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In-vitro metabolism of the new anxiolytic agent, RWJ-50172, in rat hepatic S9 fraction and microbial transformation in fungi, *Cunninghamella* sp.

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Abstract

The in-vitro biotransformation of the anxiolytic agent, RWJ-50172 was studied after incubation with rat hepatic S9 fraction in the presence of an NADPH-generating system, and incubating with *Cunninghamella echinulata* in soy-bean medium. Unchanged RWJ-50172 (80% of the sample in rat; 86% in fungi) plus 6 metabolites (M1–M6) were profiled, quantified and tentatively identified on the basis of API-MS/MS data. The metabolic pathways for RWJ-50172 are proposed, and the four metabolic pathways are: pyrido-oxidation (pathway A), phenylhydroxylation (B), dehydration (C) and reduction (D). Pathway A formed hydroxy-pyrido-RWJ-50172 (M1, 10% of the sample in both rat and fungi) as the only major metabolite, which further dehydrated to form dehydro-RWJ-50172 in trace quantities in rat. Pathway B produced hydroxyphenyl-RWJ-50172 (M2) in small amounts (4%) in rat, and in conjunction with step A formed dihydroxy-RWJ-50172 as a trace metabolite in rat. Step D produced a minor benzimidazole-reduced metabolite in fungi. RWJ-50172 is substantially metabolized by this rat hepatic S9 fraction and fungi.

Introduction

RWJ-50172, 7-fluoro-1,2-dihydro-3-oxo- *N*-(2,6-difluorophenyl)pyrido[1,2- *a*]benzimidazole-4-carboxamide (Figure 1), is a new anxiolytic agent. It was synthesized by Johnson & Johnson Pharmaceutical Research & Development, L.L.C. (Spring House, PA) (Maryanoff et al 1995, 1996, 1999; Reitz et al 1998; Scott et al 1999; Jordan et al 2002). RWJ-50172 and its analogues, RWJ-51204, RWJ-51297, RWJ-51521, RWJ-52844 and RWJ-53050, bind with high affinity to the benzodiazepine site on GABA-A receptors (Maryanoff et al 1995; Scott et al 1999; Dubinsky et al 2002; Jordan et al 2002). Based on their in-vivo properties, including a lack of sedation, they have the potential to be clinically useful drugs for treating anxiety (Reitz et al 1998; Scott et al 1999; Dubinsky et al 2002). The benzodiazepine drugs have generally been held to be the most effective class of drug for treating anxiety disorders, although they cause side effects such as sedation and motor and memory impairment, etc. (Woods et al 1992, 1995). The in-vitro metabolism of RWJ-50172 and its analogues were investigated, to select the metabolically stable compounds for further anxiolytic studies. The in-vitro and in-vivo metabolism of RWJ-51204 (Wu et al 1998a, 1999a), RWJ-51297 (McKown et al 2000), RWJ-51521 (Wu et al 2000), RWJ-52763 (Wu et al 2001a), RWJ-52844 (Wu et al 1999b) and RWJ-53050 (Wu et al 1998b, 2001b) in the rat, dog and man have been investigated and reported previously. The objectives of the current study were to investigate the in-vitro metabolism of RWJ-50172 in rat hepatic S9 fraction and fungi, *Cunninghamella echinulata*, using LC/API-MS and MS/MS techniques. This resulted in the profiling, quantification, characterization and identification of unchanged RWJ-50172 and six metabolites. Preliminary results of this study have been reported previously (Wu et al 1996).

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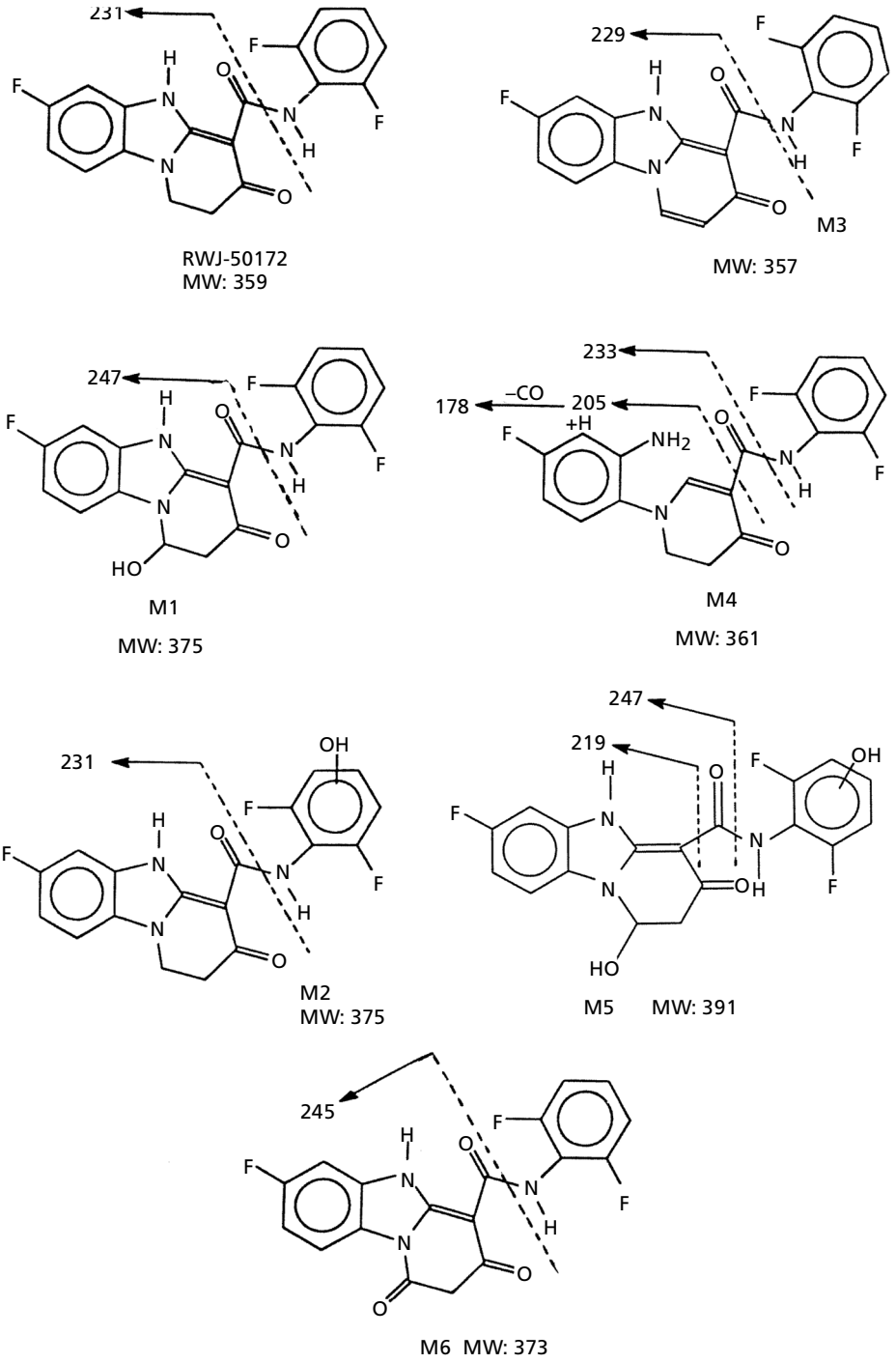

Figure 1 Structures and MS/MS product ions for RWJ-50172 and its metabolites.

Materials and Methods

Chemicals

RWJ-50172 was obtained from The CNS Research Team, Johnson & Johnson Pharmaceutical Research & Development, L.L.C. (Spring House, PA) with purity 97% (API-MS/MS/MS, HPLC). HPLC-grade solvents were obtained from the Fisher Scientific Co. (Fairlawn, NJ) and glass-distilled solvents were purchased from Burdick & Jackson Laboratories, Inc. (Muskegon, MI). The incubation components for S9, Tris, potassium chloride, magnesium chloride, $NADP^+$ and glucose-6-phosphate, were purchased from Sigma (St Louis, MO).

Hepatic S9 fraction

The rat hepatic S9 fraction was generated from a male Crl:CD (SD)IGSBR VAF/Plus rat, 200–250 g, fed with certified rodent diet meal at room temperature at Johnson & Johnson Pharmaceutical Research & Development, LLC (Spring House, PA).

Rat hepatic S9 incubation

Chilled, freshly-made components were added to each flask (on ice) in the following order: 1.15% KCl in 0.05 M Tris buffer (pH 7.4), 5 mM $MgCl_2$, 5 mM glucose-6-phosphate, 0.5 mM $NADP^+$, test substrate (hepatic S9) and RWJ-50172 spike, to obtain a final volume of 5 mL and a RWJ-50172 concentration of 100 $\mu g\,mL^{-1}$. After the addition of the last component, each flask was incubated in a 37 °C Dubnoff Metabolic Shaker Incubator (Precision Scientific, Chicago, IL). Samples were removed at 0, 30 and 60 min. Control flasks were incubated without rat subcellular fraction, to determine drug stability under incubation conditions.

Microbial culture and transformation

Cultures of *Cunninghamella echinulata* (American Type Culture Collection, Rockville, MA) were maintained on potato dextrose agar slants (Remel, Lenexa, KS) and kept at 4 °C. The spores and mycelia were transferred to potato dextrose agar plates to grow for 48 h at room temperature. The sterile soybean meal medium was added to the agar plate of *Cunninghamella echinulata* by stirring to form a suspension. The spore suspension was transferred to a sterile medium flask with 25 mL of medium containing soybean flour, glucose, potassium dibasic phosphate and yeast extract. The cultures were incubated for 24 h at 27 °C by shaking at 250 rev min^{-1}, and then 20 mg of RWJ-50172 in 0.5 mL of DMSO was added. Control experiments consisted of cultures without RWJ-50172 or RWJ-50172 in culture medium without fungi. Samples (5 mL) were obtained at 0, 24, 48 and 72 h and each mixed with 5 mL methanol. One mL of aqueous methanolic solution, after diluting with 4 mL of water, was extracted with ether (10 mL). The ether extract residue, after reconstituting in methanol (0.25 mL), was analysed by HPLC (C18 column; detector: UV 270 nm; isocratic gradient elution (90% H_2O and 10–100% MeOH in 20 min): H_2O–MeOH with 0.02% ammonium acetate; flow rate: 1 mL min^{-1}, and followed by Sciex ionspray-MS and MS/MS analysis.

Sample storage

Immediately following removal from the incubator, samples were transferred to pre-labelled storage vials, deactivated by the addition of ethyl acetate and placed in a dry-ice–acetone bath to terminate and freeze the reaction. Samples were stored at approximately −20 °C.

Metabolite profiling, quantifying and identification

Following ethyl acetate (3 mL) extraction of each ammonium-hydroxide-basified (pH ~ 9) hepatic S9 incubate (1 mL), the extract residue was reconstituted in buffer (0.5 mL of acetonitrile–water (50:50, v/v) with 5 mM ammonium acetate buffer, pH 4.0) and then analysed via 20 μL flow-injection using the LC (C18)/ PE Sciex API III-Plus MS (Perkin-Elmer Sciex Instuments, Thornhill, ON), a triple quadrupole mass spectrometer, interfaced to a Hitachi HPLC solvent delivery system (L-6200 A Intelligent pump) via an ionsprayer using nitrogen as a curtain and nebulizing gas and argon as a collision gas for MS/MS analysis. The mobile phase for this system was the same buffer as described for the residue reconstitution, at a flow rate of 0.1 mL min^{-1}. For each sample, the relative percentage of unchanged RWJ-50172 and metabolites were estimated using the integrated chromatograms generated by the Sciex API-III Q1 scan MS (TIC, total ion chromatogram). These data were not absolutely quantitative, due to potential differences in the degree of ionization of each analyte. However, they were reproducible. Unchanged RWJ-50172 and its metabolites were elucidated on the basis of MS and MS/MS data.

Methyl derivatization

Each extract residue was dissolved in 1 mL of methanol, added with an excess amount of ethereal diazomethane, and left at room temperature overnight, followed by evaporation to yield a residue. Each residue was analysed for further confirmation of metabolites using the same method as described above.

Results and Discussion

The in-vitro and microbial biotransformation of RWJ-50172 was conducted in rat hepatic S9 fraction and fungi, *Cunninghamella echinulata*, respectively. Unchanged RWJ-50172 (80% of the sample in rat; 86% in fungi) and a total

Table 1 Metabolism of RWJ-50172 in rat hepatic S9 and fungi.

Analyte	Rat hepatic S9 (%)		Fungi (%)	
	30 min	60 min	24 h	72 h
RWJ-50172	88	80	92	86
M1	7	10	6	10
M2	3	4	—	—
M3	<1	<1	—	—
M4	<1	<1	2	3
M5	—	<1	—	—
M6	1	2	—	—

Data are derived from the integrated ion chromatograms via Q1 scan MS determinations.

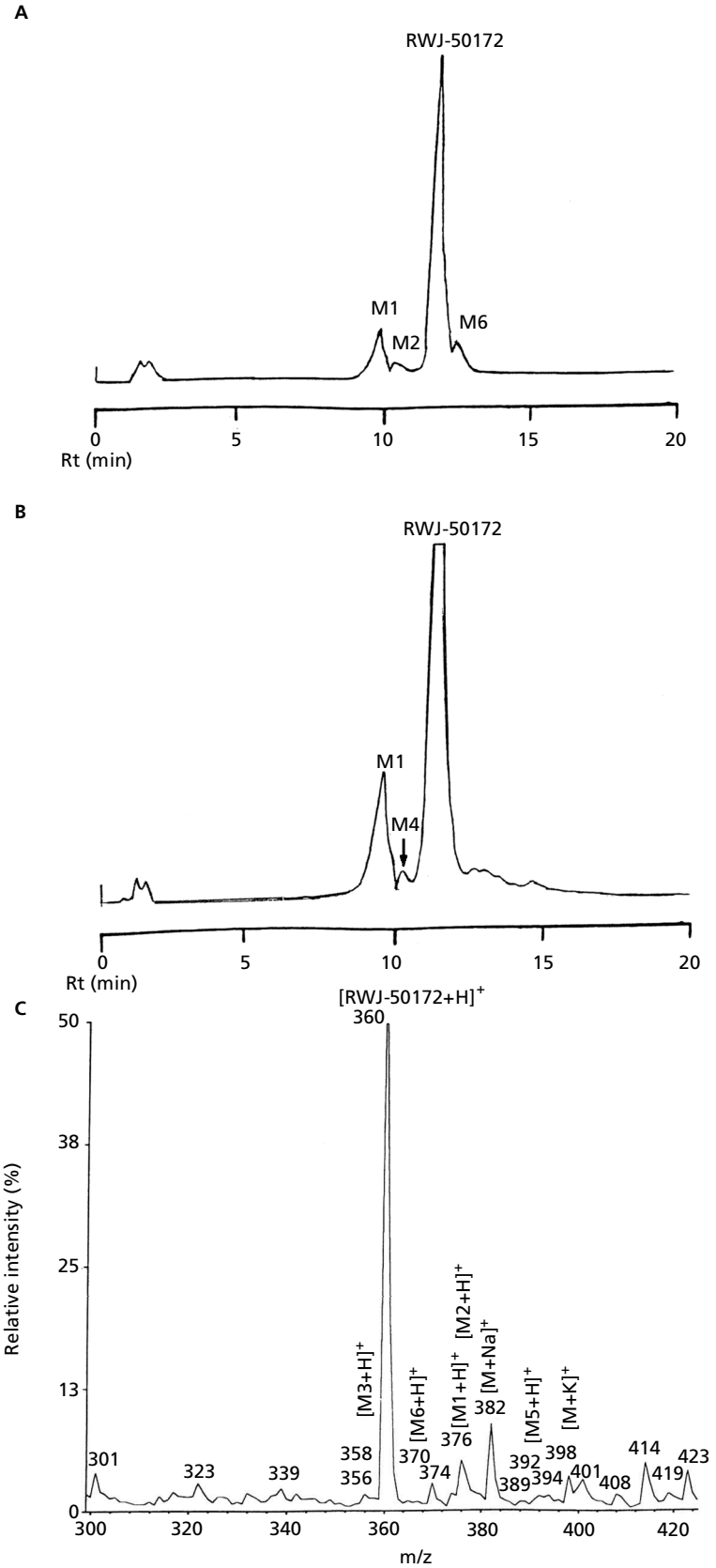

Figure 2 HPLC profiles of rat hepatic S9 60-min (A) and fungal 72-h (B) incubation of RWJ-50172 and its metabolites and MS profile of RWJ-50172 in rat hepatic S9 (60-min) fraction (C).

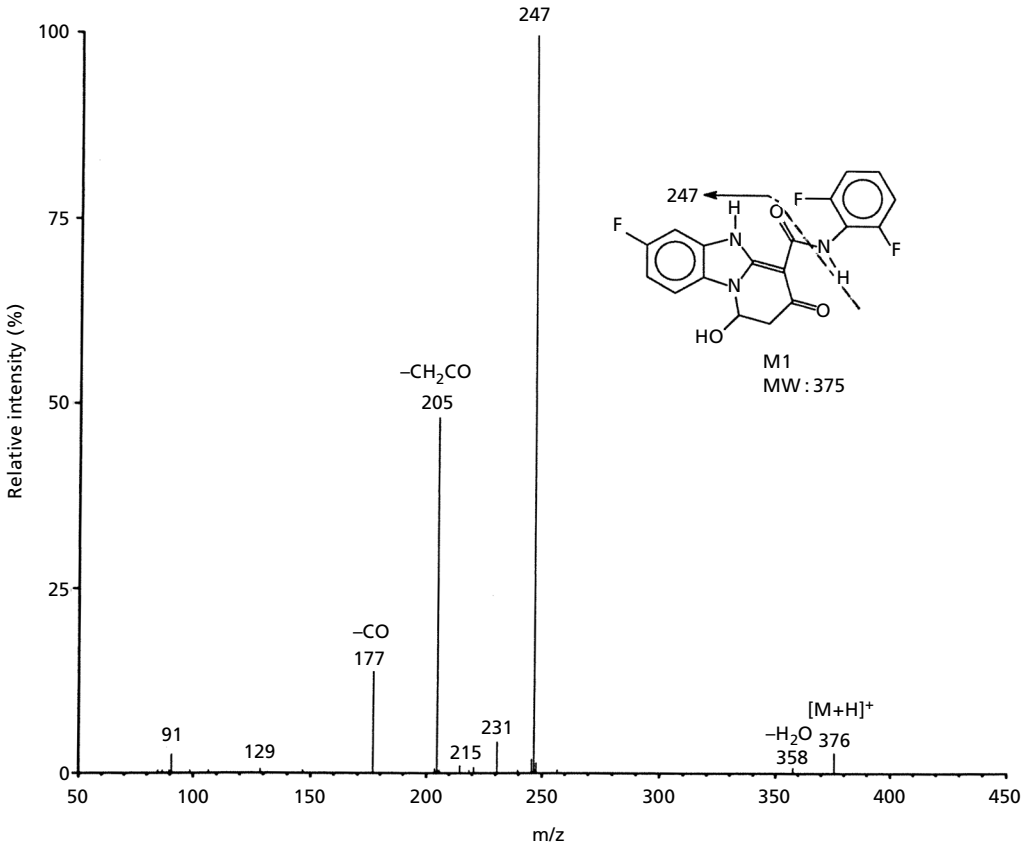

Figure 3 Representative API-MS/MS spectrum of metabolite 1.

of 6 metabolites (M1–M6), were profiled, quantified, characterized and tentatively identified in the 30- and 60-min hepatic incubates and 72-h fungal incubates based on API ionspray-MS and MS/MS data. The structures of RWJ-50172 and its metabolites, and their MS data are presented in Figure 1, and the percent of unchanged RWJ-50712 and each metabolite are shown in Table 1. Control incubates revealed unchanged RWJ-50172 only.

The representative metabolic profiles for the 60-min incubate of rat S9 and the 72-h incubate of fungi are presented in Figure 2.

The representative mass spectrum for metabolite 1 is presented in Figure 3. Metabolite 2 was further derivatized to form a methyl ether, and confirmed by mass spectral data (Figure 1). The isolation, profiling, quantification, characterization and identification of unchanged RWJ-50172 and each metabolite are discussed below.

Unchanged RWJ-50172 (MW 359, HPLC retention time 11.8 min) was isolated, and identified from all incubates (0, 30 and 60 min in rat; 0–72 h in fungi) by solvent extraction and MS and MS/MS techniques in comparison with authentic RWJ-50172 (Figures 1 and 2). Mass spectral analysis of RWJ-50172 revealed an intense protonated molecular ion at m/z 360 ($[M + H]^+$) (Figures 1 and 2). The MS/MS analysis of m/z 360 exhibited prominent product ions (% relative abundance) at m/z 249 (31%), 231 (100%) and 230 (2%) (Figure 1). Unchanged RWJ-50172 was present in major quantities (80% of the sample) in the 60-min S9 incubate (Table 1).

Metabolite 1 (MW 375, HPLC retention time 9.6 min) was present as a major metabolite in rat S9 and fungi (10% of the sample) (Table 1). The MS and MS/MS ($[M + H]^+$) spectral data showed a protonated molecular ion at m/z 376 (3%) and diagnostic product ions at m/z 358 (MH^+-H_2O, 1%), 347 (100%), 231 (6%), 215 (2%), 205 (48%) and 177 (13%) (Figures 1–3). The MS data clearly assigned M1 as OH-pyrido-RWJ-50172. It remained unchanged by the reaction of diazomethane.

Metabolite 2 (MW 375, HPLC retention time 10.2 min) was present in small amounts in the 60-min incubate of rat S9 (4%) (Table 1). The structure of M2 was tentatively identified on the basis of MS and MS/MS data, and methyl derivatization. The MS data displayed an apparent protonated molecular ion at m/z 376 (Figures 1 and 2). The MS/MS analysis of the protonated molecular ion revealed important product ions at 358 (MH^+-H_2O, 1%), 348 (MH^+-CO, 2%), 231 (100%), 220 (6%), 205 (15%) and 177 (4%) (Figure 1). The MS and MS/MS spectra of M2 methyl ether showed an intense protonated molecular ion at m/z 390 and MS/MS of m/z 390 provided an informative product ion at m/z 231 (100%). M2 was identified as OH-phenyl-RWJ-50172.

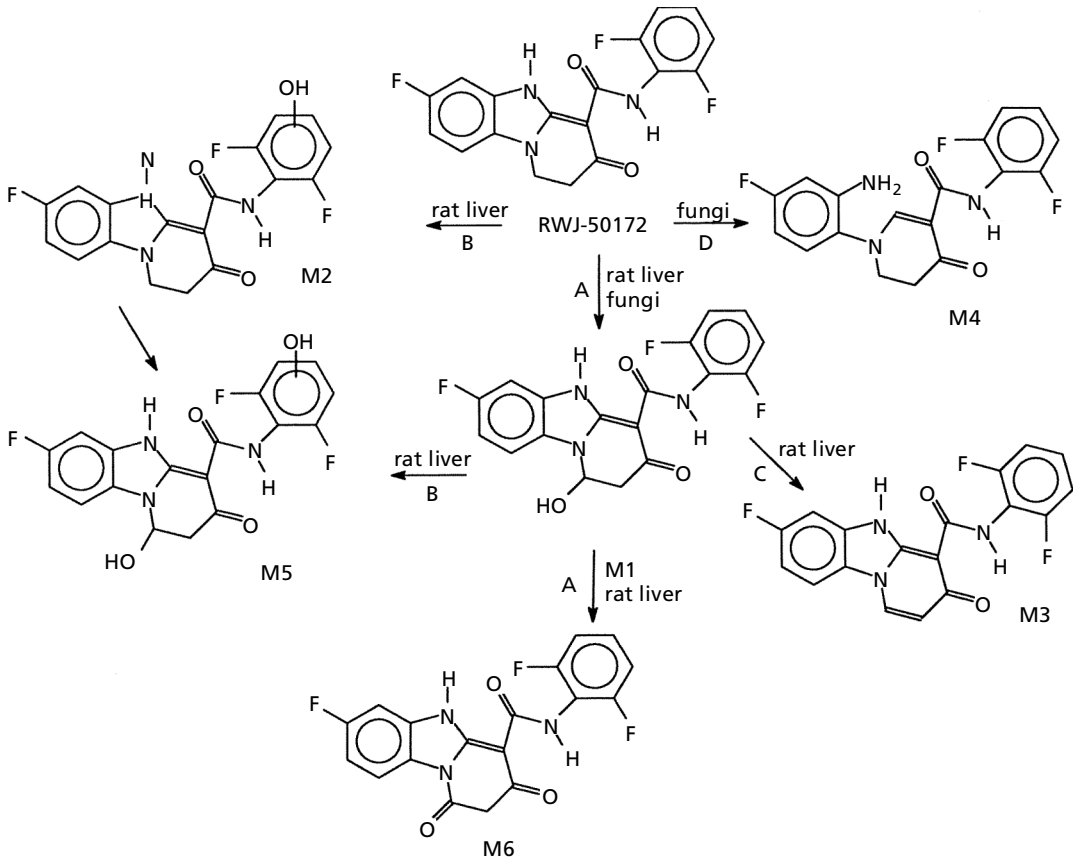

Figure 4 Proposed metabolic pathways for RWJ-50172 in the rat and fungi.

Metabolite 3 (MW 357) was detected in trace amounts of rat S9 (<1%) (Table 1). The MS data for this metabolite gave an apparent protonated molecular ion at m/z 358 (Figure 2), and MS/MS analysis of the protonated molecular ion (m/z 358) exhibited prominent as well as informative product ions at m/z 341 (MH^+-NH_3, 1%), 340 (MH^+-H_2O, 3%), 317 (2%), 257 (80%), 247 (12%), 229 (100%), 197 (8%) and 185 (32%), together with a protonated molecular ion 358 (21%) (Figure 1). The MS data of M3 characterized the metabolite as dehydro-RWJ-50172.

Metabolite 4 (MW 361, HPLC retention time 10.1 min) was found as a minor metabolite in the 72-h incubate of fungi (3%) (Table 1). The ionspray-MS and MS/MS analysis of this metabolite contained a protonated molecular ion at m/z 362 in MS spectrum, and important product ions at m/z 344 (MH^+-H_2O, 1%), 233 (100%), 232 (77%), 205 (1%) and 178 (205-CO + H, 2%), along with a protonated molecular ion at m/z 362 (1%) in MS/MS spectrum (Figure 1). On the basis of the MS data, metabolite 4 was tentatively proposed to be reduced pyrido-RWJ-50172.

Metabolite 5 (MW 391) was present in trace amounts (<1%) in rat S9 incubate (Table 1). This metabolite showed a protonated molecular ion at m/z 392 in ionspray-MS (Figure 2), and significant product ions at m/z 247 (8%), 219 (23%), 205 (2%) and 175 (2%), together with a protonated molecular ion at m/z 392 (100%) (Figure 1). The structure of metabolite 5 was tentatively proposed to be OH-phenyl-OH-pyrido-RWJ-50172.

Metabolite 6 (MW 373, HPLC retention time 12.5 min) was detected in trace amounts in the rat S9 incubate (2%) (Table 1, Figure 2). An apparent protonated molecular ion at m/z 374 exhibited in MS spectrum indicated a molecular weight of 2 amu less than metabolite 1, OH-pyrido-RWJ-50172. The loss of 2 amu could be explained by the formation of oxo-pyrido group via further oxidation of M1. The MS/MS analysis of protonated molecular ion (m/z 374) provided informative product ions at m/z 342 (MH^+-MeOH, 1%), 277 (1%), 263 (2%) and 245 (100%), along with an apparent protonated molecular ion (6%) (Figure 1).

Conclusion

The in-vitro metabolism and microbial transformation of RWJ-50172 was conducted in the rat hepatic S9 fraction, and fungal incubation. Unchanged RWJ-50172, plus six metabolites, were profiled, quantified, characterized and tentatively identified on the basis of MS data. API ionspray-MS and MS/MS exhibited apparent protonated molecular ions, and prominent as well as important

product ions for the structural elucidation of RWJ-50172, its metabolites and methyl derivatives. Formation of these metabolites in the rat hepatic S9 and fungi can be explained by four pathways: pyrido-oxidation (A), phenyl-hydroxylation (B), dehydration (C) and reduction (D). Pathway A appeared to be the most important pathway, forming major and trace metabolites, OH-pyrido-RWJ-50172 (M1, 10% of the sample in both rat and fungi) and oxo-pyrido-RWJ-50172 (M6, 2% in rat) and, in conjunction with pathway C, produced a trace dehydro-RWJ-50172 (M3, <1%). Pathway B produced a minor metabolite, hydroxy-phenyl-RWJ-50172 (M2, 4% in rat) and, in conjunction with step A, formed dihydroxy-RWJ-50172 (M5, <1%) (Table 1). Pathway D formed a minor benzimidazole-cleaved product, reduced RWJ-50172 (M4, 3%) in fungal incubation. The proposed in-vitro metabolic pathways for RWJ-50172 in rat hepatic S9 fraction and fungal incubation are depicted in Figure 4. In conclusion, our results indicated that RWJ-50172 is substantially metabolized in rat hepatic S9 fraction and fungi.

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