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Liquid chromatography–tandem mass spectrometry identification of metabolites of three phenylcarboxyl derivatives of the 5-HT_{1A} antagonist, *N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridyl) *trans*-4-fluorocyclohexanecarboxamide (FCWAY), produced by human and rat hepatocytes

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Abstract

We have previously described fluorine-18 radiolabeled FCWAY [*N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridyl) *trans*-4-fluorocyclohexanecarboxamide] as a high affinity ligand for imaging the 5-HT_{1A} receptor in vivo. In a search for radiopharmaceuticals with unique imaging applications using positron emission tomography (PET), we have also developed three new phenylcarboxamide analogues of FCWAY. Two of these analogues were generated by replacing the fluorocyclohexane carboxylic acid with fluorobenzoic acid (FBWAY) or with 3-methyl-4-fluorobenzoic acid (MeFBWAY). The final analogue was generated by replacing the pyridyl group with a pyrimidyl group and the fluorocyclohexane carboxylate with fluorobenzoic acid (FPWAY). We evaluated the metabolic profile of these compounds using either human or rat hepatocytes to produce metabolites and LC–MS/MS to identify these metabolites. We also compared the metabolic rate of these compounds in human or rat hepatocytes. These in vitro metabolism studies indicate that hydrolysis of the amide linkage was the major metabolic pathway for FPWAY and FBWAY in human hepatocytes, whereas aromatic oxidation is the major metabolic pathway for MeFBWAY. The comparative metabolic rate in human hepatocytes was FPWAY>FBWAY>MeFBWAY. In rat hepatocytes, aromatic oxidation was the major metabolic pathway for all three analogs and the rate of this process was similar for all of the analogues. These in vitro metabolic studies demonstrated species differences prior to the acquisition and interpretation of in vivo results.
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Keywords: FCWAY analogues; 5-HT antagonists

1. Introduction

Positron emission tomography (PET) is an external imaging technique with the unique ability to measure receptor density changes. A target molecule to probe the 5-HT_{1A} receptor using a radiolabeled analog recently became available with the development of the 5-HT_{1A} silent antagonist, WAY100635 [1–3]. A successful radiolabeled probe will permit the determination of 5-HT_{1A} receptor density by

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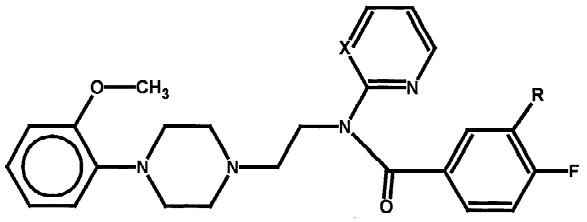
external imaging using PET. However, in order to determine the 5-HT_{1A} receptor density by external imaging, it is important to know the chemical form of the radioligand in the brain and in the blood [4,5]. Since PET detects coincidence radiation, which contains no information about the chemical form, the metabolic profile of the radiolabeled ligand must be determined by independent methods. With knowledge of the chemical form of the radiolabel, any metabolite can be isolated and synthesized, then tested for blood–brain-barrier penetration and affinity to the 5-HT_{1A} receptor [6]. Penetration of the blood–brain-barrier and binding to the 5-HT_{1A} receptor by metabolites will confound the pharmacokinetic analysis and the determination of the 5-HT_{1A} receptor density. Furthermore, knowledge of the chemical identity of metabolites can lead to the development of a simple extraction procedure that makes the determination of the plasma time activity curve of the parent compound (the input function) more sensitive than measurements using thin layer chromatography or high pressure liquid chromatography [5,8,9]. We previously developed a high affinity compound, *trans* 4-FCWAY [*N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridyl) *trans*-4-fluorocyclohexanecarboxamide], which is suited for measurements of receptor density [7,9]. We have also developed a number of compounds with lower binding potentials and therefore more susceptible to endogenous serotonin [10]. The goal of this work is to determine the metabolite profile of three lower affinity analogues of FCWAY and to understand the metabolic kinetics by comparison of the metabolic rate in vitro. Two of the analogues are prepared by substitution of the fluorocyclohexyl carboxylate with 4-fluorophenylcarboxyl (FBWAY) or 3-methyl, 4-fluorophenyl-carboxyl (MeFBWAY). The third analogue has *N*-2-pyrimidine substituted for the *N*-2-pyridine and 4-fluorophenylcarboxyl in place of cyclohexane-carboxyl (FPWAY) (Fig. 1).



(1) FPWAY R = H, X = N
(2) FBWAY R = H, X=CH
(3) MeFBWAY R = Me, X=CH

Fig. 1. Structures of FPWAY, FBWAY and MeFBWAY.

2. Experimental

2.1. Chemical syntheses

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.

FBWAY [14,15] and MeFBWAY [7] were prepared according to the published procedure. FPWAY was prepared in the following method.

2.1.1. {2-[4-(2-Methoxyphenyl)-1-piperazinyl]-ethyl}-N-(2-pyrimidinyl)amine

To a 100-ml round-bottomed flask, 1.9 g (8.1 mmol) of 2-[4-(2-methoxyphenyl)-1-piperazinyl]-ethylamine (obtained from Med-Life System, Upper Darby, PA, USA), 1.1 g of sodium carbonate (10.0 mmol), 1.1 g of 2-chloropyrimidine (9.6 mmol), and 50 ml of ethanol were added. The mixture was refluxed overnight and the solution was cooled and filtered. The solvent was evaporated under reduced pressure and the residue was re-dissolved in 5 ml methylene chloride. The solution was loaded onto a 5.5×20 cm silica gel column and eluted with ethyl acetate–methanol–triethylamine (90:10:1) to give 2.0 g of product (yield: 79%). ^{1}H NMR ($CDCl_3$) δ 2.69 (m, 6H), 3.10 (m, 4H), 3.53 (q, 2H), 3.86 (s, 3H), 5.75 (t, 1H, broad), 6.51 (t, 1H), 6.84–7.05 (m, 4H), 8.28 (d, 2H). MS (EI) 313 (M^+), 298, 218, 205, 190, 162, 149, 134, 120, 108, 70.

2.1.2. 4-Fluoro-N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-(2-pyrimidinyl)benzamide (FPWAY)

To a solution of the above compound (570 mg, 1.8 mmol) in 10 ml 1,2-dichloroethane was added 0.30

ml (2.5 mmol) of 4-fluorobenzoyl chloride and 0.35 ml (2.5 mmol) of triethylamine. The mixture was refluxed overnight. The solvent was evaporated and residue redissolved in 2 ml ethyl acetate and chromatographed on a silica gel column eluting with ethyl acetate containing 0.5% triethylamine to give 760 mg of product (yield: 96%). ^{1}H NMR ($CDCl_3$) δ 2.62–2.72 (m, 4H), 2.80–2.89 (m, 6H), 3.83 (s, 3H), 4.38 (t, 2H), 6.80–6.95 (m, 7H), 7.40 (dd, 2H), 8.39 (d, 2H). MS (EI) 435 (M^+), 420, 312, 244, 218, 205, 190, 162, 149, 134, 123, 95, 70.

2.1.3. N-{2-[4-(2-Methoxyphenyl)-1-piperazinyl]-ethyl}-4-nitro-N-(2-pyrimidinyl)benzamide

To a solution of {2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-*N*-(2-pyrimidinyl)amine (400 mg, 1.3 mmol) in 10 ml 1,2-dichloroethane was added 350 mg (1.9 mmol) of 4-nitrobenzoyl chloride and 0.26 ml (1.9 mmol) of triethylamine. The mixture was refluxed overnight. The solvent was evaporated and residue redissolved in 2 ml ethyl acetate and chromatographed on a silica gel column eluting with ethyl acetate containing 0.5% triethylamine. The fractions containing the product were pooled together and the solvent evaporated to give 610 mg of product (yield: 86%). ^{1}H NMR ($CDCl_3$) δ 1.1–1.4 (m, 2H), 1.6–2.0 (m, 4H), 2.0–2.3 (m, 2H), 2.5–2.7 (m, 6H), 3.0 (s, 4H, broad), 3.85 (s, 3H), 3.98 (t, 2H), 4.3–4.7 (md, 1H, fluorine coupling), 6.8–7.0 (m, 4H), 7.2–7.4 (m, 4H), 7.8 (td, 1H), 8.55 (dd, 1H). MS (EI) 440 (M^+), 425, 311, 278, 249, 218, 205, 190, 162. Anal. ($C_{25}H_{33}FN_4O_2F$) calcd, C, 68.16; H, 7.55; N, 12.72; found, C, 68.02; H, 7.82, N, 12.52. HPLC purity by UV (254 nm): 97.3%.

2.1.4. 4-[F-18]-Fluoro-N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-(2-pyrimidinyl)benzamide F-18-FPWAY)

To a 10-ml test tube containing 3 μmol of potassium carbonate in 15 μl of water and 6 μmol of Kryptofix in 30 μl of acetonitrile, 100–200 μl of $[^{18}F]^-$ (20–40 mCi) were added. The water was removed with argon flow three times using anhydrous acetonitrile on a 105 °C heating block.

To the above test tube, 3 mg of the above compound in 0.2 ml DMSO were added and heated in a microwave oven for 2 min. The solution was then cooled and diluted with 0.3 ml HPLC solvent (45% acetonitrile–water) and injected onto a reversed-phase semi-prep HPLC column (Beckman C-18, 10×250 mm). The radioactive product was collected and trapped on a C-18 Sep-Pak. The final product was eluted with 0.3 ml ethanol (yield: 25–35% EOB). Radiochemical purity: 99.0%.

2.2. LC–MS

All experiments were performed with a Finnigan LCQ MS (San Jose, CA, USA) coupled with 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 0.5 ml/min flow-rate and a gradient of 0–65% acetonitrile over 10 min followed by isocratic elution at 65% acetonitrile for an additional 12 min. The entire column eluent was introduced into the ESI-MS with a standard high flow tune method. Ion detection was achieved with the Finnigan 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).

2.3. Incubation of WAY100635 analogues with hepatocytes

In vitro metabolism studies of FPWAY, FBWAY, MeFBWAY 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). After washing the cells with the medium, the viable cell concentration was adjusted to 1.0 million per ml and the resulting cell suspension incubated at 37 °C for 15 min prior to the introduction of the test compound. To 1 ml of cell suspension, 10 μl of stock solution of FPWAY, FBWAY and MeFBWAY (2.0 mg/ml in 10% ethanol in water) were added to give a final concentration of 20 μg/ml. The suspension was maintained at 37 °C; 100 μl of cell suspension were removed and added

to 100 μl acetonitrile at 10, 30, 60 and 120 min. 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 compounds, the same procedure was used with the exception of the addition of 0.5 mCi (~0.1 μg) of [^{18}F]-labeled WAY analogue in 20 μl 20% ethanol to the cell suspension and corresponding unlabeled compound (20 μg/ml). Supernatant (20 μl containing about 5 μCi) was injected into the HPLC with a radioactivity flow detector (Flow-count, Bioscan, Washington, DC, USA) followed by MS. The detection limit for the parent compounds was about 1 pmol on-column injection.

2.4. Extraction of FPWAY from plasma

Monkey blood from the imaging study containing [^{18}F]FPWAY and metabolites was centrifuged at 17,000 *g* for 10 min. The plasma (150 μl) was added to 450 μl buffer (pH 12.5, 125 m*M* KCl–NaOH buffer) and 1.2 ml organic solvent (hexane–EtOAc, 4:1), vortexed for 2 min, and then centrifuged at 2500 *g* for 5 min to separate the two phases. The test tube was put in dry ice, which froze the aqueous phase. The organic phase was transferred. The unmetabolized parent compound in the organic phase was counted in a gamma counter. Homogeneity of the radioactivity component was confirmed by HPLC and Radio TLC. The extraction efficiency of the parent was determined by adding 10 μCi [^{18}F]FPWAY to 150 μl plasma and extracting according to the above method.

3. Results and discussion

The use of hepatocytes to evaluate metabolism of new compounds offers a rapid method of producing metabolites, evaluating species variability, and providing a biological matrix to evaluate extraction procedures. Because liquid chromatographic–electrospray tandem mass spectrometric method is a powerful technique that allows structural identification of metabolites from complex biological matrices [11,12], we employed this method to identify metabolites generated in vitro by rat and human hepatocytes. By using the radiolabeled analogues, we are able to evaluate the metabolic rate differences between the compounds.

3.1. Identification of metabolites produced by rat hepatocytes

The metabolites of the FCWAY analogues (Fig. 1, FPWAY, FBWAY, MeFBWAY) were clearly observed at 10 min and increased through 2 h. The LC–MS chromatogram for all three WAY analogues after a 1-h incubation with rat hepatocytes is shown in Figs. 2 and 3. In addition to parent (**P**), eight metabolites were indicated. The major radioactive metabolite is identified as the product of aromatic ring oxidation (**O**).

Interpretation of the LC–MS/MS data allowed determination of structural features of the metabolites. The structural assignments were confirmed in some cases by comparison of HPLC retention time and MS/MS data from synthesized standards. The LC–MS/MS of authentic parent compound gave a major fragment at *m*/*z* 244 (FPWAY), 243 (FBWAY), 257 (MeFBWAY), respectively, which is consistent with cleavage of the C–N bond between the acyclic ethyl bridge and the piperazine [9].

The major peak (**1**−**O** $[M+H]^+$ *m*/*z* 452, **2**−**O** $[M+H]^+$ *m*/*z* 451 and **3**−**O** $[M+H]^+$ *m*/*z* 465) in Fig. 2, which is 16 mass units more than the parent WAY analogue, suggests an oxidation product. Because the parent and the oxidation products have the same MS/MS fragment at *m*/*z* 244 (FPWAY), 243 (FBWAY), 257 (MeFBWAY) respectively, the oxidation must have occurred in the 2-[4-(2-methoxyphenyl) piperazino] moiety.

This oxidation metabolite (**O**) was observed to conjugate with glucuronic acid to form Glu-t-OFWAY (**G**) in the rat hepatocyte incubation. Because metabolite (**O**) was extracted with 20% ethyl acetate in hexane from pH 9 buffer but not from pH 12.5 buffer, the structure should be a phenol. The regiochemistry of the oxidation could not be determined. The (**O**) metabolite was indicated to be a phenol in the FCWAY metabolite study [9]. In the case of FPWAY, this oxidation product (**1**−**O**) can undergo subsequent hydrolysis to form the amide-hydrolysis product (**OH**).

A demethylation product (**M**), resulting from

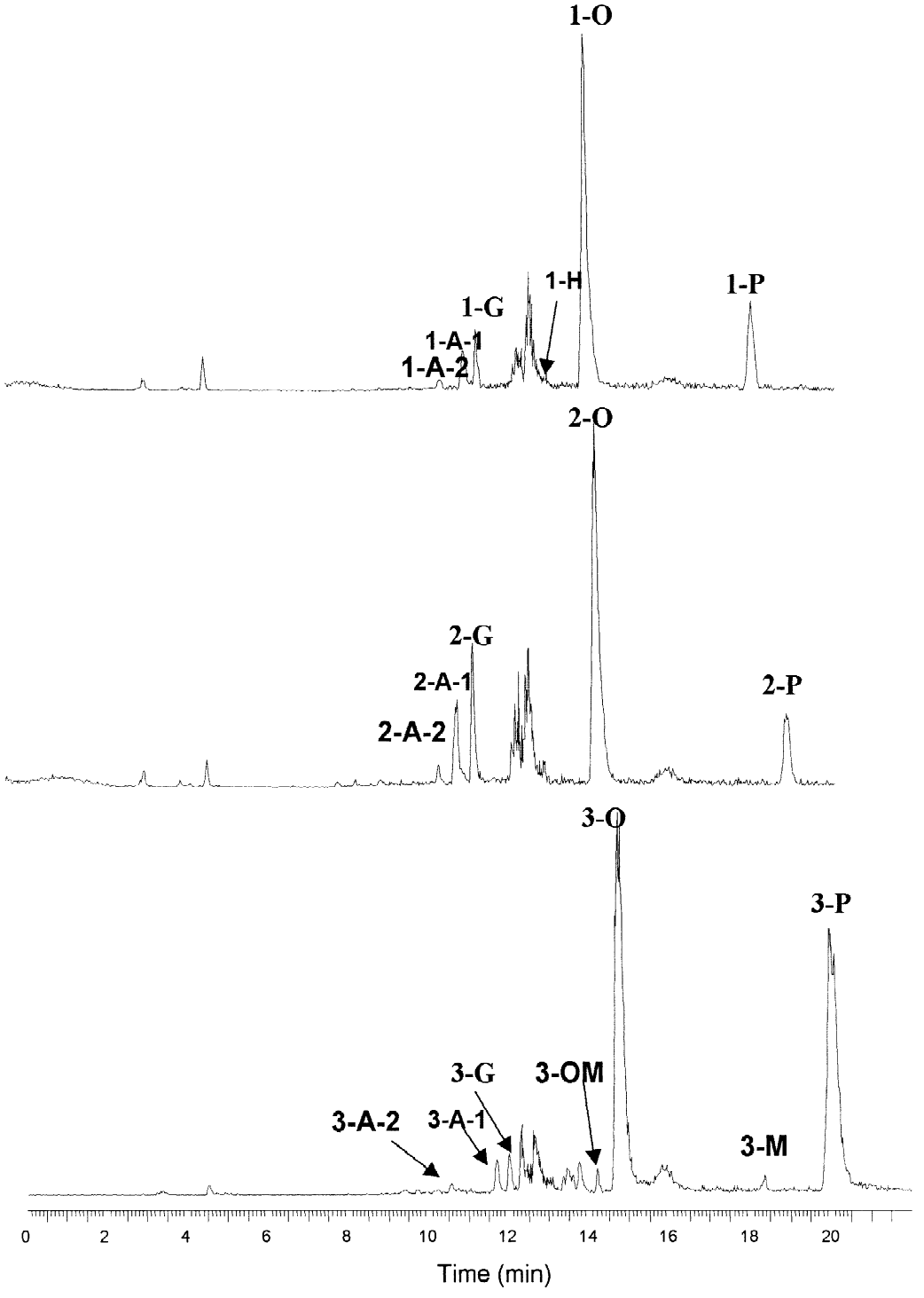


Fig. 2. LC–MS (positive ionization) of FPWAY (top), FBWAY (middle) and MeFBWAY (bottom) metabolites produced by 1 h rat hepatocyte incubation. The peaks were labeled on the basis of identification of the metabolites by MS/MS and/or by co-chromatography of synthesized standards.

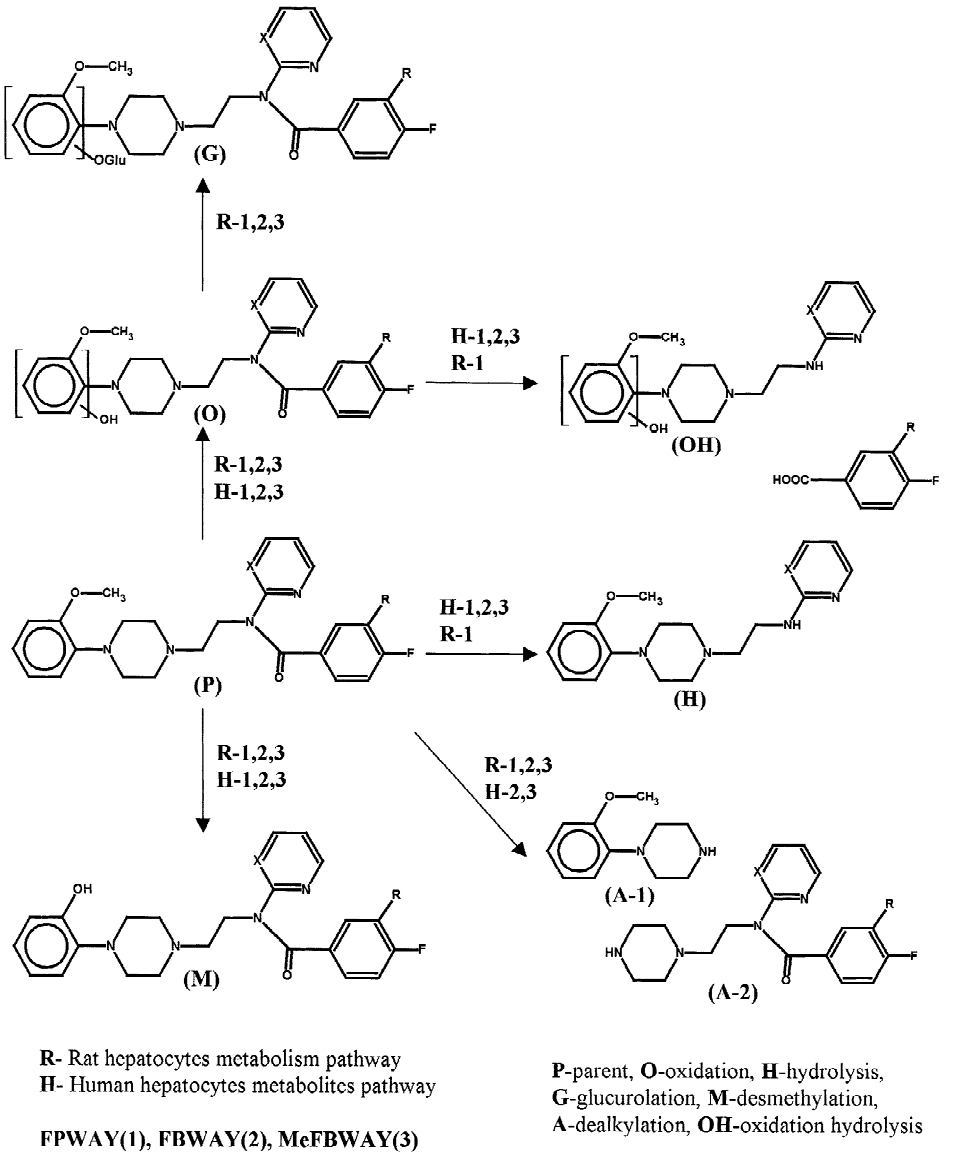


Fig. 3. Metabolic pathways of FPWAY, FBWAY and MeFBWAY.

cleavage of the aryl methyl ether, was observed from rat hepatocytes. The metabolite showed $[M+H]^+$ 422 (**1**−**M**) which is 14 mass units less than the parent compound with the same MS/MS fragment as that of parent.

Two other nitrogen dealkylation metabolites resulting from cleavage at either nitrogen of the piperazine ring were also identified, 1-(2-methoxylphenyl) piperazine (**A**−**1**) and *N*-[2-(piperazino)-ethyl]-*N*-(2-pyridyl) *trans*-4-fluoro carboxamide (FBWAY) (**A**−**2**). The dealkylation metabolite (**A**−**1**) was confirmed by comparison with authentic standard (1-(2-methoxyphenyl) piperazine, Aldrich). The desaryl product **A**−**2** has the same MS/MS fragment as the parent compound.

The amide-hydrolysis product (**1**−**H**) was also found for FPWAY (**1**) after incubation with rat hepatocyte. No amide hydrolysis was observed for

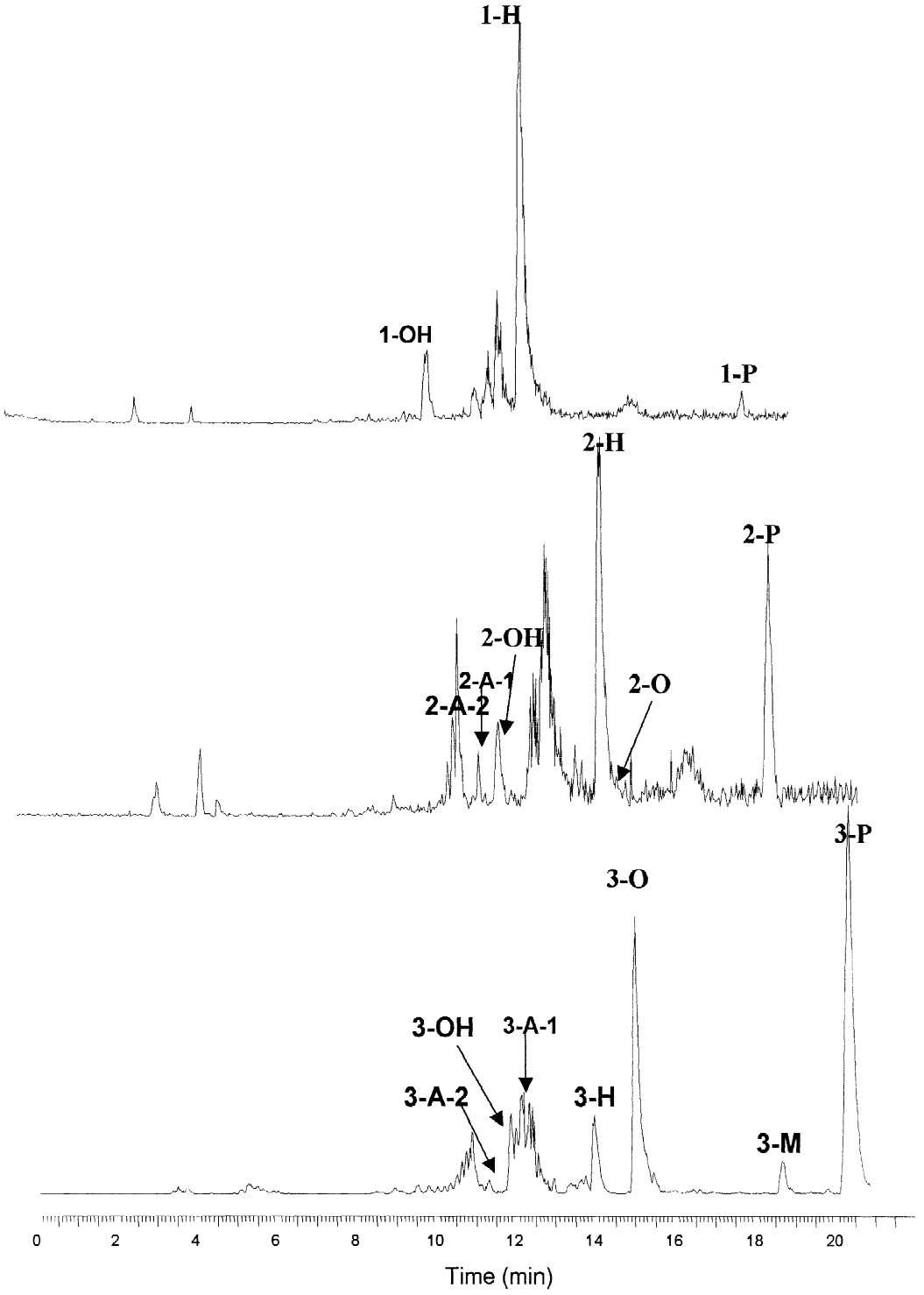


Fig. 4. LC–MS (positive ionization) of FPWAY (top), FBWAY (middle) and MeFBWAY (bottom) metabolites produced by 1 h human hepatocyte incubation. The peaks were labeled on the basis of identification of the metabolites by MS/MS and by co-chromatography of synthesized standards.

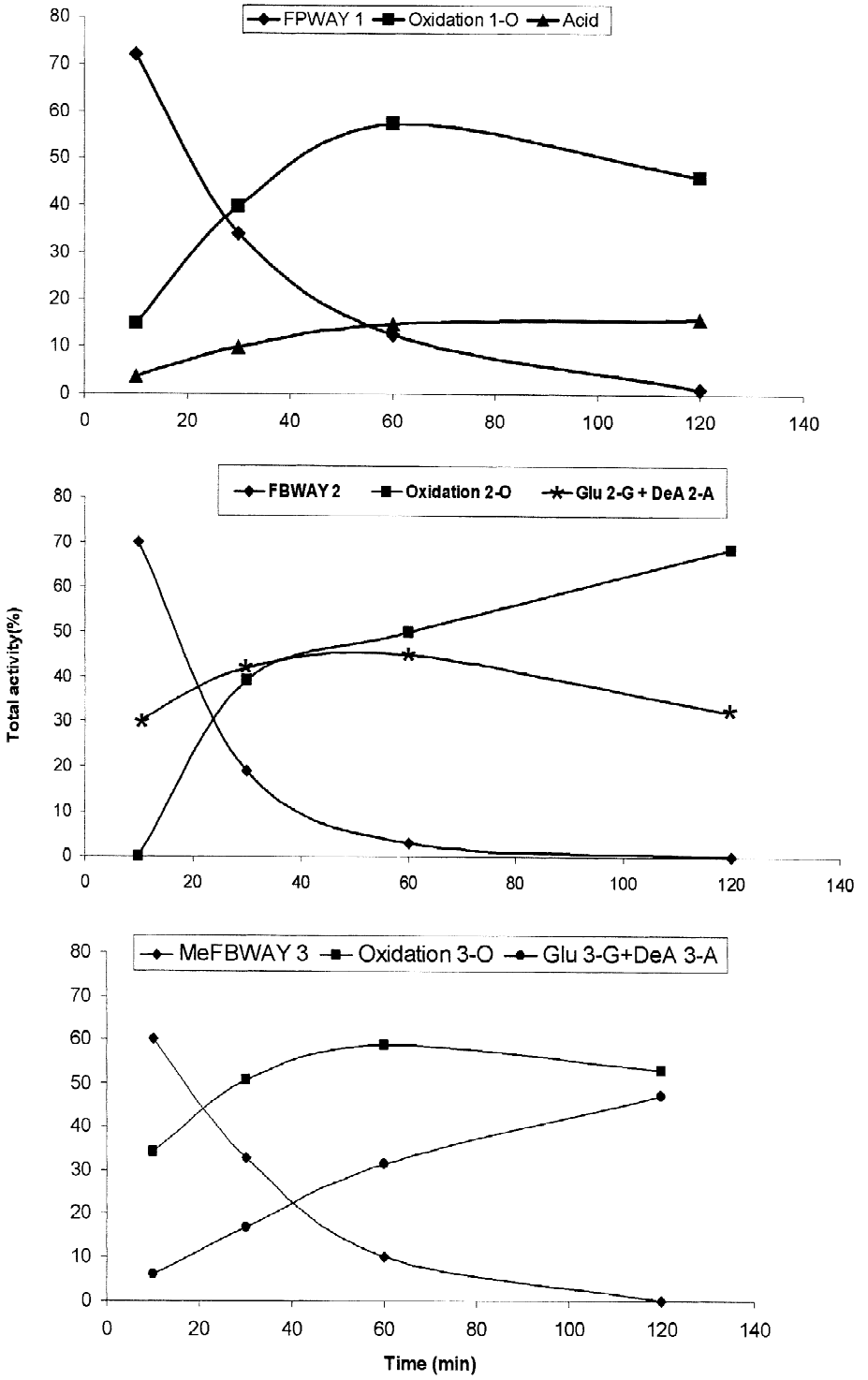


Fig. 5. FPWAY (top), FBWAY (middle) and MeFBWAY (bottom) metabolites present after incubation with rat hepatocytes. Parent (diamond), phenol (square), glucuronide and dealkylation (star), amide-hydrolysis (triangle).

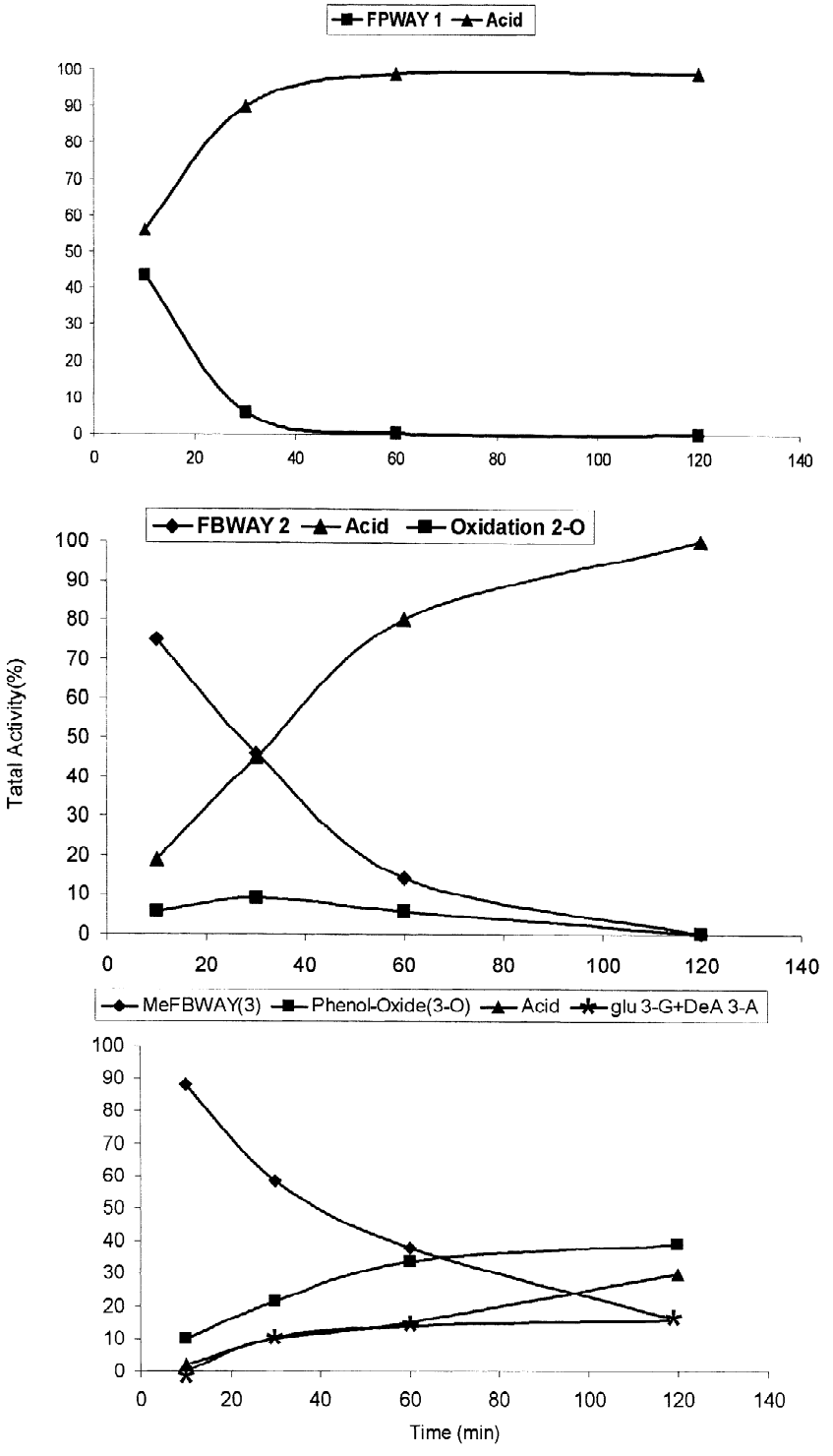


Fig. 6. FPWAY (top), FBWAY (middle) and MeFBWAY (bottom) metabolites present after incubation with human hepatocytes. Parent (diamond), phenol (square), glucuronide and dealkylation (star), amide-hydrolysis (triangle).

FBWAY and MeFBWAY (**2** and **3**) or their oxidation metabolites(**2**,**3**−**O**) when incubated with rat hepatocyte.

3.2. Identification of metabolites produced by human hepatocytes

Amide hydrolysis products were the major metabolites for FPWAY and FBWAY after incubation with human hepatocytes (Fig. 4). The major metabolite peak for FBWAY was identified as WAY100634 (*N*-{2-[4-(2-methoxyphenyl)-piperazino]ethyl}-2-pyridinamine) $[M+H]^+$ m/z 313 (**2**−**H**) resulting from amide hydrolysis of the parent compound. It was identified based on co-chromatography and MS data of synthesized standard [16].

Compound **OH** was also observed in human hepatocyte metabolites. It can be formed directly by oxidation of WAY100634 or by hydrolysis of the oxidation product (**O**).

The oxidation (**O**), demethylation product (**M**) and dealkylation (**A**−**1** m/z 193 and **A**−**2**) metabolites for all three WAY analogues were the same as found in rat hepatocytes.

For MeFBWAY, aromatic oxidation (**3**−**O**) is still the major metabolite when incubated with human hepatocytes while the amide hydrolysis product (**3**−**H** and **3**−**OH**) are also found. A summary of the metabolic pathways is given in Fig. 3.

The hepatocyte metabolism study can expose species differences. For these compounds, the rat model system displays a different metabolism pathway than does human hepatocytes. Our results with human hepatocytes would predict only minor issues with metabolites in any mathematical modeling that requires a blood input function. However, for those researchers that may wish to conduct small animal imaging studies, for example in knockout mice or other rodent models, there may be significant uptake of metabolites into brain tissue.

3.3. Comparison of metabolism rates

Parent and major metabolites were determined as a function of incubation time with rat and human hepatocytes by integration of the peaks in the radiochromatogram (Figs. 5 and 6). Integration of total ion chromatographic peaks is not quantitative without correction for component sensitivity. Since only radiolabeled metabolites affect the input function in in vivo studies, any metabolite that no longer carries the radiolabel is not relevant.

The concentration of the three parent (Fig. 7, top) compounds (FPWAY, FBWAY, and MeFBWAY) and the corresponding major metabolite, oxidation product (Fig. 7, bottom), were quantitatively similar throughout the 120-min incubation with rat hepatocytes because the metabolic pathway in rat hepatocytes was the same for all three compounds. The aromatic-ring oxidation was the major metabolism pathway for all three compounds. However, FPWAY did show significantly more amide hydrolysis than either FBWAY or MeFBWAY.

The quantitative time course of the parent and major metabolites in human hepatocytes based on integration of the radiochromatograms is shown in Fig. 6. The results highlight the much more rapid

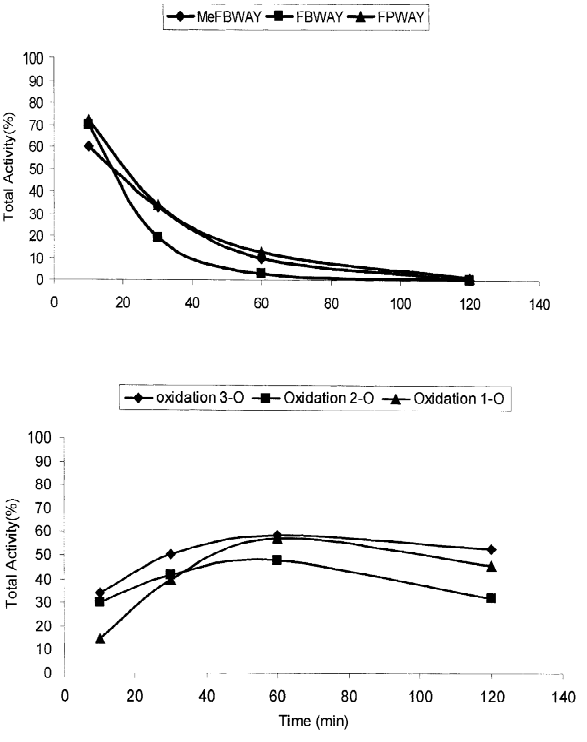


Fig. 7. Comparison of parent (top) and major metabolite phenol (bottom) after incubation with rat hepatocytes. MeFBWAY (diamond), FBWAY (square), FPWAY (triangle).

amide hydrolysis of FPWAY compared to the other analogues.

In human hepatocytes, a more significant difference in metabolism was noted among the compounds (Fig. 8). While FPWAY and FBWAY had a similar metabolic pathway of predominant amide hydrolysis, MeFBWAY was subjected to primarily aromatic ring oxidation. The comparative metabolic rate in human hepatocytes was FPWAY > FBWAY > MeFBWAY.

The pyrimidyl substitution for pyridyl in FPWAY apparently made the amide function much more labile to metabolic hydrolysis. When the pyridyl group was present the difference in metabolic hydrolysis rate between fluorobenzoyl and 3-methyl-4-fluorobenzoyl cannot be explained easily on electronic or structural arguments. Since the amide hydrolysis rate was significantly reduced in MeFBWAY, aromatic oxidation of MeFBWAY was the major metabolite in both rat and human hepatocytes in spite of the relatively different metabolism for the other two WAY analogues. The differences in metabolism in human hepatocytes are important because the acid is known to cross the blood–brain-barrier [13] whereas the biodistribution of the oxidation product is untested [5].

3.4. Parent extraction

With the identification of the radiolabeled metabolites, a simple method for the extraction of F-18 labeled parent compound (**1**) was developed. Because the major F-18 labeled metabolites include phenolic (**O**) 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

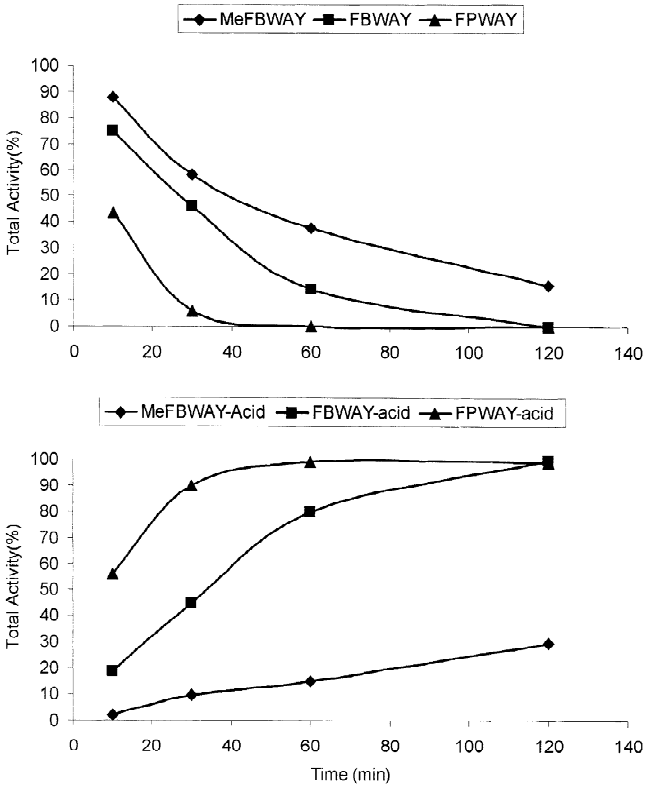


Fig. 8. Comparison of parent (top), and amide-hydrolysis (bottom) after incubation with human hepatocytes. MeFBWAY (diamond), FBWAY (square), FPWAY (triangle).

extraction of the parent was demonstrated by HPLC of both extracted phases. The parent was the only compound detected in the organic phase. The extraction efficiency for the pure parent compound from plasma was 94.5±3.5% ($n=6$).

4. Conclusion

While MeFBWAY showed similar metabolic rates and metabolites in both rat and human hepatocytes, FPWAY and FBWAY show dramatically different metabolic pathways. Because the major radiolabeled metabolites were phenolic and acidic, a simple, yet sensitive, extraction procedure was developed to determine the concentration of parent compound in plasma.

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