oil (52%). Pure *trans* (**5a**) and *cis* (**6b**) isomers were obtained from a small portion of the isomeric mixture by semi-preparative HPLC [Beckman Ultrasphere C_{18}, 25×1.0 cm, 5 μm particle size), eluted with acetonitrile–5 m*M* NaH_2PO_4 (41:59), 5 m*M* triethylamine.

2.2. LC–MS

All experiments were performed with a Finnigan LCQ MS system (San Jose, CA, USA) coupled with a HP series 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). HPLC utilized a YMC-pack ProC_{18} reversed-phase column (150×4.6 mm I.D., YMC, c/o Waters, Milford, MA, USA),

eluting with 50 m*M* ammonium acetate and acetonitrile at a 0.5 ml/min flow-rate and a gradient of 0–65% acetonitrile over 10 min followed by isocratic elution at 65% for an additional 10 min. The entire column eluent was introduced into the ESI-MS source with a standard high-flow tune method. Ion detection was achieved with the Finnigan ESI instrument 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). By using the entire column eluent, FCWAY could be determined with a detection limit of 1 pmol on-column.

2.3. Incubation of FCWAY with hepatocytes

The cryopreserved hepatocytes from male Sprague–Dawley rats and from male human liver tissue (In Vitro Technologies, Baltimore, MD, USA) were used for in vitro metabolism studies of **2a** and **2b**. The cells, which were stored in liquid nitrogen, were thawed rapidly at 37°C in a water bath and gradually diluted with cell culture medium (RPMI Medium 164O media; Life Technologies, Rockville, MD, USA). After washing the cells with the medium, the viable cell concentration was adjusted to $1.0 \cdot 10^6$ per ml and the resulting cell suspension incubated at 37°C for 15 min prior to the introduction of test compound. From stock solution of **2a** and **2b** (2.0 mg/ml in 10% EtOH in water), 10 μl was added into 1 ml of cell suspension, 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, 1, 2, 3 h. Each suspension was centrifuged at 5000 rpm for 5 min. The metabolites in 20 μl supernatant were analyzed by LC–MS.

For the analysis of radiolabeled compounds, 0.5 mCi of [^{18}F]-**2a** and **2b** in 20 μl ethanol was added to 1.0 ml suspension of cells and corresponding unlabeled compound, prepared as described in the preceding paragraph. A single time point, 2 h, was chosen for analysis, which was performed in the same manner as before. The fractions were collected and analyzed off-line. [^{18}F] Radioactivity was measured by gamma counting using a Cobra Auto-Gamma counter (Packard Instrument Company, Downers Grove, IL, USA).

2.4. In vivo metabolism study

Analysis of plasma metabolism was performed in normal male Sprague–Dawley rats weighing about 250 g. A dose of 2 mg of **2a** or **2b** in 100 μl 40% EtOH in saline solution was administered by tail vein injection. The rats were sacrificed 30 min post-injection by carbon dioxide inhalation. A blood sample was then removed and centrifuged at 14 000 rpm for 10 min. The plasma was drawn off and 500 μl portion diluted with an equal volume of acetonitrile and centrifuged at 14 000 rpm for 5 min to precipitate plasma proteins. A 20-μl volume of supernatant was injected for LC–MS analysis.

2.5. Extraction of FCWAY from plasma

Blood containing [^{18}F]t-FCWAY and metabolites was centrifuged at 3600 rpm for 10 min. To 150 μl plasma was added 450 μl buffer (pH 12.5, 125 m*M* KCl–NaOH buffer) and 1.2 ml organic solvent (hexane–EtOAc, 4:1). After mixing and centrifuged at 3600 rpm for 5 min, the aqueous phase was frozen in dry ice. The organic phase was collected. The unmetabolized parent compound in the organic phase was counted in a gamma counter. Homogeneity of the radioactivity component was confirmed by HPLC.

3. Results and discussion

The use of hepatocytes to evaluate metabolism of new compounds offers a rapid way of producing metabolites, evaluating species variability, and providing a biological matrix to evaluate extraction procedures. Because LC–ESI-MS–MS is a powerful technique that allows structural identification of metabolites from complex biological matrices [9,10], we employed this method to identify metabolites generated in vitro by rat and human hepatocytes. We developed a gradient HPLC method to separate the

polar metabolites and even the geometric isomers **5a** and **5b**.

3.1. Identification of metabolites produced by rat hepatocytes

t-FCWAY (**2a**) metabolites were clearly observed at 0.5 h and increased through 2 h. The LC–MS chromatogram at 2 h for rat hepatocytes is shown in Fig. 2a. In addition to parent (**2a**), eight metabolites were identified. Mass spectral data and proposed structures are displayed in Table 2. The radioactivity study (Fig. 1) shows one major and several minor metabolites. The major radioactivite metabolite is identified as **3a,** a product of aromatic ring oxidation.

Interpretation of the LC–MS–MS data allow determination of structural features of the metabolites. The basis for assigning structures is the interpretation of MS–MS data from synthesized standards. The LC–MS–MS of authentic FCWAY (**2a** or **2b**) of the $[M+H]^+$ m/z 441 molecular ion gave a major fragment at m/z 249 which is consistent with cleavage of the C–N bond between the acyclic ethyl bridge and the piperazine. This results in a cation of the structure 4-fluoro[ethyl-*N*-(2-pyridyl)]cyclohexanecarboxamide (**I**) (Table 2).

Major peak (**3a** $[M+H]^+$ m/z 457) in Fig. 2a, which is 16 u more than the parent FCWAY, was suggested as an oxidation product. Because parent (**2a**) and **3a** have the same MS–MS fragment (m/z 249), the oxidation must have occurred in the 2-[4-(2-methoxyphenyl)piperazino] moiety.

This oxidation metabolite (**3a**) was observed to be further conjugated with glucuronic acid to form Glut-OFWAY (**4a**) in the rat hepatocyte incubation. MS–MS of **4a** $[M+H]^+$ m/z 633 give the fragments m/z 457(oxidation product, **3a**) and m/z 249 {4-fluoro-[ethyl-*N*-(2-pyridyl)]cyclohexanecarboxamide}. The m/z 249 fragment supports that oxidation happened in the 2-[4-(2-methoxylpheny)piperazino] side chain. Because metabolite (**3a**) was extracted with 20% ethyl acetate in hexane from pH 9 buffer, but not from pH 12.5 buffer, it was concluded that the structure was a phenol, though the regiochemistry of the functionality could not be determined.

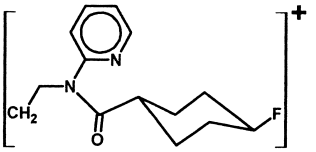
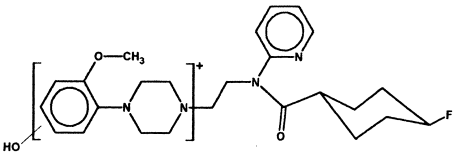
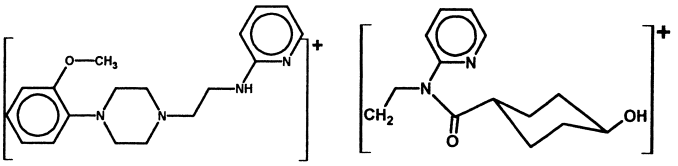
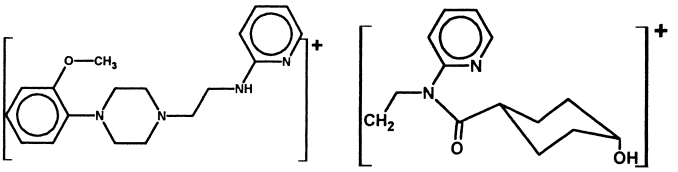
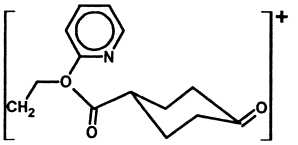
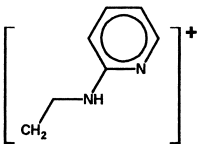
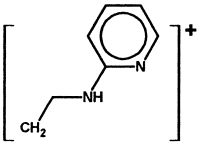
Alcohol and ketone metabolites (**5a**, **5b** and **5c**) resulted from the replacement of the 4-fluoro with oxygen. Defluorination of t-[^{18}F]FCWAY were detected indirectly in the radiochromatograms (Fig. 1) by the elution of the released [^{18}F]fluoride near the solvent front. The mechanism for the formation of those defluorination metabolites needs further clarification. The alcohols, *N*-{2-[4-(2-methoxylphenyl)piperazino]ethyl}-*N*-(2-pyridyl) *trans*- and *cis*-4-hydroxycyclohexanecarboxamide (**5a** and **5b**), were identified based on a molecular ion of m/z 439 and MS–MS fragments m/z 313 (descyclohexylcarbonyl of **5a**) and m/z 247 [ethyl-*N*-(2-pyridyl)4-hydroxycyclohexanecarboxamide]. Compound **5a** was the major defluorination metabolite. The structures (**5a** and **5b**) were further confirmed by co-chromatography and mass spectral data of synthesized standards. A ketone analogue (**5c**) was also observed at m/z 437 with MS–MS m/z 245 [ethyl-*N*-(2-pyridyl)4-keto-cyclohexanecarboxamide].

Demethylation product (**6a**), resulting from cleavage of the aryl methyl ether, was observed from rat hepatocytes. It was identified from $[M+H]^+$ m/z 427 (14 u less than the parent compound) with the same MS–MS fragment of m/z 249 as that of parent **2a**.

Two other nitrogen dealkylation metabolites were also identified, 4-(2-methoxylphenyl)piperazine (**7**) and *N*-[2-(piperazino)ethyl]-*N*-(2-pyridyl) *trans*-4-fluorocyclohexanecarboxamide (**8a**), resulting from cleavage at either nitrogen of the piperazine ring. Dealkylation metabolite (**7**), $[M+H]^+$ m/z 193, was formed by loss of ethyl-*N*-(2-pyridyl)cyclohexanecarboxamide from FCWAY (**2a**). Its structure was confirmed by comparing with synthesized standard. The desaryl product **8a** $[M+H]^+$ m/z 353, *trans*-*N*-[2-(piperazino)ethyl]-*N*-(2-pyridyl)4-fluorocyclohexanecarboxamide, has the same MS–MS fragment of m/z 249 {4-fluoro-[ethyl-*N*-(2-pyridyl)]cyclohexanecarboxamide, **I**} as FCWAY (**2a**).

cis-FCWAY (**2b**) has a nearly identical metabolic profile (Fig. 3a) as *trans* (**2a**) when incubated with rat hepatocyte. The oxidation, demethylation and dealkylation metabolites have the same *cis* form as the parent **2b**. The metabolic defluorination results in formation of 4-hydroxy products produces a greater amount *trans* (**5a**) compared to *cis* (**5b**) without regard to the configuration of fluorine in the parent.

Table 2
t-FCWAY metabolites produced by rat and human hepatocytes

Metabolite [M+H] (m/z)	Metabolite structure	MS–MS fragments (m/z)	Possible structures
441	**2a**	249	 **I**
457	**3a**	249	**I**
633	**4a**	457 249	 **+I**
439	**5a**	313 247	
439	**5b**	313 247	
437	**5c**	245	
427	**6a**	249	**I**
193	**7**		
335	**8a**	249	**I**
313	**9**	121	
329	**10**	121	

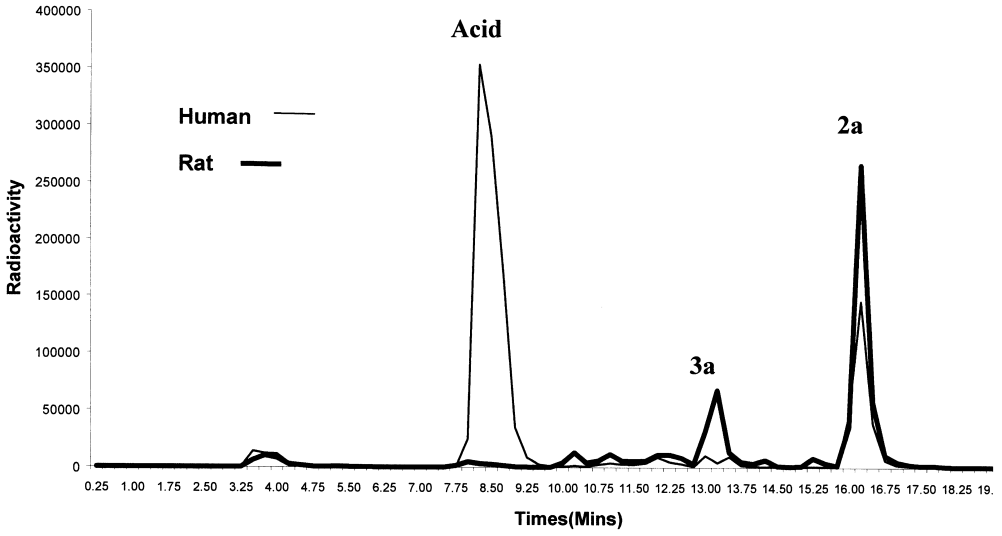


Fig. 1. Radiochromatography of [^{18}F]t-FCWAY (0.5 mCi plus 20 μg nonradioactive t-FCWAY in 1 ml incubation solution) metabolites produced by 2 h rat and human hepatocyte incubation.

3.2. Identification of metabolites produced by human hepatocytes

t-FCWAY (**2a**) metabolites from human hepatocytes provided the positive ion chromatogram displayed in Fig. 2b. The major metabolite peak $[M+H]^+$ m/z 313 was identified as WAY100634 (**9**) resulting from amide hydrolysis of the parent compound. It was identified based on co-chromatography and MS data of synthesized standard. This major metabolite, **9**, no longer carries the radiolabel. The radiochromatogram in Fig. 1 displayed a different major component which can be observed by LC–MS only in the negative ion mode. This component was identified as 4-fluorocyclohexanecarboxylic acid by ESI negative ion $[M-H]^-$ m/z 145. The oxidation (**3a** m/z 457), defluorination (**5a** and **5b** m/z 439, **5c** m/z 437) and dealkylation (**7** m/z 193 and **8a** m/z 335) metabolites were the same as found in rat hepatocytes.

Compound **10** (m/z 329) was also observed in human hepatocyte metabolites. It can be formed directly by oxidation of WAY100634 (**8a**) or by hydrolysis of the oxidation product (**3a** m/z 457). Osman et al. [7] reported an unknown radioactive metabolite more polar than [O-methyl-^{11}C]-WAY100634 in a human metabolite study. This unknown metabolite was suggested to be the oxidation product of [O-methyl-^{11}C]WAY100634. The MS results support this suggestion by identification of this oxidation product (**10**) and WAY100634 (**9**) from the incubation of WAY100635 (**1**) in human hepatocytes.

The oxidation product (**3a** or **3b**) was also identified in human hepatocytes metabolism. We did not observe the formation of demethylation product (**6a** or **6b**) by incubation of human hepatocytes.

3.3. Analysis of rat in vivo metabolites

Fig. 3b shows the LC–MS chromatogram of rat plasma metabolites at 30 min after intravenous injection of no-carrier-added **2b**. The metabolite profile is similar to that of the in vitro hepatocytes (Fig. 3a), although the relative ratios of metabolites are different.

In agreement with previously published work [5], we did not find 4-fluorocyclohexanecarboxylic acid or **9** in rat hepatocyte metabolites identified by LC–MS.

It was reported that the extraction efficiency of [^{18}F]-labeled FCWAY was low (<85%) because of its rapid defluorination [1]. This was verified by

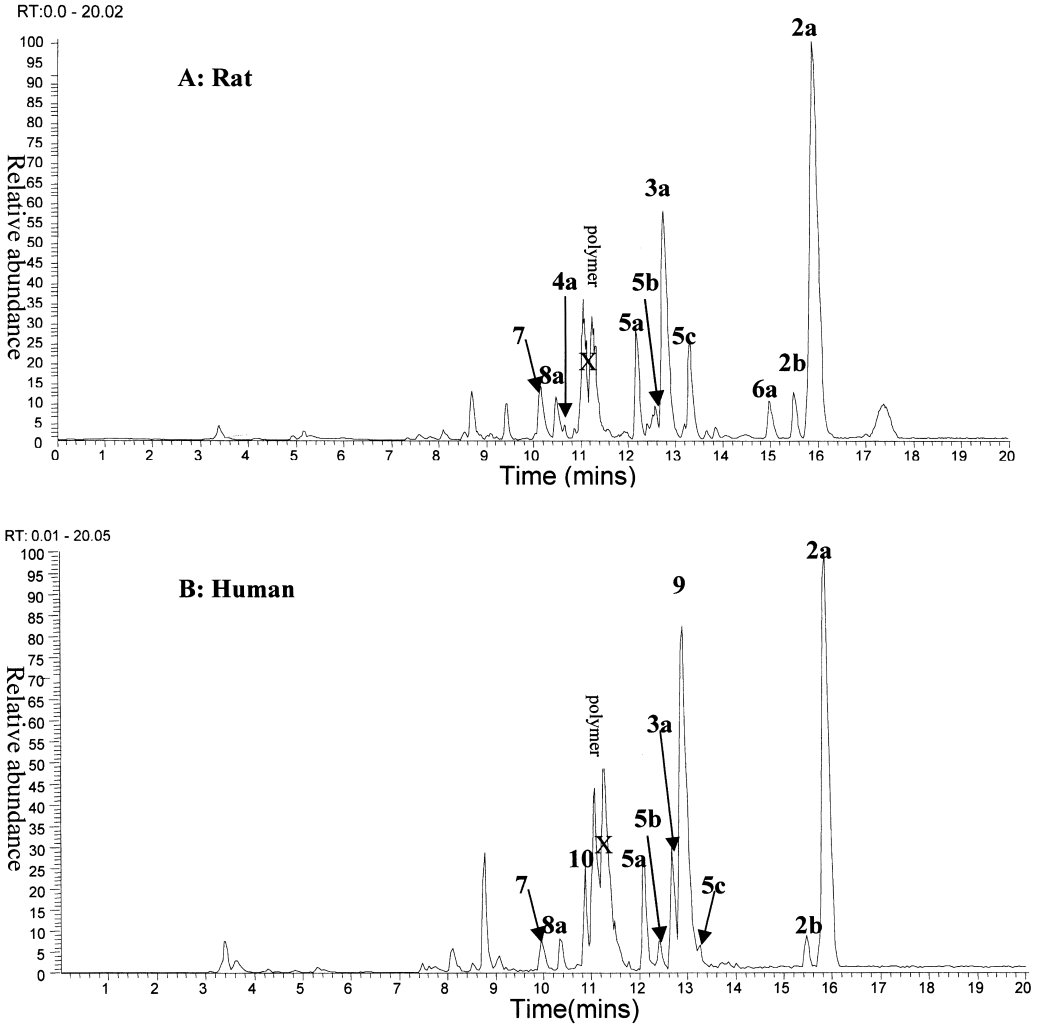


Fig. 2. LC–MS (positive ionization) of t-FCWAY (**2a**) metabolites produced by 2 h rat (a) and human (b) hepatocyte incubation. The peaks were labeled on the basis of identification of the metabolites by MS–MS and by co-chromatography of synthesized standards.

identification of the defluorination metabolites in rat hepatocytes.

3.4. Development of the extraction procedure for FCWAY from plasma

The structural identity of metabolites provides a window to explore the ability of the metabolites to cross the blood–brain barrier. Radioactive metabolites can be prepared and subjected to biodistribution studies. In addition, structural identity can be exploited for the development of an efficient extraction system for quantitation of unmetabolized radioligand in plasma or other biological fluid. Intrepretation of PET imaging data for this ligand [^{18}F]FCWAY requires mathematical modeling. For the modeling, a time activity curve for the amount of parent radioligand in the plasma is required. Traditionally, the concentration of parent ligand is determined by a single extraction of the plasma by an organic solvent and then HPLC or TLC analysis of the extract for the proportion of parent ligand. The percent extraction of radioactivity times the percent of parent in the extract gives the fraction of parent in the plasma.

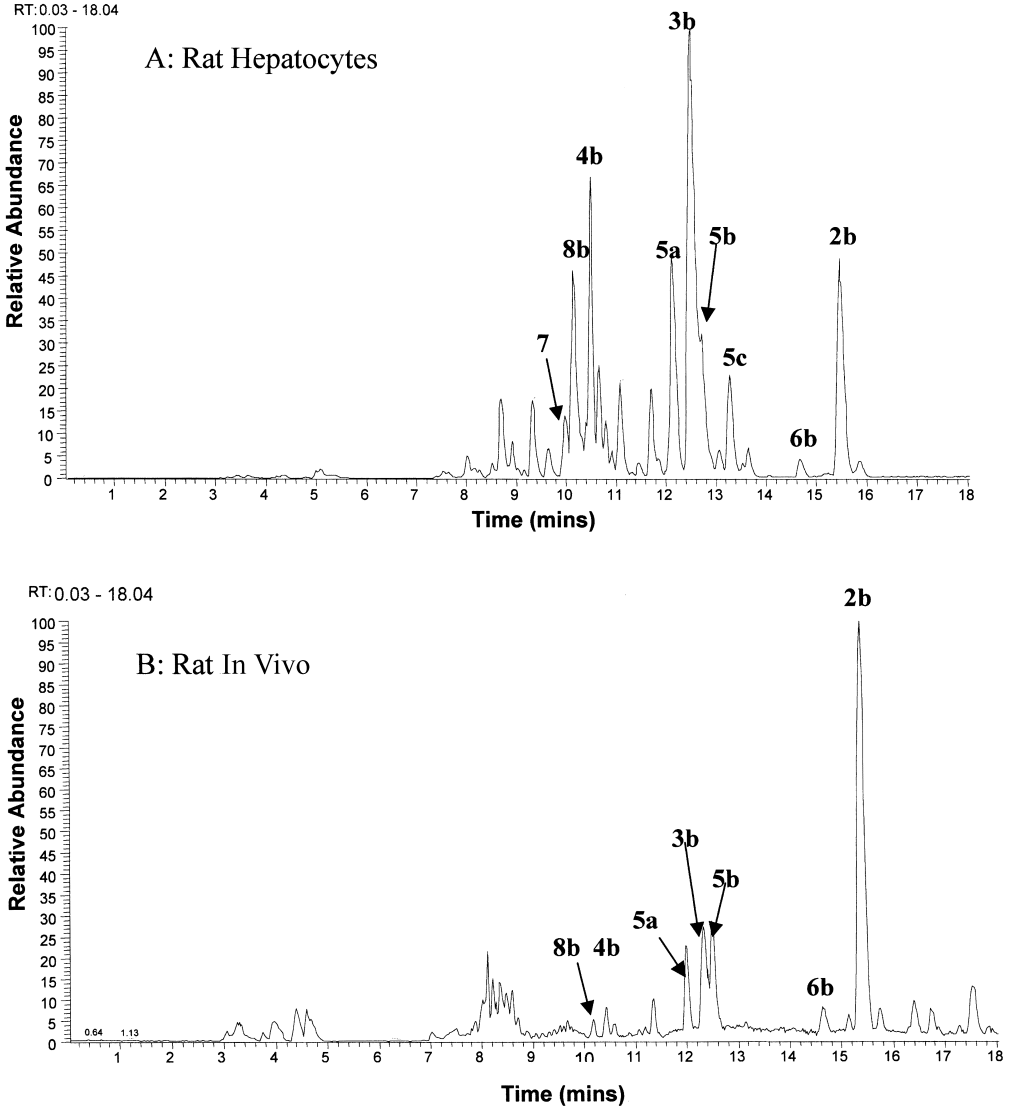


Fig. 3. (A) LC–MS (positive ionization) of c-FCWAY (**2b**) metabolites produced by 2 h rat hepatocyte incubation. (B) LC–MS (positive ionization) of in vivo rat plasma metabolites after 30 min administration of c-FCWAY (**2b**). The peaks were labeled on the basis of identification of the metabolites by MS–MS and by co-chromatography of synthesized standards.

With radioactivity counting and correction for radioactivity decay, the actual concentration of radioactive parent ligand can be calculated. The HPLC analysis limits the number of samples during the time course of an imaging study because the decay of this radioisotope is quite time limiting. An extraction system that only extracts parent ligand will obviate the need for HPLC and allow analysis of more samples, more replicates, and result in higher statistical precision. This is important because of the low count rate encountered in TLC or HPLC fractions. A one step extraction procedure can produce much higher precision in the input function.

With the identification of the radiolabeled metabolites, a simple method for the extraction of [^{18}F]-labeled parent compound (**2a** or **2b**) was developed. Because the major [^{18}F]-labeled metabolites include phenolic (**3a** or **3b** and **6a** and **6b**) and carboxylate

functions, they were easily separated by proper choice of pH. Using a strongly basic aqueous buffer (125 m*M* KCl–NaOH, pH 12.5), the parent can be extracted by the organic phase (hexane–EtOAc, 4:1) and the metabolites remained in a basic aqueous phase. The selectivity of extraction of the parent was demonstrated by LC–MS of both extracted phases. The parent was only detected in the organic phase. The extraction efficiency of parent was 93.5%.

4. Conclusion

Based on the proposed structural identity of the metabolites, we constructed the metabolic pathway for rat and for human hepatocytes (Fig. 4). Both species show common pathways of oxidation, defluorination, and dealkylation. In addition, human hepatocytes show amide hydrolysis. For in vitro hepatocyte incubation, oxidation with formation of a phenol is the major metabolic pathway for rat and amide hydrolysis is the major metabolic pathway for humans.

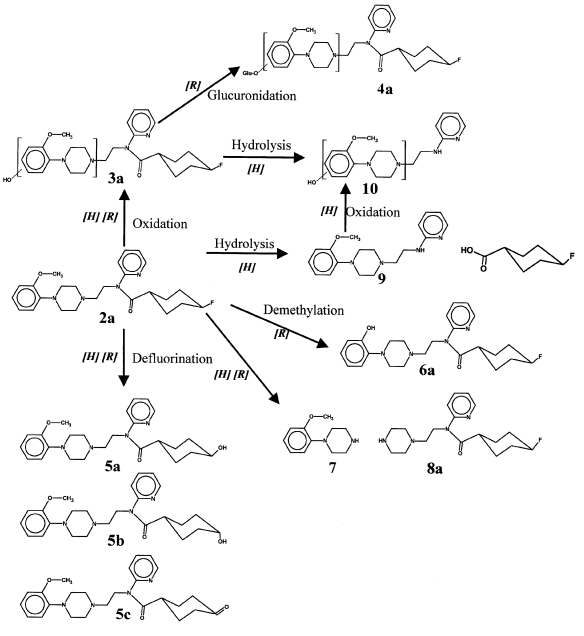


Fig. 4. Summary of t-FCWAY (**2a**) in vitro metabolism pathway by human [H] and rat [R] hepatocytes.

We developed a procedure to extract parent FCWAY from pH 12.5 buffer, leaving the major radiolabeled metabolites, 4-fluorocyclohexanecarboxylic acid and the phenol (**3a**) in the aqueous phase layer. Therefore, we can determine parent concentration in plasma using a single extraction.

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