Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
31 (2003) 95–102
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

# In vitro metabolism of the new anxiolytic agent, RWJ-52763 in human hepatic S9 fraction-API-MS/MS identification of metabolites

Wu-Nan Wu *, Linda A. McKown, Allen B. Reitz

*Johnson & Johnson Pharmaceutical Research & Development, L.L.C., Welsh McKean Rds, PO Pox 776, Spring House, PA 19477, USA*

Received 11 June 2002; received in revised form 9 September 2002; accepted 16 September 2002

**Abstract**

The in vitro metabolism of the anxiolytic agent, RWJ-52763 was studied after incubation with human hepatic S9 fraction in the presence of an NADPH-generating system. Unchanged RWJ-52763 (64% of the sample) plus six metabolites (M1–M6) were profiled, quantified, and tentatively identified on the basis of API-MS/MS data. The metabolic pathways for RWJ-52763 are proposed, and the two metabolic pathways are: (1) *N*/*O*-dealkylation, and (2) phenylhydroxylation. Pathway 1 formed a major *N*-dealkylated metabolite, *N*-desethoxy-RWJ-52763 (M1, 22% of the sample) and 2 minor *N*/*O*-dealkylated metabolites, *O*-desmethyl-RWJ-52763 (M2; 2%) and *N*,*N*-didesethoxymethyl-RWJ-52763 (M3; 3%). Pathway 2 produced two hydroxyphenyl metabolites, hydroxydifluorophenyl-RWJ-52763 (M4; 4%) and hydroxyphenyl-pyrido-RWJ-52763 (M5; 3%) in small amounts, and in conjunction with step 1 formed a minor *N*-desethoxymethyl-M4 (M6; 1%). RWJ-52763 is substantially metabolized by this human hepatic S9.


*Keywords:* RWJ-52763; Anxiolytic agent; Human hepatic S9; In vitro metabolism; API-MS/MS

## 1. Introduction

RWJ-52763, 6-*N*,*N*-dimethoxyethyl-1,2-dihydro-3-oxo-*N*-(2,6-difluorophenyl)pyrido[1,2-*a*]benzimidazole-4-carboxamide (Fig. 1), is a new anxiolytic agent. It was synthesized by Johnson & Johnson Pharmaceutical Research & Development, LLC, Spring House, PA, USA [1–4]. RWJ-52763 and its analogs, RWJ-50172, RWJ-51204, RWJ-51297, RWJ-51521, RWJ-52844, and RWJ-53050 bind with high affinity to the benzodiazepine site on GABA-A receptors [3,4]. The in vitro and in vivo metabolism of RWJ-50172 [5,6], RWJ-51204 [7,8], RWJ-51297 [9], RWJ-51521 [10], RWJ-52844 [11], and RWJ-53050 [12,13] in the rat, dog and human have been investigated and reported previously. The objectives of the current study were to investigate the in vitro metabolism of RWJ-52763 in human hepatic S9 fraction using LC/API-MS and MS/MS techniques. This resulted in the profiling, quantification, characterization, and identification of unchanged RWJ-52763 and

* Corresponding author. Tel.: +1-215-628-5562; fax: +1-215-628-7822.
*E-mail address:* wwu@prius.jnj.com (W.-N. Wu).

0731-7085/02/$ - see front matter 
PII: S0731-7085(02)00597-6

six metabolites. Preliminary results of this study have been reported previously for the identification of six metabolites [14].

## 2. Experimental section

### 2.1. Materials

RWJ-52763 was obtained from The CNS Research Team, Johnson & Johnson Pharmaceutical Research & Development, L.L.C. (Spring House, PA, USA) with purity >97% (API-MS/MS/MS, HPLC). HPLC-grade solvents were obtained from the Fisher Scientific Co. (Fairlawn, NJ, USA) and glass-distilled solvents were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI, USA). The incubation components for S9, Tris, potassium chloride, magnesium chloride, $NADP^+$ and glucose-6-phosphate, were purchased from Sigma (St. Louis, MO, USA).

### 2.2. Hepatic S9 fraction

The human hepatic S9 fraction was purchased from XenoTech, L.L.C. (Kansas City, KS, USA). It was obtained from a mixed gender pool of 15, Lot # 082897 A, 20 mg protein per ml.

### 2.3. 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-52763 spike, to obtain a final volume of 5 ml and a RWJ-52763 concentration of 100 μg/ml. After the addition of the last component, each flask was incubated in a 37 °C Dubnoff Metabolic Shaker Incubator (Precision Scientific, Chicago, IL, USA). Samples were removed at 0 and 60 min. Control flasks were incubated without human subcellular fraction or human subcellular fraction only, to determine drug stability under incubation conditions.

### 2.4. Sample storage

Immediately following removal from the incubator, aliquots 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. Sample were stored at approximately −20 °C.

### 2.5. Metabolite profiling, quantifying, and identification

Following ethyl acetate (2 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 analyzed via 20 μl flow-injection using the PE Sciex API III-Plus MS (Perkin–Elmer Sciex Instuments, Thornhill, Ontario, Canada), 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. For each sample, the relative percentage of unchanged RWJ-52763 and metabolites were estimated using the integrated chromatograms generated by the Sciex API-III Q1 scan MS (TIC, total ion chromatogram). Unchanged RWJ-52763 and its metabolites were elucidated on the basis of MS, MS/MS and MRM data.

## 3. Results and discussion

The in vitro biotransformation of RWJ-52763 was conducted in human hepatic S9 fraction. Unchanged RWJ-52763 (64% of the sample) plus six metabolites (M1–M6), were profiled, quantified, characterized, and tentatively identified in the 60 min incubate on the basis of API ionspray-MS and MS/MS data. The structures of RWJ-52763 and its metabolites, and their MS data are presented in Fig. 1, and the percent of unchanged

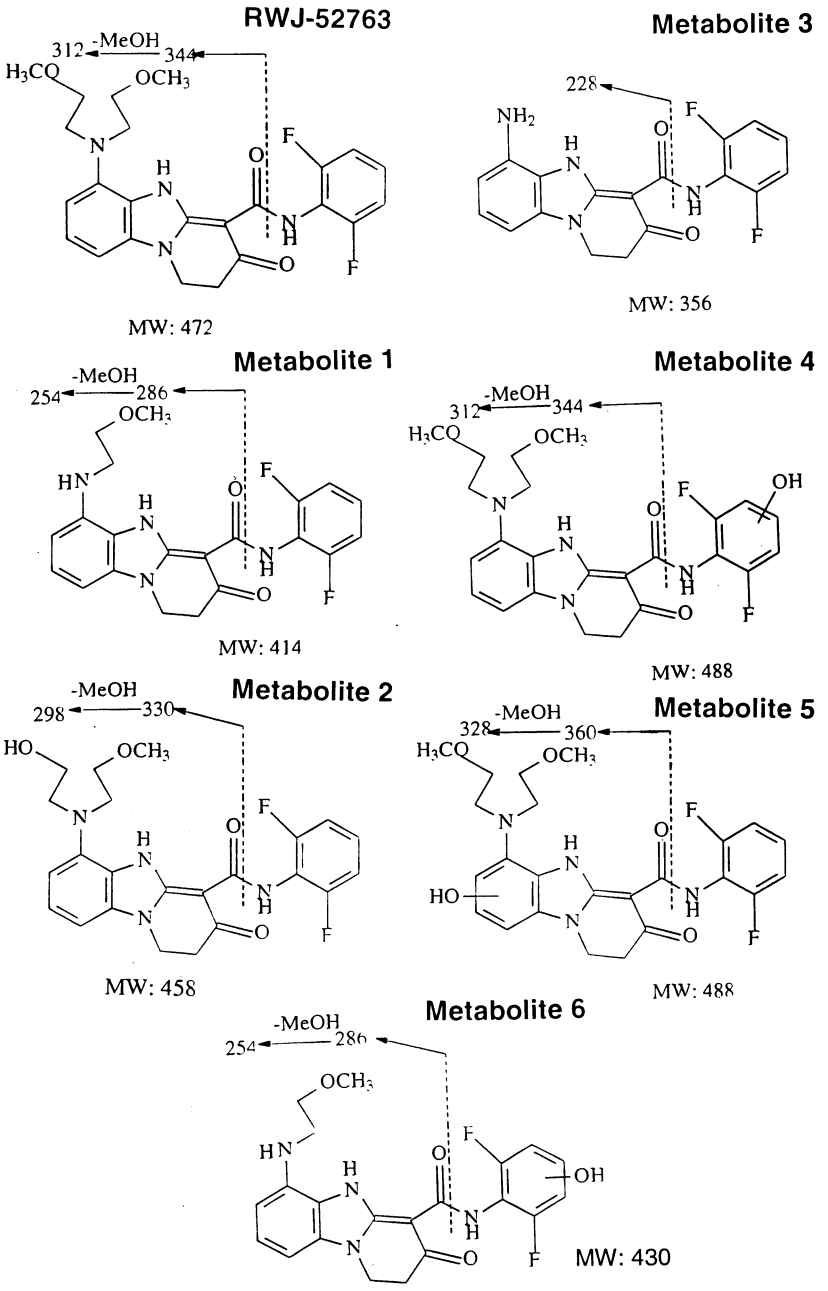


Fig. 1. Structures and MS/MS product ions for RWJ-52763 and metabolites.

RWJ-52763 and each metabolite are shown in Table 1. Control incubates revealed unchanged RWJ-52763 only. The representative metabolic profile using Q1 scan MS (TIC) for the 60 min incubate of human hepatic S9 is presented in Fig. 2. Representative MS/MS spectra for unchanged RWJ-52763 and metabolites 1 are presented in Fig. 3. The isolation, profiling, quantification,

Table 1
Metabolism of RWJ-52763 in human hepatic S9

| Analyte | Human S9 60 (min) |
|---|---|
| RWJ-52763 | 64 |
| M1 | 22 |
| M2 | 2 |
| M3 | 3 |
| M4 | 4 |
| M5 | 3 |
| M6 | 1 |

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

characterization, and tentative identification of unchanged RWJ-52763 and each metabolite are discussed below.

Unchanged RWJ-52763 was identified from all incubates (0 and 60 min) by solvent extraction and MS and MS/MS techniques in comparison with authentic RWJ-52763 (Figs. 1–3). Mass spectral analysis of RWJ-52763 revealed intense protonated molecular and ammonia-adduct ions at *m/z* 473 ($[M+H]^+$) and 490 ($[M+NH_4]^+$), respectively (Figs. 1 and 2). MS/MS analysis of *m/z* 473 revealed prominent product ions at *m/z* 362 (2%), 344 (100%), 330 (1%), 312 (38%), 280 (5%), and 254 (3%) (Figs. 1–3). Unchanged RWJ-52763 was present in major quantities (64% of the sample) in the 60 min S9 incubate (Table 1).

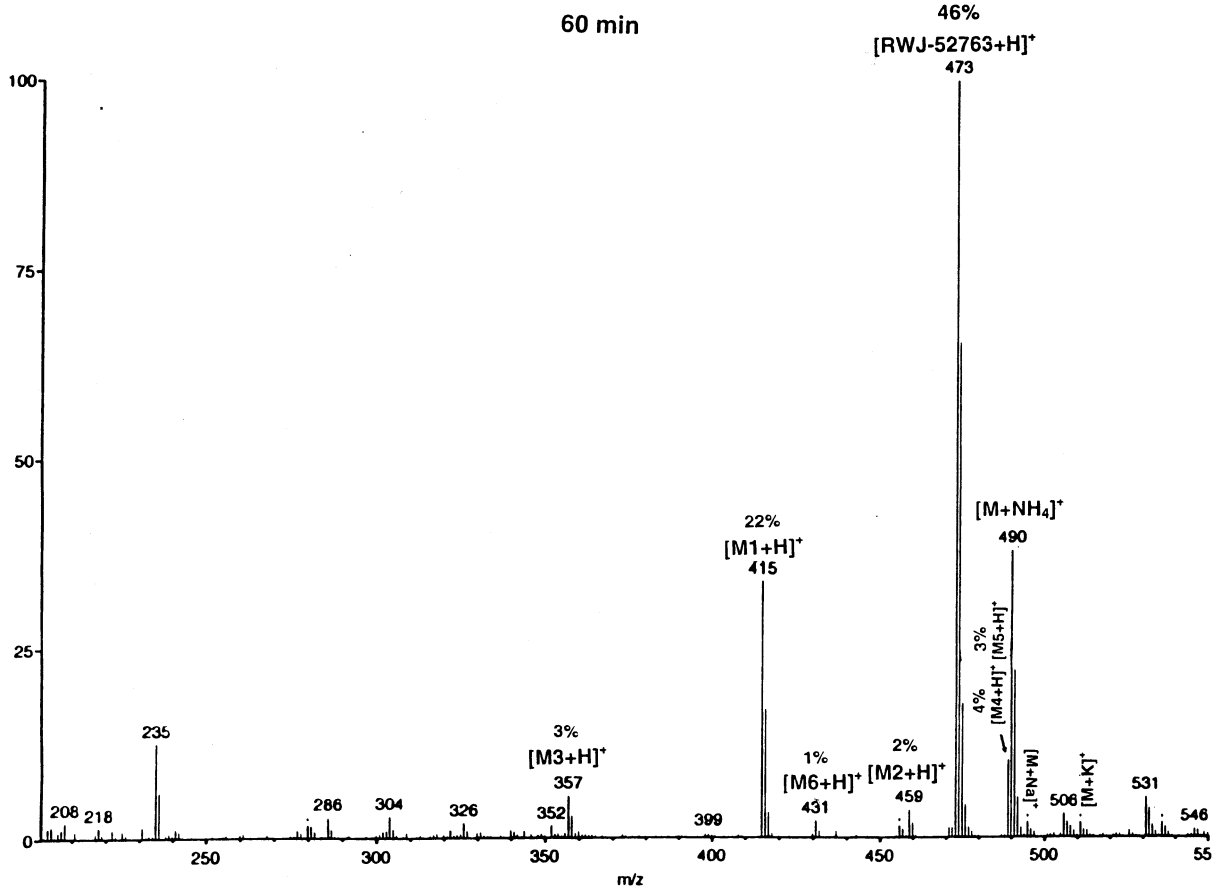


Fig. 2. Q1 scan MS profiles of human hepatic S9 incubate of RWJ-52763.

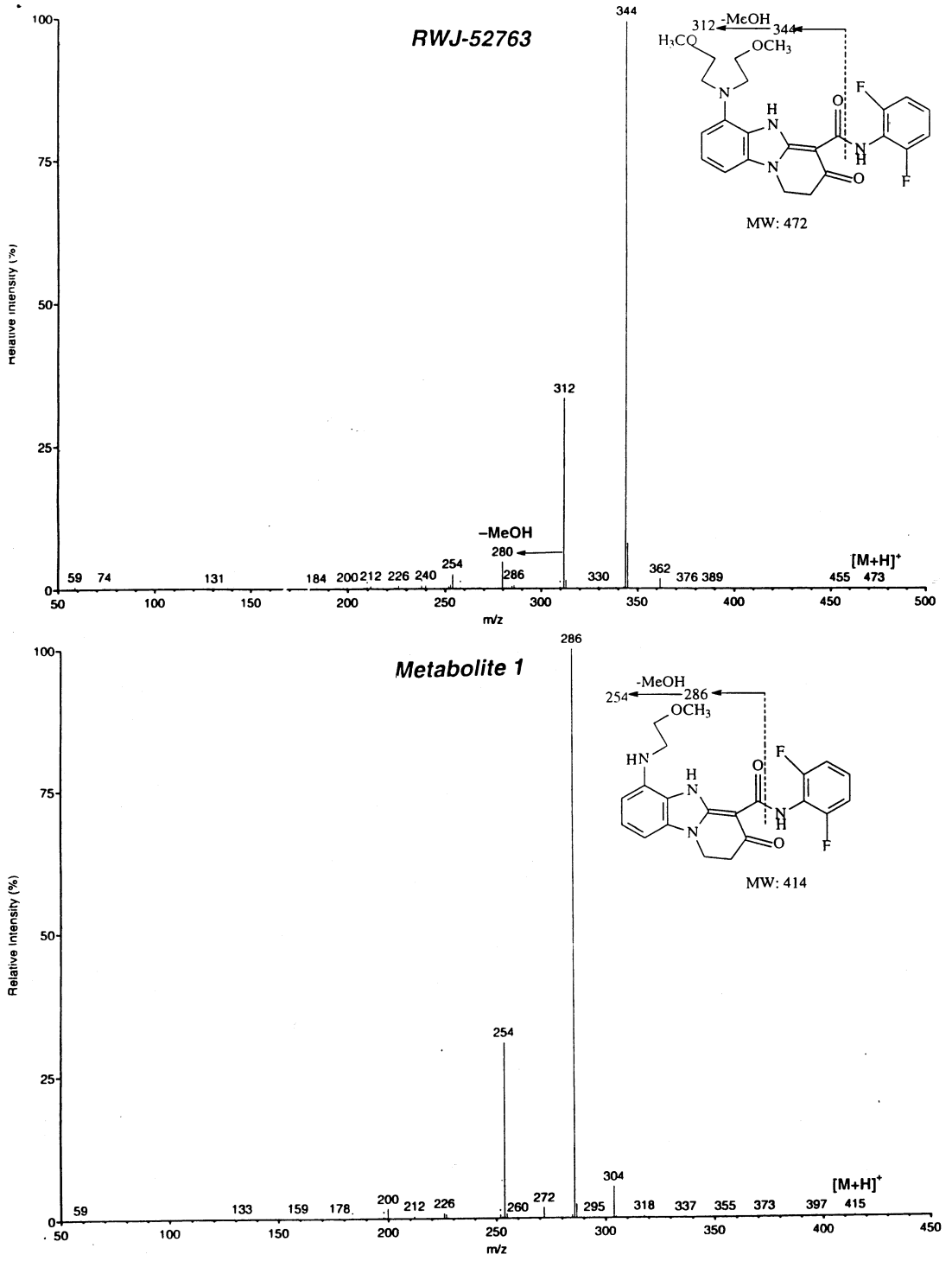


Fig. 3. Representative API-MS/MS spectra of RWJ-52763 and metabolite 1.

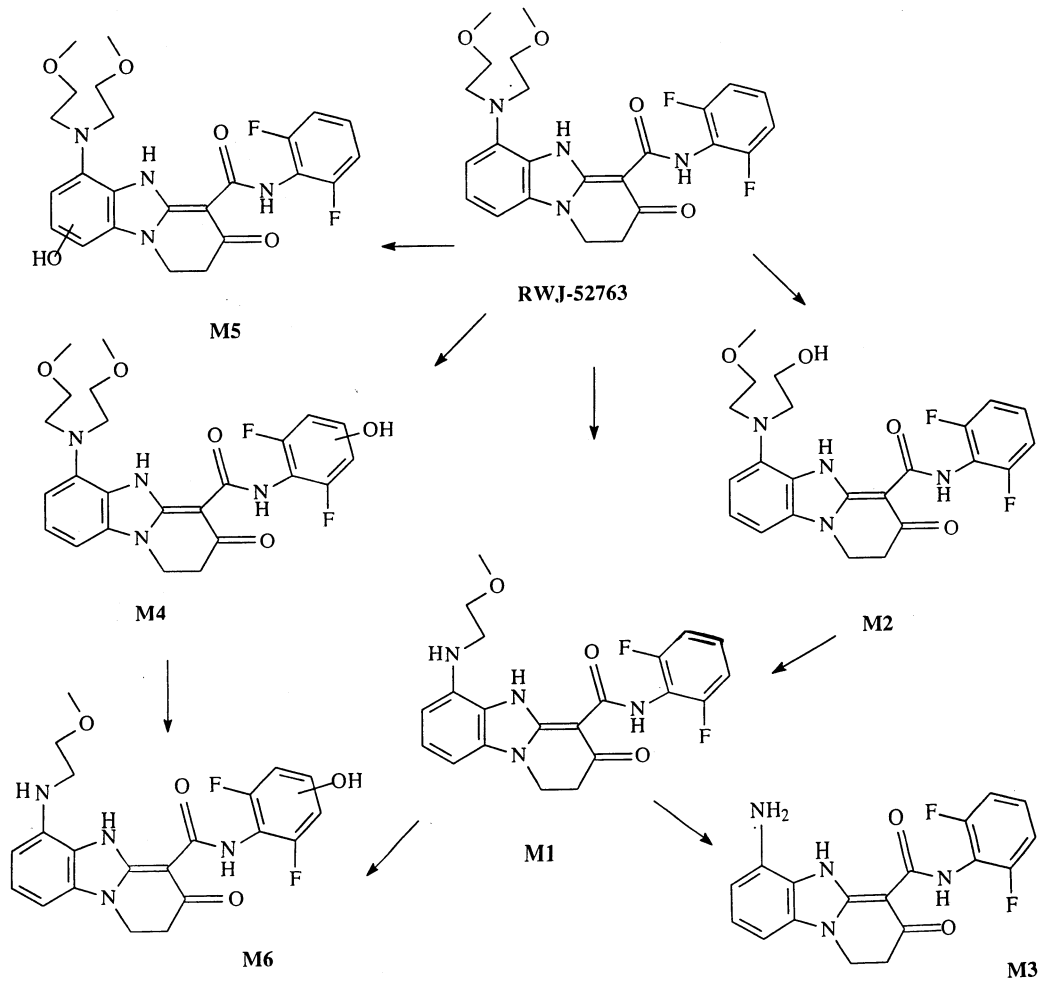


Fig. 4. Proposed in vitro metabolic pathways for RWJ-52763 in human hepatic S9 fraction.

Metabolite 1 was present as a major metabolite (22% of the sample) (Table 1). The MS and MS/MS ($[M+H]^+$) spectral data showed a protonated molecular ion at *m/z* 415 (1%) and diagnostic product ions at *m/z* 304 (6%), 286 (100%), 272 (2%), and 254 (30%) (Figs. 1–3). The MS data tentatively assigned M1 as *N*-desmethoxyethyl-RWJ-52763.

Metabolite 2 was present in minor amounts in the 60 min Incubate (2%) (Table 1). The structure of M2 was tentatively identified as *O*-desmethyl-RWJ-52763 on the basis of MS and MS/MS data. The MS data showed an apparent protonated molecular ion at *m/z* 459 (Figs. 1 and 2). MS/MS analysis of the protonated molecular ion revealed important product ions at 348 (3%), 330 (100%), 312 (330-$H_2O$, 4%), 298 (8%), and 254 (3%), together with a protonated molecular ion at *m/z* 459 (1%) (Fig. 1).

Metabolite 3 was identified in minor amounts (3%) (Table 1). The MS data for this metabolite gave an apparent protonated molecular ion at *m/z* 357 (Fig. 2), and MS/MS analysis of the protonated molecular ion (*m/z* 357) exhibited a protonated molecular ion and informative product ions at *m/z* 246 (14%), 228 (100%), and 196 (2%) with a

protonated molecular ion, $m/z$ 357 (2%) (Fig. 1). MS data of M3 tentatively characterized the metabolite as *N*,*N*-didesmethoxyethyl-RWJ-52763.

Metabolite 4 was detected as a minor metabolite in the 60 min incubate (4%) (Table 1). The ionspray-MS and MS/MS analysis of this metabolite contained a protonated molecular ion at $m/z$ 489 in MS spectrum, and important product ions at $m/z$ 473 ($MH^+ - CH_4$, 2%), 344 (100%), 312 (344-MeOH, 8%), and 286 (2%), along with a protonated molecular ion at $m/z$ 489 (1%) in MS/MS spectrum (Fig. 1). On the basis of the MS data, metabolite 4 was tentatively proposed to be hydroxydifluorophenyl-RWJ-52763.

Metabolite 5 was present in minor quantities (3%) in 60 min S9 incubate (Table 1). This metabolite showed a protonated molecular at $m/z$ 489 in ionspray-MS (Fig. 2), and significant product ions at $m/z$ 473 ($MH^+ - CH_4$, 3%), 360 (100%), 328 (360-MeOH, 5%), and 286 (328-$CH_2$=CO, 2%), together with a protonated molecular ion at $m/z$ 489 (2%) (Fig. 1). The structure of metabolite 5 was tentatively proposed to be OH-benzimidazole-RWJ-52763.

Metabolite 6 was detected in trace amounts in the 60 min incubate (1%). An apparent protonated molecular ion at $m/z$ 431 showed in MS spectrum indicated a molecular weight of 16 amu more than M1. This could be explained by the formation of phenolic group at the phenyl moiety of M1. MS/MS analysis of protonated molecular ion ($m/z$ 431) provided informative product ions at $m/z$ 302 (49%), 286 (100%), 270 (286-$CH_4$, 2%), 254 (286-MeOH, 20%), and 228 (286-CH=$CH_2$OMe, 5%), along with a protonated molecular ion (2%) (Fig. 1).

## 4. Conclusion

The in vitro metabolism of RWJ-52763 was conducted in the human hepatic S9 fraction. Unchanged RWJ-52763 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, fragment product ions for the structural elucidation of RWJ-52763 and its metabolites. Formation of these metabolites in the human hepatic S9 can be explained by two metabolic pathways: (1) *N*/*O*-dealkylation, and (2) phenyl-hydroxylation. Pathway 1 appeared to be the most important pathway, forming one major and two minor metabolites, *N*-desmethoxyethyl-RWJ-52763 (M1, 22% of the sample), *O*-desmethyl-RWJ-52763 (M2; 2%), and *N*,*N*-didesmethoxyethyl-RWJ-52763 (M3, 3%). Pathway 2 produced two minor phenylhydroxylated metabolites, hydroxydifluorophenyl-RWJ-52763 (M4, 4%) and hydroxybenzimidazole-RWJ-52763 (M5; 3%), and in conjunction with pathway 1 formed a trace metabolite, hydrox-M1 (M6, 1%). The proposed in vitro metabolic pathways for RWJ-52763 in human hepatic S9 fraction are depicted in Fig. 4. In conclusion, RWJ-52763 is substantially metabolized in human hepatic S9 fraction.

## References

[1] B.E. Maryanoff, W. Ho, D.F. McComsey, A.B. Reitz, P.P. Grous, S.O. Nortey, R.P. Shank, B. Dubinsky, R.J. Taylor, Jr, J.F. Gardocki, J. Med. Chem. 38 (1995) 16–20.
[2] B.E. Maryanoff, D.F. McComsey, W. Ho, R.P. Shank, B. Dubinsky, Bioorg. Med. Chem. Lett. 6 (1996) 333–338.
[3] B.E. Maryanoff, S.O. Nortey, J.J. McNally, P.J. Sanfilippo, D.F. McComsey, B. Dubinsky, R.P. Shank, A.B. Reitz, Bioorg. Med. Chem. Lett. 9 (1999) 1547–1552.
[4] M.K. Scott, D.A. Demeter, S.O. Nortey, B. Dubinsky, R.P. Shank, A.B. Reitz, Progress in Med. Chem., Elsevier Science, Amsterdam, Vol. 36 1999, pp. 169–200.
[5] W.N. Wu, L.A. McKown, J.L. Melton, D.M. Isaacson, A.R. Takacs, A.B. Reitz, ISSX Proceedings 1(1996) Abstract # 344, The Seventh North American ISSX Meeting.
[6] . W.N. Wu, L.A. McKown, J.L. Melton, A.B. Reitz, J. Pharm. and Biomed. Analy. (2002, in submission).
[7] W.N. Wu, L.A. McKown, A.B Reitz, A.R. Takacs, Pharm. Sci$^{TM}$. 1 (1998) PS36.
[8] W.N. Wu, L.A. McKown, ISSX Proceedings 15(1999) Abstract #174, The Ninth North American ISSX Meeting.
[9] L.A. McKown, W.N. Wu, A.B. Reitz, Drug Metab. Rev. 32 (2000) 252 (The Tenth North American ISSX Meeting Abstract # 231).
[10] W.N. Wu, L.A. McKown, A.B. Reitz, Drug Metab. Rev. 32 (2000) 252 (The Tenth North American ISSX Meeting Abstract # 232).

[11] W.N Wu, L.A. McKown, M.D. Moyer, A.B. Reitz, ISSX Proceedings 15 (1999) Abstract # 425, The Ninth North American ISSX Meeting.
[12] W.N. Wu, L.A. McKown, A.J. Streeter, A.R. Takacs, A.B. Reitz, The 12th International Symposium on Microsomes and Drug Oxidations (1998) Abstract # 107.
[13] W.N. Wu, L.A. McKown, A.R. Takacs, The 12th International Symposium on Microsomes and Drug Oxidations (1998) Abstract# 108.
[14] W.N. Wu, L.A. McKown, A.B. Reitz, Drug Metab. Rev. 33 (2001) 122 (The Sixth International ISSX Meeting Abstract#241).