JPP Journal of Pharmacy and Pharmacology

JPP 2003, 55: 631–637

Received October 2, 2002
Accepted February 5, 2003
DOI 10.1211/002235702973
ISSN 0022-3573

Hepatic biotransformation of the new calcium-mimetic agent, RWJ-68025, in the rat and in man – API-MS/MS identification of metabolites

Wu-Nan Wu, Linda A. McKown, Philip J. Rybczynski and Keith Demarest

Abstract

The in-vitro biotransformation of a new calcium-mimetic agent and benzenemethanamine analogue, RWJ-68025, was studied after incubation with rat and human hepatic S9 fractions in the presence of an NADPH-generating system. Unchanged RWJ-68025 (44–48% of the sample) plus 12 metabolites were profiled, quantified, and tentatively identified on the basis of API (ionspray)-MS and MS/MS data, and ethyl derivatization for phenolic and carboxylic metabolites. Four metabolic pathways for RWJ-68025 were proposed: pathway 1, O-demethylation; pathway 2, phenyl oxidation; pathway 3, methyl oxidation; and pathway 4, N-dealkylation/acetylation. Pathway 1 formed a major metabolite, O-desmethyl-RWJ-68025 (M1; RWJ-68311; 26% in rat; 16% in human fraction). Pathway 2 produced one major (M2; 12–17% in rat and human fraction) and two minor phenolic metabolites (M4 and M5; all <1% in both species), and in conjunction with step 1, formed hydroxy-M1 (M3; 4–5% in both species). Pathways 3 and 4 formed seven minor oxidized metabolites (M6–M12). RWJ-68025 was extensively metabolized in the rat and human hepatic S9 fractions.

Introduction

RWJ-68025, 1-*R*-phenyl-2-*R*-(1-(3-methoxyphenyl)-*R*-ethylamino)methylcyclo-propane, is a new orally active agonist of the extracellular calcium receptor (Figure 1) (Fray et al 1987; Nemeth & Carafolei 1990; Brown 1991; Brown et al 1993; Brown & Hebert 1997; Nemeth et al 1998). Like elevated extracellular calcium, RWJ-68025 stimulated the mobilization of intracellular calcium and inhibited cAMP accumulation in cells which possessed the calcium-sensing receptor (CaSR) (Fray et al 1987; Brown 1991; Brown et al 1993; Pollak et al 1993). RWJ-68025 inhibited bone resorption in neonatal mouse bone organ culture (Nemeth & Carafolei 1990; Raisz 1992; Brown et al 1993; Pollak et al 1993; Riccardi et al 1996; Brown & Hebert 1997; Nemeth et al 1998). NPS R-568 (Norcalcin, 3-methoxyphenyl-*R*-ethylamino-2-chlorophenylpropane), an analogue of RWJ-68025, is an orally active agonist at the CaSR for the potential treatment of osteoporosis that inhibits parathyroid hormone (PTH) secretion in-vitro and in rats and man in-vivo (Raisz 1992; Brown et al 1993; Fox 1994; Heath 1995; Fox et al 1999a, b). RWJ-68025 was first synthesized at Johnson & Johnson Pharmaceutical Research & Development, L.L.C. (Raritan, NJ). Preliminary results on the in-vitro metabolism of RWJ-68025 in rat and human hepatic S9 fractions were reported by Wu et al (2000). The in-vitro metabolism of a diastereomeric pair of RWJ-69113 and RWJ-69115, two analogues of RWJ-68025, in rat, dog and human hepatic S9 fractions has been presented (Wu et al 2001). The objectives of this paper were to report the in-vitro metabolism of RWJ-68025 in rat and human hepatic S9 fractions using API-ionspray-MS, MS/MS, and derivatization techniques. This resulted in the profiling, quantification, characterization, and identification of unchanged RWJ-68025 plus 12 metabolites.

Johnson & Johnson Pharmaceutical Research & Development, L.L.C., Spring House, PA, USA

Wu-Nan Wu, Linda A. McKown

Johnson & Johnson Pharmaceutical Research & Development, L.L.C., Raritan, NJ, USA

Philip J. Rybczynski, Keith Demarest

Correspondence: W. N. Wu, Johnson & Johnson Pharmaceutical Research & Development, L.L.C., Spring House, PA, USA.
E-mail: wwu@prdus.jnj.com

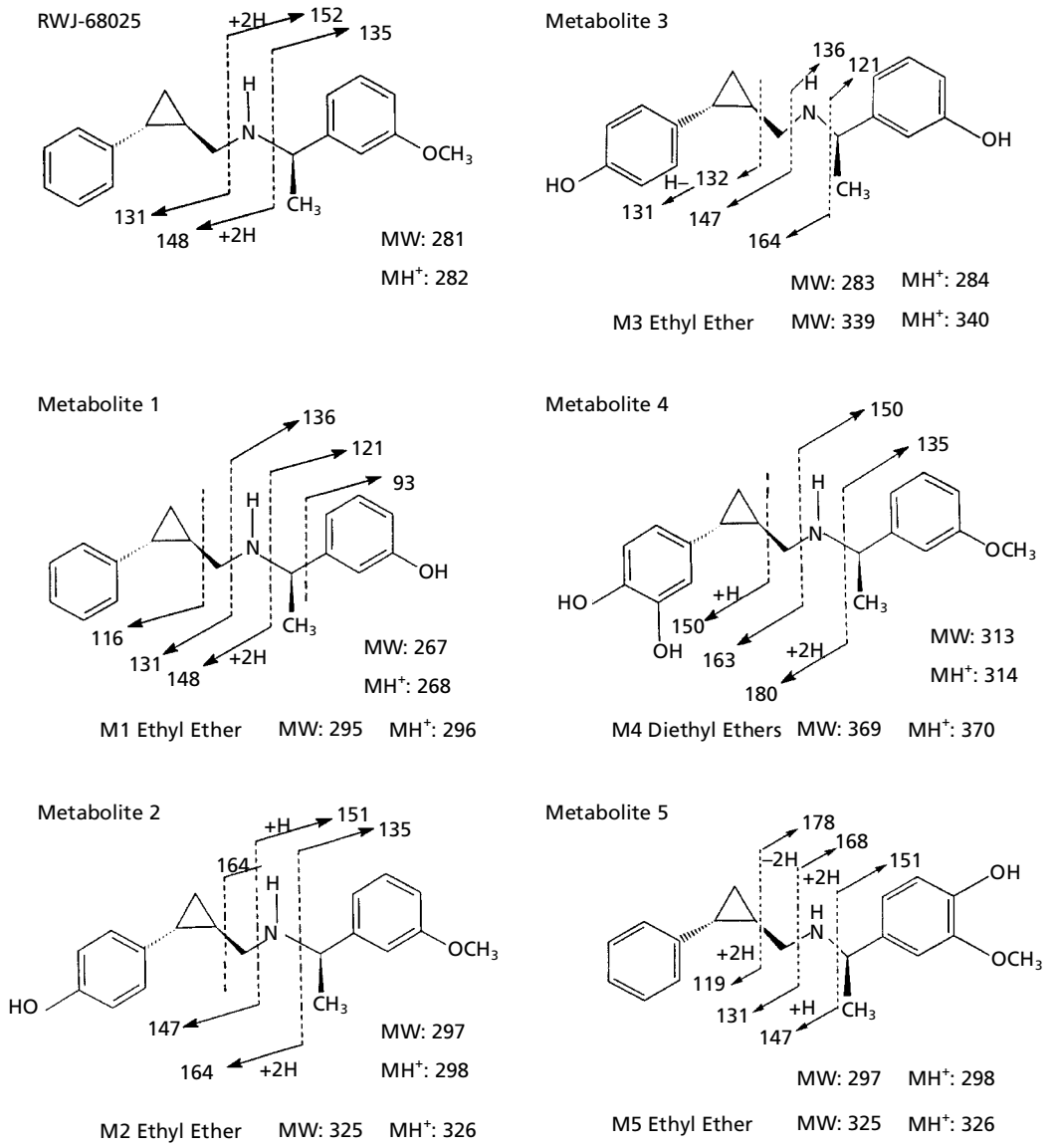

Figure 1 Structures and MS/MS product ions for RWJ-68025, metabolites and ethyl derivatives.

Materials and Methods

Chemicals

RWJ-68025 and its metabolite, metabolite 1 (M1, RWJ-68311), were synthesized at Johnson & Johnson Pharmaceutical Research & Development, L.L.C. (Raritan, NJ) with purity >97% (API-MS/MS, HPLC). HPLC-grade solvents were obtained from the Fisher Scientific Co. (Fairlawn, NJ) and glass-distilled solvents were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI). The incubation components for S9 fractions, Tris, potassium chloride, magnesium chloride, $NADP^+$ and glucose-6-phosphate, were purchased from Sigma (St Louis, MO). 1-Ethyl-3-nitro-1-nitrosoguanidine was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI).

Hepatic S9 fractions

The rat hepatic S9 fraction was generated from a male, Crl:CD (SD)IGS BR VAF/Plus rat at Johnson & Johnson Pharmaceutical Research & Development, L.L.C. (Spring House, PA). The human hepatic S9 fraction was purchased from Xenotech (Kansas City, KS).

Rat and human 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-68025, to obtain a final volume of 5 mL and an RWJ-68025 concentration of 100 $\mu g\,mL^{-1}$. After the addition of the last component, each flask was incubated

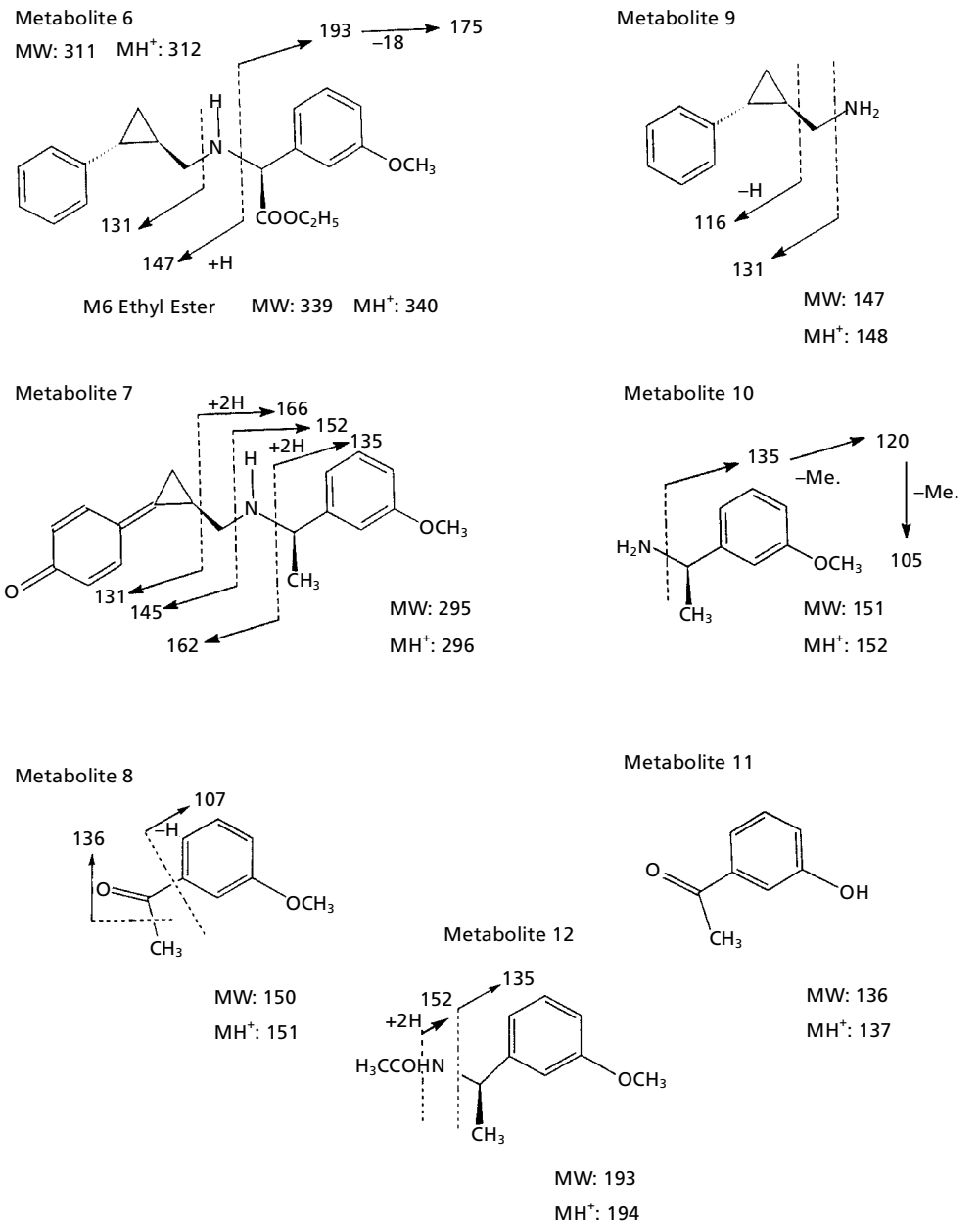

Figure 1 (*Continued*).

in a 38 °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.

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) incubate (1 mL), the extract residue was reconstituted in buffer (0.5 mL acetonitrile/water (50/50, v/v) with 5 mM ammonium acetate buffer, pH 4.0) and then analysed via a 20-μL flow-injection using the PE Sciex API III-Plus MS (Perkin-Elmer Sciex Instruments, Thornhill, Ontario, Canada), a triple quadruple mass spectrometer, interfaced to a Hitachi HPLC (C18 column) 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 isocratic 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-68025 and its 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-68025, its metabolites, and ethyl derivatives were elucidated on the basis of MS and MS/MS data.

Ethyl derivatization

Each extract residue was dissolved in 0.5 mL methanol, added with an excess amount of ethereal diazoethane generated from 1-ethyl-3-nitro-1-nitrosoguanidine, 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 biotransformation of RWJ-68025 was conducted in rat and human hepatic S9 fractions. Unchanged RWJ-68025 (60-min incubate, 44–48% of the sample) and a total of 12 metabolites (M1–M12), were profiled, quantified, characterized, and tentatively identified in the 30-and 60-min incubates (Table 1), based on API ionspray-MS and MS/MS data. The structures of RWJ-68025, its metabolites, ethyl derivatives, and their MS data are presented in Figure 1, and the percent of unchanged RWJ-68025 and each metabolite are shown in Table 1. Control incubates revealed unchanged RWJ-68025 only. The representative metabolic profile using Q1 scan MS (TIC) for the 60-min incubate of human hepatic S9 is presented in Figure 2. Representative mass spectrum for M1 is presented in Figure 3. Metabolites 1–6 were further derivatized to form ethyl ethers and ester, and confirmed by the MS/MS data (Figure 1).

Unchanged RWJ-68025 was isolated, and identified from all incubates (0, 30 and 60 min) by solvent extraction and MS and MS/MS techniques in comparison with authentic RWJ-68025 (Figures 1 and 2). The Q1 mass spectral analysis of RWJ-68025 revealed an intense protonated molecular ion at m/z 282 (MH^+) (Figures 1 and 2) which, followed by MS/MS analysis, exhibited prominent product ions at m/z (% relative abundance) 164(1), 152(3), 148(13), 135(100), 131(97), 129(3), 120(1), 105(3), and 91(2), together with 282(MH^+, 11) (Figure 1). Unchanged RWJ-68025 was present in major quantities (44–48% of the sample) in the 60-min S9 incubates of both species (Table 1).

Metabolite 1 was present as a major metabolite (16–26% of the sample) in the phenolic fraction of both species (Table 1). The MS and MS/MS (MH^+) spectral data showed an intense protonated molecular ion at m/z 268, and diagnostic product ions at m/z (%) 148(1), 136(2), 131(100), 129(7), 121(71), 116(30), 103(3), and 91(14), along with 268(MH^+, 1) (Figures 1–3). The MS data clearly assigned M1 as O-desmethyl-RWJ-68025 by comparison with synthetic sample (RWJ-68311). It was further derivatized as an ethyl ether (MS/MS of MH^+, m/z 296(14): 166(2), 149(100), 148(20), 131(91), 129(2), 121(14), and 91(1)) (Figure 1) by the reaction of diazoethane.

Metabolite 2 was present in major amounts in the 60-min incubates of both species (12–17%) (Table 1). The structure of M2 was tentatively identified on the basis of MS and MS/MS data. The MS data displayed an apparent protonated molecular ion at m/z 298 (Figure 1), which was analysed by MS/MS technique to reveal important product ions at m/z (%) 164(3), 152(10), 151(11),

Table 1 Hepatic metabolism of RWJ-68025 in the rat and human hepatic S9 fractions.

Analyte	Rat S9 fraction		Human S9 fraction	
	30 min	60 min	30 min	60 min
RWJ-68025	49	44	63	48
M1, RWJ-68311 (O-desmethyl-RWJ-68025)	27	26	9	16
M2 (OH-Ph-RWJ-68025)	13	12	16	17
M3 (OH-Ph-O-desmethyl-RWJ-68025)	2	4	2	5
M4 (diOH-Ph-RWJ-68025)	–	< 1	–	< 1
M5 (OH-methoxy-Ph-RWJ-68025)	–	< 1	–	–
M6 (carboxy-RWJ-68025)	–	< 2	–	–
M7 (quinone)	< 2	< 2	3	3
M8	< 1	< 1	< 1	< 1
M9	4	4	5	6
M10	< 2	2	< 2	2
M11	< 1	< 1	< 1	< 1
M12	–	< 1	–	–

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

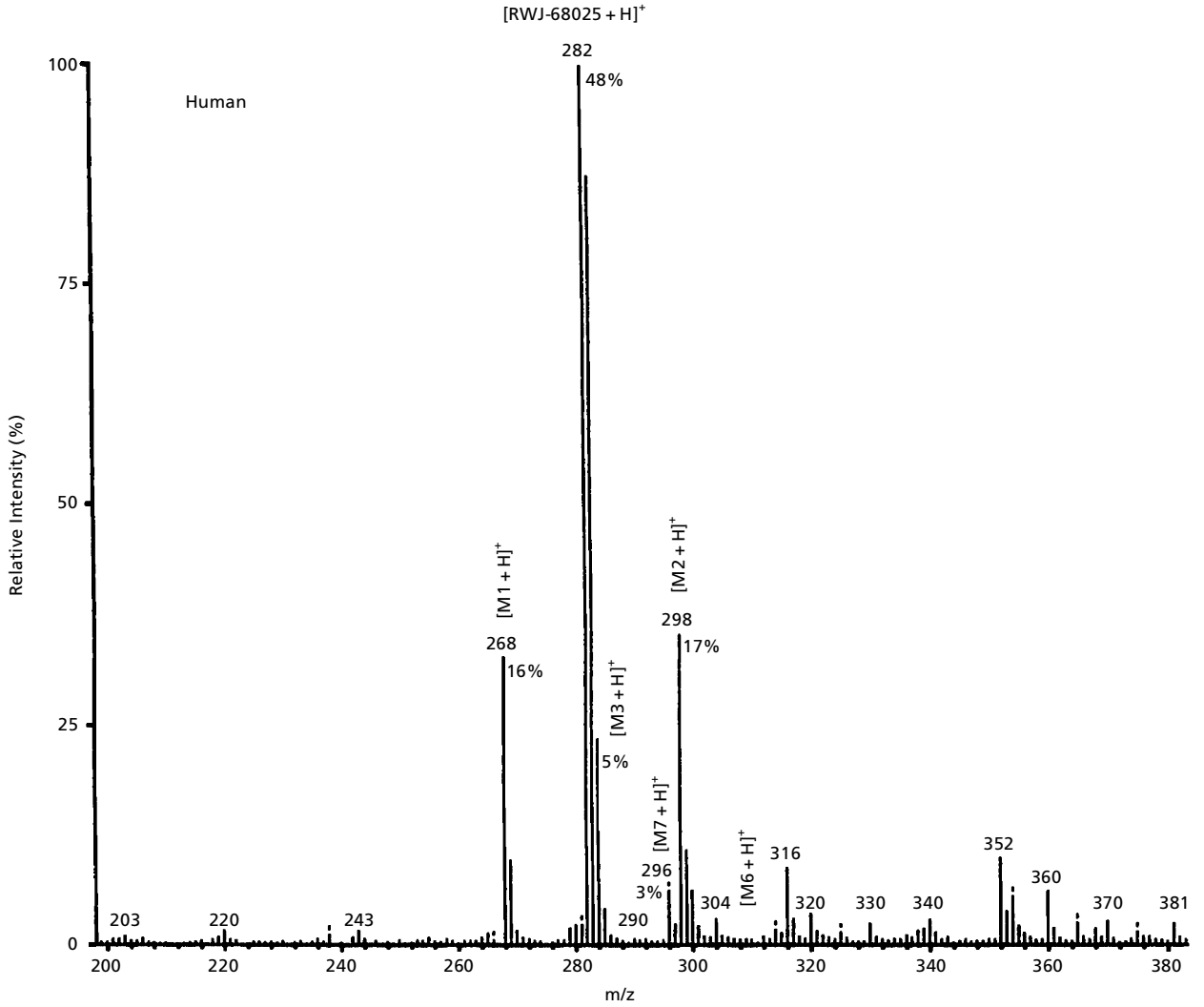

Figure 2 API-Q1 scan MS of human hepatic incubate of RWJ-68025 (60 min).

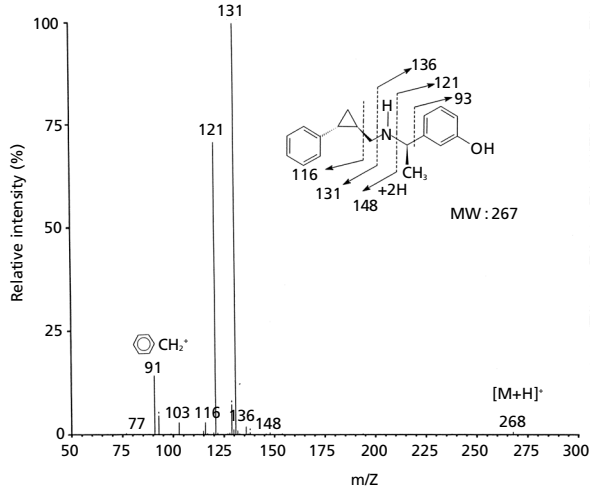

Figure 3 API-MS/MS spectrum of metabolite 1.

147(100), 135(68), 131(11), 119(3), 107(1), and 91(1) with 298(MH^+, 5) (Figures 1 and 2). M2 was identified as hydroxy-phenyl-RWJ-68025 from a phenolic fraction. It formed an ethyl ether (MS/MS of MH^+: 326(3), 179(10), 175(100), 151(1), 147(18), 135(8), 119(4), 107(2), and 91(1)) (Figure 1) by diazoethane reaction.

Metabolite 3 was detected in minor amounts (4–5%) (Table 1). The MS data for this metabolite gave an apparent protonated molecular ion at m/z 284 (Figure 1), and MS/MS analysis of the protonated molecular ion exhibited prominent as well as informative product ions at m/z (%) 164(11), 147(19), 136(100), 132(86), 131(89), 121(29), and 106(2), together with a protonated molecular ion 284(23) (Figure 1). The MS data of M3 characterized the metabolite as hydroxyphenyl-O-desmethyl-RWJ-68025 (OH-M1). Ethylation of M3 produced a diethyl ether, which displayed an intense protonated molecular ion at m/z 340 in the MS spectrum and product ions at m/z (%) 175(100), 165(20), 149(25), 147(22), and 131(28) in the MS/MS spectrum.

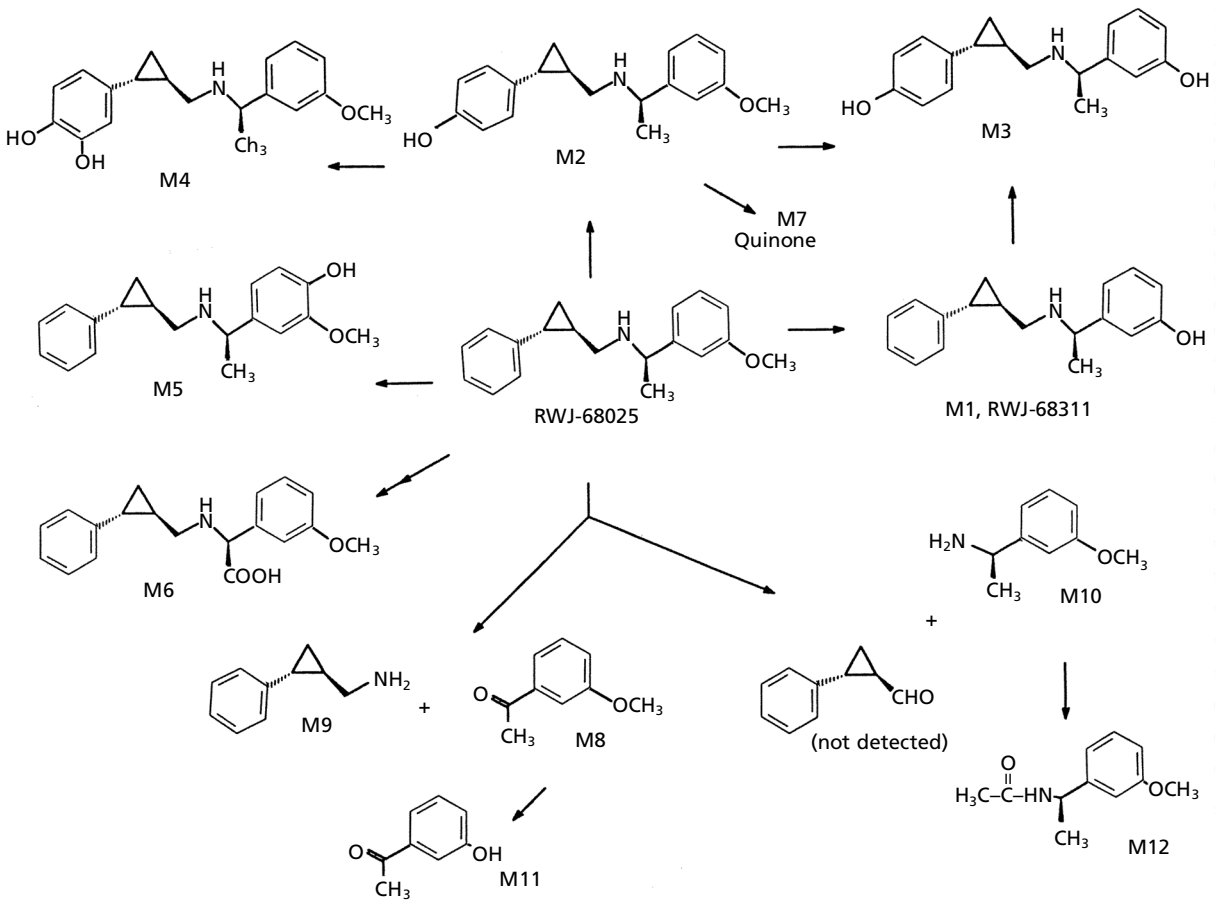

Figure 4 Proposed in-vitro metabolism of RWJ-68025 in rat and human hepatic S9 fractions.

Metabolite 4 was found as a minor metabolite in the 60-min rat incubate (< 1%) (Table 1). The ionspray-MS and MS/MS analysis of this metabolite contained a protonated molecular ion at m/z 314 in MS spectrum, and important product ions at m/z (%) 249(3), 235(3), 163(61), 150(4), 147(50), 145(11), 135(100), and 117(14), along with a protonated molecular ion at m/z 314(9) in the MS/MS spectrum (Figure 1). On the basis of the MS data, the structure of metabolite 4 was tentatively proposed to be dihydroxyphenyl-RWJ-68025. It was further derivatized as a diethyl ether, which was confirmed by MS data (MH^+: 370; MS/MS: m/z (%) 370(28), 236(5), 219(100), 165(24), and 135(14)) (Figure 1).

Metabolite 5 was present in trace amounts (< 1%) in rat incubate (Table 1). This metabolite showed a protonated molecular at m/z 298 in ionspray-MS (Figure 1), and significant product ions at m/z (%) 178(2), 168(1), 151(7), 147(100), 146(6), 131(52), and 119(3), together with a protonated molecular ion (m/z 298(11)) in the MS/MS data (Figure 1). The structure of metabolite 5 was tentatively proposed to be hydroxy-methoxyphenyl-RWJ-68025, which was further converted to an ethyl ether by diazoethane derivatization. The MS/MS data of M5 ethyl ether showed m/z (%) MH^+: 326(15), 179(42), 147(100), 133(27), and 117(2) (Figure 1).

Metabolite 6 was detected in trace amounts in the 60-min rat incubate (< 2%). An apparent protonated molecular ion at m/z 312 exhibited in MS spectrum, which could be explained by the formation of carboxy group via further methyl oxidation of parent compound. It was diazoethane-derivatized as an ethyl ester, which was further confirmed by MS/MS analysis of protonated molecular ion (m/z 340) providing prominent as well as informative product ions at m/z 193(100), 175(55), 165(3), 149(5), 147(3), and 131(5), along with an apparent protonated molecular ion 340(5) (Figure 1).

Metabolite 7 was detected in minor amounts (2–3%) in 60-min incubates of all species and showed a protonated molecular ion at m/z 296, which exhibited important product ions at m/z (%) 166(13), 162(5), 145(1), 135(100), 131(26), 107(3) and 91(1) in the MS/MS spectrum (Figure 1). On the basis of MS data, M7 was tentatively proposed to be a quinone metabolite.

Metabolite 8 was profiled as a trace N-dealkylated metabolite (< 1%) in all 60-min incubates. The Q1 scan MS and MS/MS showed a protonated molecular ion at m/z (%) 151(100) and product ions at m/z (%) 136(MH^+-Me, 6), 119(MH^+-MeOH, 51), 107(2), and 91(24) (Figure 1). The structure of metabolite 8 was tentatively assigned as methoxyphenyl methyl ketone, based on the MS data.

Metabolite 9 was identified as a minor N-dealkylated metabolite in both species (4–6%) (Table 1), which revealed an apparent molecular ion at m/z 148 (Figure 1). The MS/MS analysis of m/z 148 displayed the diagnostic product ions at m/z (%) 131(100), 129(8), 120(2), 116(3), and 91(9) (Figure 1). The structure of metabolite 9 was tentatively elucidated as phenyl cyclopropylmethyl amine on the basis of MS data.

Metabolite 10 was found as a minor metabolite in both species (2%) (Table 1), which gave a protonated molecular ion at m/z 152. The MS/MS spectrum of the metabolite provided important fragment ions at m/z 135(100), 120(7), 105(8), and 92(2) (Figure 1). These data were consistent with the structure of 3-methoxyphenyl ethyl amine tentatively assigned for M10.

Metabolite 11 was profiled as a trace metabolite (<1%) in all 60-min incubates (Table 1). The Q1 scan MS of the metabolite showed an apparent protonated molecular ion at m/z 137 (Figure 1) 14 amu less than M8 and the MS/MS spectrum displayed the prominent product ions at m/z 121(MH^+-CH_4, 100), 108(2), 106 (MH^+-MeO, 1), and 103(121-H_2O, 2), along with a protonated molecular ion at m/z 137(17) (Figure 1). Metabolite 11 was tentatively assigned to be O-desmethyl-M8.

Metabolite 12 was profiled as a trace metabolic product (<1%) in the rat incubate (Table 1), which gave an apparent protonated molecular ion at m/z 194 and diagnostic product ions at m/z 179(MH^+-Me, 4), 166(MH^+-CO, 10), 164(MH^+-OCH_2, 10), 163(MH^+-OMe, 8), 135(100), 105(4), and 91(3) from the MS/MS analysis (Figure 1). These MS data tentatively assigned metabolite 12 to be an N-acetylated M10 metabolite.

In general, 60-min hepatic S9 incubations of both species produced higher percent sample of metabolites than those of 30-min incubations (Table 1). Of all metabolites, only metabolite 1 (RWJ-68311) was synthesized and was shown to be less biologically active than the parent drug.

Conclusions

Formation of RWJ-68025 and its twelve metabolites in the rat and human hepatic S9 can be explained by four metabolic pathways: pathway 1, O-demethylation; pathway 2, phenyl oxidation; pathway 3, methyl oxidation; and pathway 4, N-dealkylation/acetylation. Pathway 1 appeared to be the most important pathway, forming a major metabolite, O-desmethyl-RWJ-68025 (M1; 26–16% in rat and human fractions). Pathway 2 produced one major metabolite, hydroxyphenyl-RWJ-68025 (M2; 12–17% in both species) and two minor phenolic metabolites (M4 and M5; <1% in all species), and in conjunction with step 1, formed hydroxy-M1 (M3; 4–5% in both species). Pathways 3 and 4 produced seven minor or trace methyl-oxidized and N-dealkylated/acetylated metabolites (M6–M12) (Table 1). The proposed in-vitro metabolic pathways for RWJ-68025 in rat and human hepatic S9 fractions are depicted in Figure 4. In conclusion, RWJ-68025 was rapidly and extensively metabolized in both rat and human hepatic S9 fractions.

References

Brown, E. M. (1991) Extracelluar calcium sensing, regulation of parathyroid cell function, and role of calcium and other ions as extracellular (first) messengers. *Phsiol. Rev.* **1**: 371–411

Brown, E. M., Hebert, S. C. (1997) Calcium-receptor-regulated parathyroid and renal function. *Bone* **20**: 303–309

Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M. A., Lytton, J., Hebert, S. C. (1993) Cloning and characterization of an extracellular Ca^{2+}-sensing receptor from bovine parathyroid. *Nature* **366**: 575–580

Fox, J. (1994) Prevention of hypocalcemia prolongs the plasma parathyroid hormone and calcitonin responses to the calcimimetic compound NPS R-568 in rats. *J. Bone Miner. Res.* **9**: S409

Fox, J., Lowe, S. H., Petty, B. A., Nemeth, E. F. (1999a) NPS R-568: a type II calcimimetic compound that acts on parathyroid cell calcium receptor of rats to reduce plasma levels of parathyroid hormone and calcium. *J. Pharmacol. Exp. Ther.* **290**: 473–479

Fox, J., Lowe, S. H., Conklin, R. L., Petty, B. A., Nemeth, E. F. (1999b) Calcimimetic compound NPS R-568 stimulates calcitonin secretion but selectively targets parathyroid gland Ca^{2+} receptor in rats. *J. Pharmacol. Exp. Ther.* **290**: 480–486

Fray, J. C. S., Park, C. S., Valentine, N. D. (1987) Calcium and control of rennin secretion. *Endocrine Rev.* **8**: 53–93

Heath, H. (1995) Inhibition of human parathyroid hormone secretion in vivo by NPS R-568, a calcimimetic drug that targets the parathyroid cell-surface calcium receptor. *International Conference on Calcium Regulating Hormones* (Meeting abstract)

Nemeth, E. F., Carafolei, E. (1990) The role of extracellular calcium in the regulation of intracellular calcium and cell function. *Cell Calcium* **11**: 319–321

Nemeth, E. F., Steffey, M. E., Hammerland, L. G., Hung, B. C. P., Van Wagenen, B. C., DelMar, E. G., Balandrin, M. F. (1998) Calcimimetics with potent and selective activity on the parathyroid calcium receptor. *Proc. Natl. Acad. Sci. USA* **95**: 4040–4045

Pollak, M. R., Brown, E. M., Chou, Y. H., Hebert, S. C., Marx, S. J., Steinmann, B., Levi, T., Seidman, C. E., Seidman, J. G. (1993) Mutations in the human Ca^{2+}-sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Cell* **75**: 1297–1303

Raisz, L. (1992) In: Coe, F. L., Favus, M. J. (eds) *Disorders of bone and mineral metabolism*. Raven Press, pp 287–311

Riccardi, D., Lee, W. S., Lee, K., Segre, G., Brown, E. M., Hebert, S. C. (1996) Location of the extracellular Ca^{2+}-sensing receptor and PTH/PTHrP receptor in rat kidney. *Am. J. Physiol.* **271**: F951–F956

Wu, W. N., McKown, L. A., Rybcyznski, P. J. (2000) In vitro metabolism of the endocrine agent, RWJ-68025, in rat and human hepatic S9 fractions. *Drug Metab. Rev.* **32**: 251 (Meeting abstract, The 10th North American ISSX Meeting)

Wu, W. N., McKown, L. A., Zeck, R., Rybczynski, P. J. (2001) In vitro metabolism of the endocrine agents, RWJ-69113 and RWJ-69115 (diastereomeric pair), in rat, dog and human hepatic S9 fractions. *Drug Metab. Rev.* **33**: 53 (Meeting abstract, The 6th Int. ISSX Meeting)