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In vitro biotransformation of the new antipsychotic agent, RWJ-46344 in rat hepatic S9 fraction: API-MS/MS/MS identification of metabolites

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Abstract

The in vitro biotransformation of the antipsychotic agent, RWJ-46344 was studied after incubation with rat hepatic S9 fraction in the presence of an NADPH-generating system. Unchanged RWJ-46344 (~ 37% of the sample) plus 12 metabolites were profiled, quantified, and tentatively identified on the basis of API (ionspray)-MS/MS/MS data. The proposed metabolic pathways for RWJ-46344 are proposed, and the six metabolic pathways are 1, *O*-dealkylation; 2, piperidinyl oxidation; 3, *N*-debenzylation; 4, phenyl hydroxylation; 5, dehydration; and 6, reduction. Pathways 1 to 3 formed *O*-desisopropyl RWJ-46344 (M3, ~ 13% of the sample) and its hydroxy-metabolite (M5, ~ 8%), hydroxy-piperidinyl RWJ-46344 (M1, ~ 5%) and a phenylpiperidinyl metabolite (M8, ~ 24%) as major and moderate metabolites. Eight minor metabolites (each < 2%) were formed via a combination of six steps. RWJ-46344 is metabolized substantially by this rat hepatic system. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: RWJ-46344; Antipsychotic agent; Rat hepatic S9; In vitro metabolism; API-MS/MS/MS

1. Introduction

RWJ-46344-002,4-[2[(1-methylethoxy)phenyl]-1-piperidinyl]methylbenzoyl-2,6-dimethylpiperidine hydrochloride (Fig. 1) is a new antipsychotic agent, first synthesized in 1995 by The R.W. Pharmaceutical Research Institute, Spring House, PA, USA [1–3]. RWJ-46344 and its analog, mazapertine, and its metabolites have undergone extensive investigation for antipsychotic activity [1–3]. The in vitro and in vivo metabolism of mazapertine in the rat, dog and human have been investigated and reported previously [4–7]. The objectives of the current study were to investigate the in vitro metabolism of RWJ-46344 in rat hepatic S9 fraction using API-MS and MS/MS techniques. This resulted in the profiling, quantification, characterization, and identification of unchanged RWJ-46344 and 12 metabolites. Preliminary results of this study have been reported previously [8].

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2. Experimental

2.1. Materials

RWJ-46344-002 was obtained from the CNS Research Team, The R.W. Johnson Pharmaceutical Research Institute (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 rat hepatic S9 fraction was generated from a male, Crl:CD®(SD)IGSBR VAF/Plus®, rat at The R.W. Johnson Pharmaceutical Research Institute (Spring House, PA, USA).

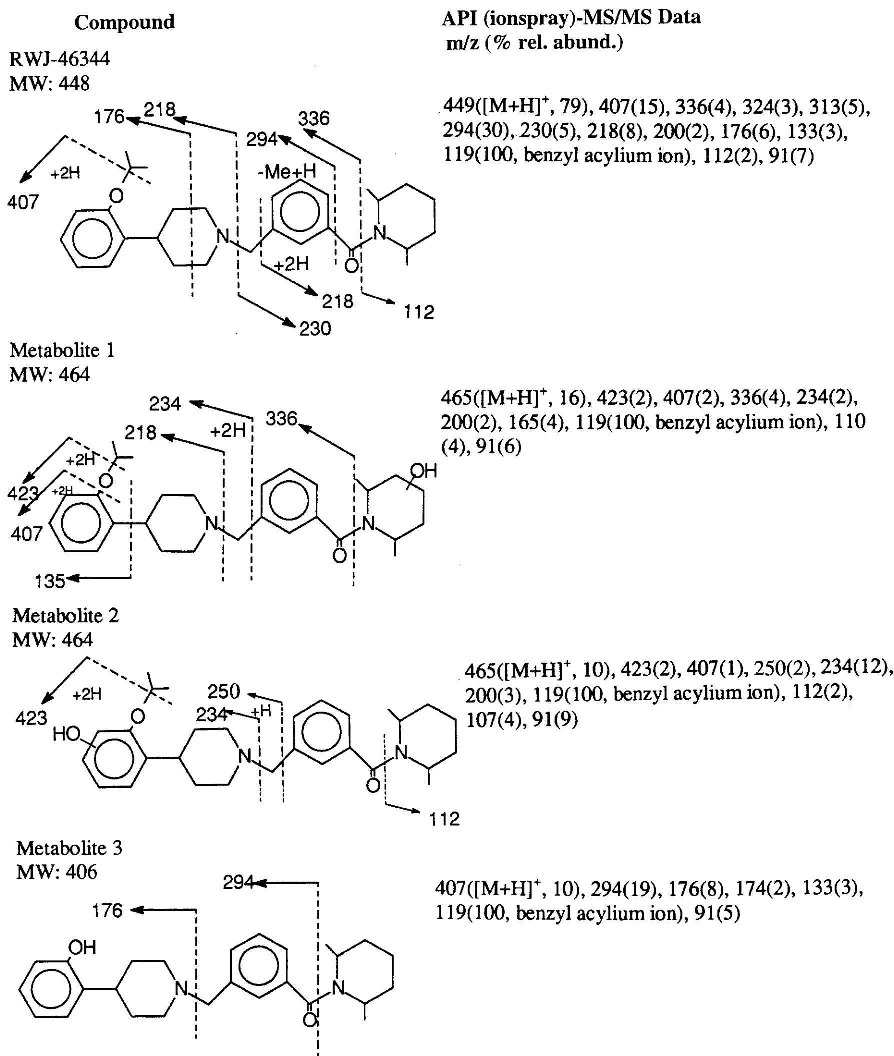


Fig. 1. Structures and MS data for RWJ-46344 and its metabolites.

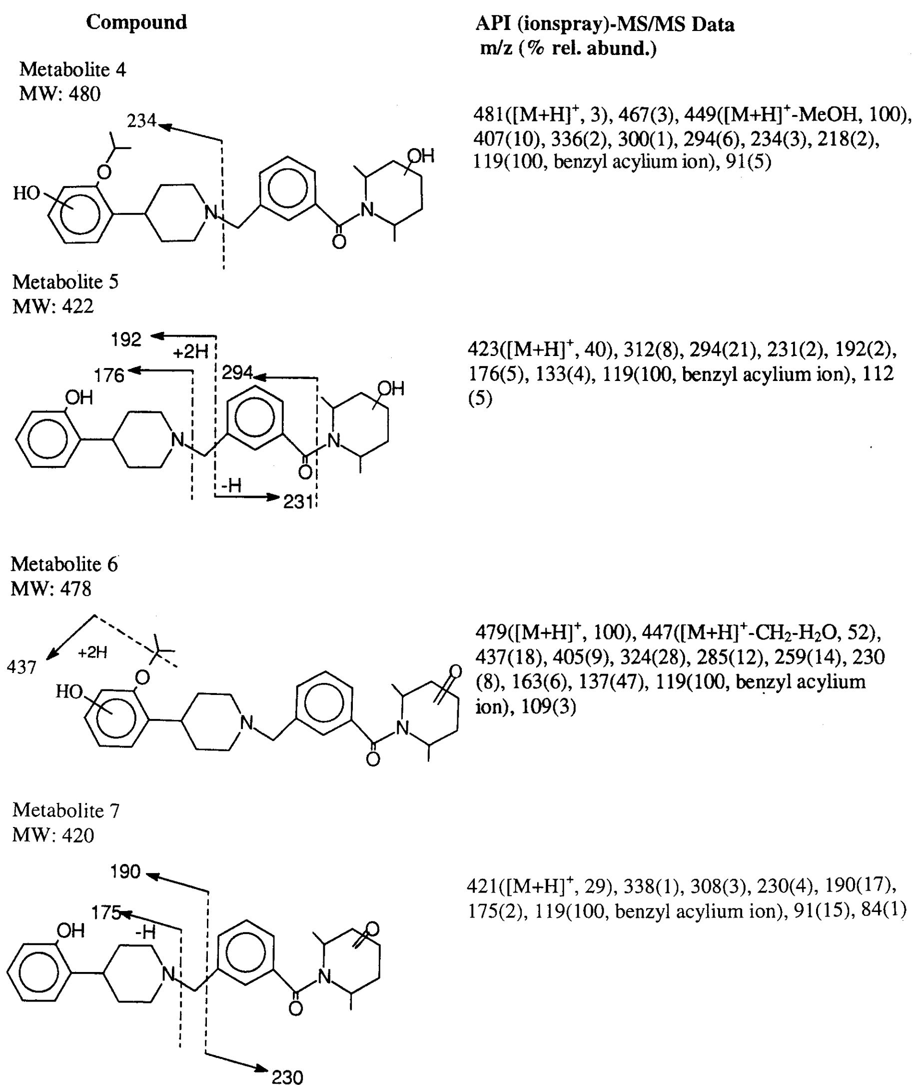


Fig. 1. (*Continued*)

2.3. 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-46344-002 spike, to obtain a final volume of 5 ml and a RWJ-46344 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, 30, and 60 min. Control flasks were incubated without rat subcellular fraction, to determine drug stability under incubation conditions.

2.4. Sample storage

Immediately after removal from the incubator, aliquots were transferred to prelabeled 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 $-20°C$.

2.5. 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 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 Instruments, Thornhill, Ont., Canada), a triple quadruple 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 reconsti-

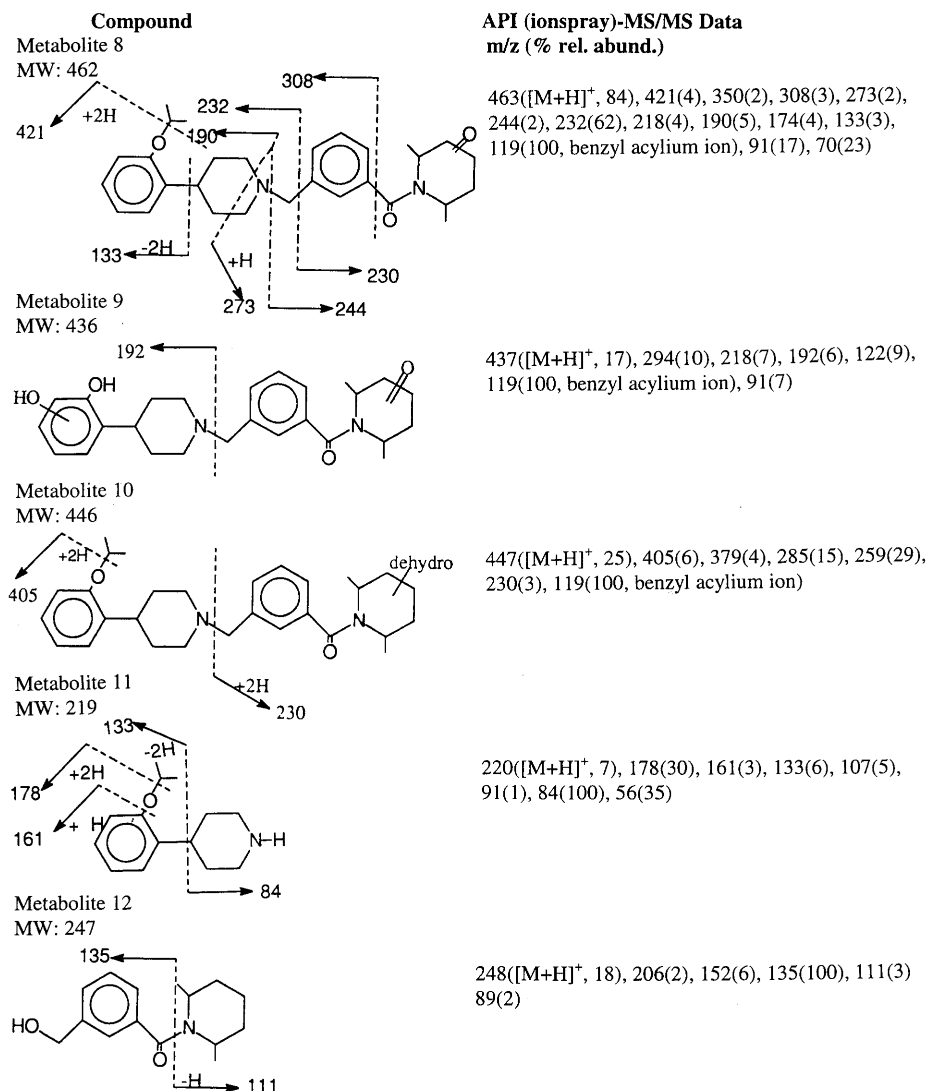


Fig. 1. (*Continued*)

Table 1
Hepatic metabolism of RWJ-46344 in the rat[a]

Analyte	Rat hepatic S9 (60 min)
RWJ-46344	37
M1	5
M2	<1
M3	13
M4	<2
M5	8
M6	<1
M7	<1
M8	<1
M9	<1
M10	1
M11	24
M12	3

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

tution, at a flow rate of 0.1 ml/min. For each sample, the relative percentage of unchanged RWJ-46344 and metabolites were estimated using the intergrated chromatograms generated by the Sciex API-III Q1 scan MS (total ion chromatogram, TIC). Unchanged RWJ-46344, its metabolites, and methyl derivatives were elucidated on the basis of MS and MS/MS data.

2.6. Methyl derivatization

Each extract residue was dissolved in 1 ml of methanol, added with excess amounts of ethereal diazomethane, left at room temperature overnight, and evaporated to yield a residue. Each residue was analyzed for further confirmation of metabolites using the same method as described above.

3. Results and discussion

The in vitro biotransformation of RWJ-46344-002 was conducted in rat hepatic S9 fractions. Unchanged RWJ-46344 (60-min incubates, ~ 37% 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 based on API ionspray-MS and MS/MS data. The structures of RWJ-46344, its metabolites, methyl derivatives, and their MS data are presented in Fig. 1, and the percentage of unchanged RWJ-46344 and each metabolite are shown in Table 1. Control incubates revealed unchanged RWJ-46344 only. The representative Q1 scan MS (TIC) metabolic profile for the 60-min incubate of rat S9 is presented in Fig. 2. Representative mass spectra for unchanged RWJ-46344, metabolites 1, 3 and 7 are presented in Fig. 3. Metabolites 2–3 were derivatized to form methyl ethers, and confirmed by mass spectral data (Fig. 1). The isolation, profiling, quantification, characterization, and identification of unchanged RWJ-46344 and each metabolite are discussed below.

Unchanged RWJ-46344 was isolated, and identified from all incubates (0, 30 and 60 min) by solvent extraction and MS and MS/MS techniques in comparison to authentic RWJ-46344 (Figs. 1–3). Mass spectral analysis of RWJ-46344 revealed intense protonated and water-adduct molecular ions at m/z 449 ($[M+H]^+$), and 467 ($[M+H_2O]^+$), respectively (Figs. 1 and 2). MS/MS analysis of m/z 449 exhibited prominent daughter ions at m/z 407, 336, 294, 230, 218, 176, 119 (100%), and 91 (Figs. 1–3). Unchanged RWJ-46344 was present in major quantities (~ 37% of the sample) in the 60-min S9 incubate (Table 1).

Metabolite 1 was present as a moderate metabolite (5% of the sample) (Table 1). The MS and MS/MS ($[M+H]^+$) spectral data showed an intense protonated molecular ion at m/z 465, and intense as well as diagnostic daughter ions at m/z 423, 407, 336, 234, 119 (100%) and 91 (Figs. 1–3). The MS data clearly assigned M1 as OH-piperidinyl-RWJ-46344. It remained unchanged by the reaction of diazomethane.

Metabolite 2 was present in small amounts in the 60-min incubates (< 1%) (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 465 (Fig. 2). MS/MS analysis of the protonated molecular ion revealed important daughter ions at 423, 407, 251, 234, 119 (100%) and 112 (Fig. 1). The MS and MS/MS spectra of M2 methyl ether showed an intense

protonated molecular ion at *m/z* 479 and the MS/MS of *m/z* 479 provided structurally informative daughter ions at *m/z* 386, 344, 294, 252, 200, 186, 162, 119 (100%). M2 was identified as OH-phenyl-RWJ-46344.

Metabolite 3 was detected in major amounts (13%) (Table 1). The MS data for this metabolite gave an apparent protonated molecular ion at *m/z* 407 (Fig. 2), and MS/MS analysis of the protonated molecular ion (*m/z* 407) exhibited prominent as well as informative daughter ions at *m/z* 294, 176, 119 (100%) and 91, together with a protonated molecular ion (Figs. 1–3). MS data of M3 characterized the metabolite as *O*-desisopropyl-RWJ-46344. Methylation of M3 produced an *O*-methyl analogue, which displayed an intense protonated molecular ion at *m/z* 421 in the MS spectrum, and daughter ions at *m/z* 259, 231, 176,119, and 91, together with an intense protonated molecular ion in the MS/MS spectrum.

Metabolite 4 was found as a minor metabolite in the 60-min incubate (<2%) (Table 1). The ionspray-MS and MS/MS analysis of the incubate extract contained a protonated molecular ion at *m/z* 481 in MS spectrum, and important daughter ions at *m/z* 467, 449 (100%), 407, 294, and 119, along with a protonated molecular ion at *m/z* 481 in MS/MS spectrum (Figs. 1 and 2). On the basis

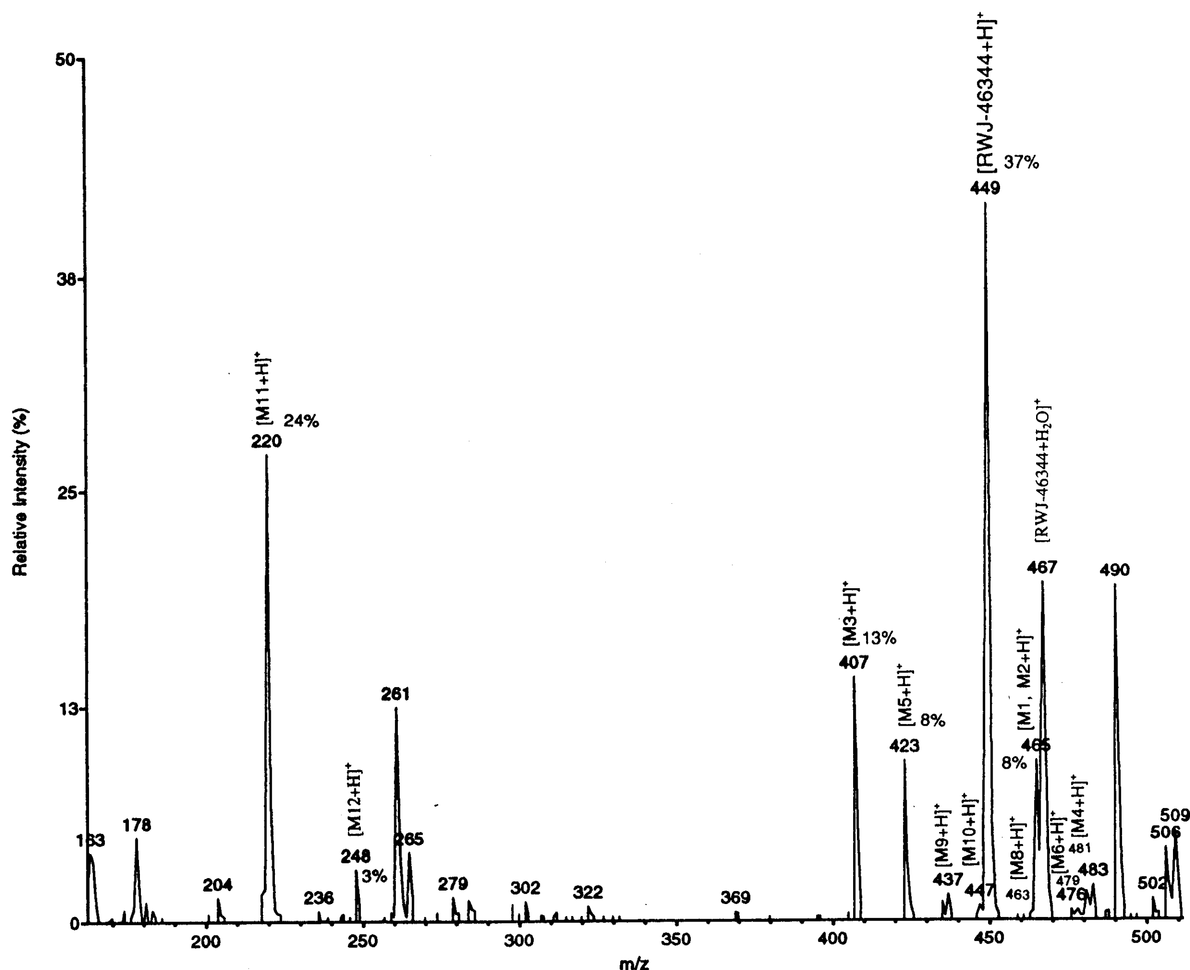
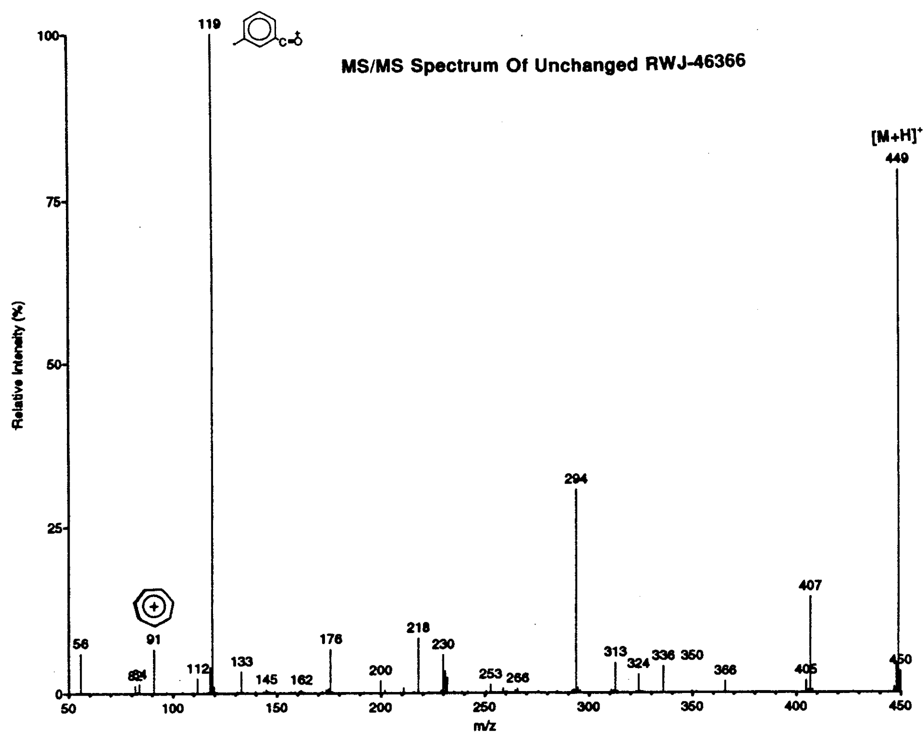


Fig. 2. API (ionspray) QI scan MS of rat hepatic S9 incubate of RWJ-46344 (60 min).



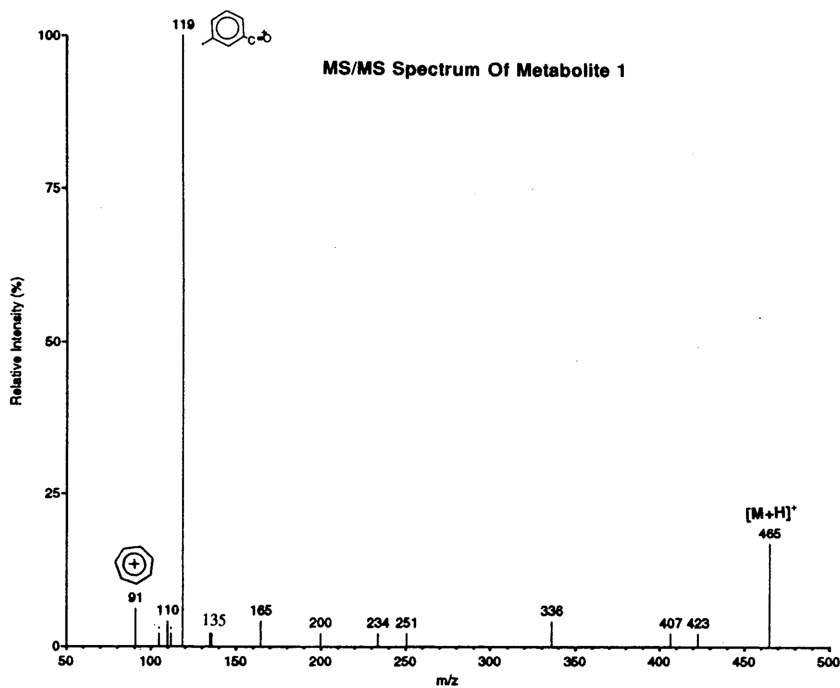
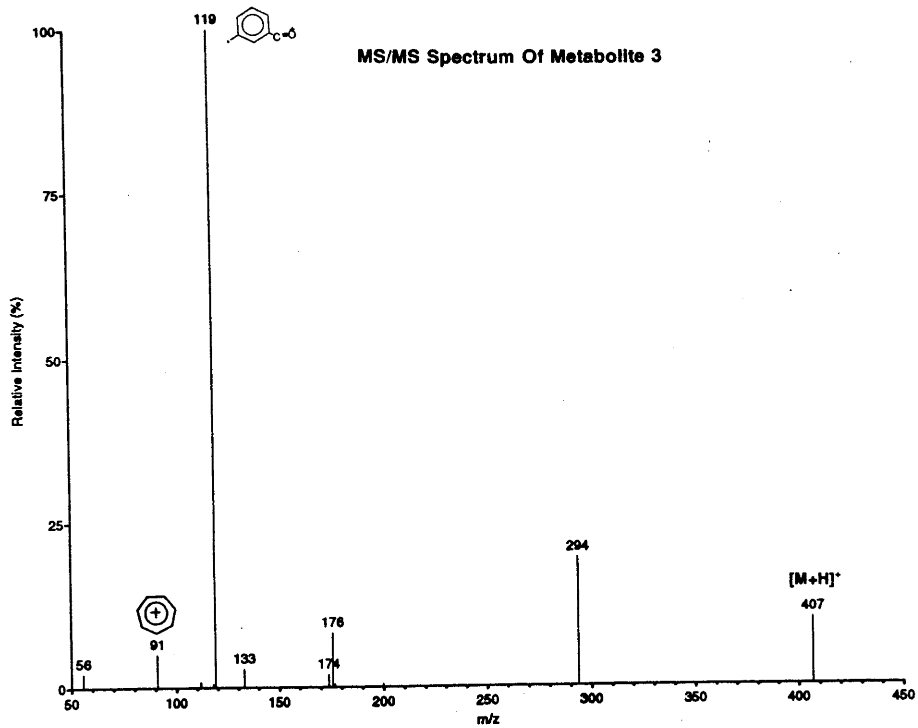


Fig. 3. Representative API-MS/MS spectra of RWJ-46344 and metabolites 1, 3 and 7.



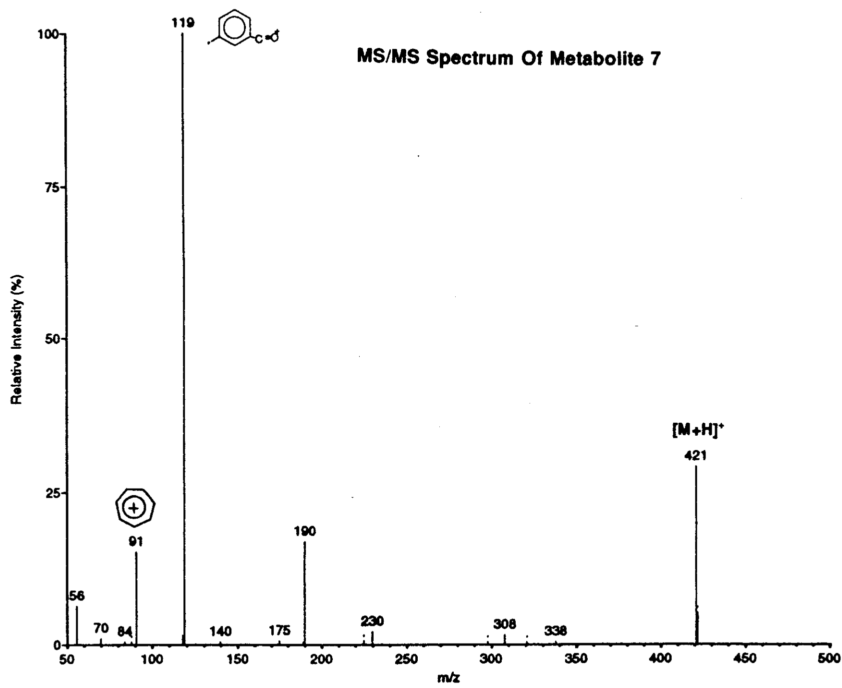


Fig. 3. (*Continued*)

of the MS data, Metabolite 4 was tentatively proposed to be OH-phenyl-OH-piperidinyl-RWJ-46344.

Metabolite 5 was present in moderate amounts (~8%) (Table 1). This metabolite showed a protonated molecular ion at *m/z* 423 in ionspray-MS (Fig. 2), and significant daughter ions at *m/z* 294, 231, 176 and 119 (100%), together with a protonated molecular ion (*m/z* 423) (Figs. 1–3). The structure of Metabolite 5 was tentatively proposed to be OH-piperidinyl-*O*-desisopropyl-RWJ-46344.

Metabolite 6 was detected in trace amounts in the 60-min incubate (< 1%). An apparent protonated molecular ion at *m/z* 479 exhibited in MS spectrum indicated a molecular weight of 2 amu less than Metabolite 4, diOH-RWJ-46344. The loss of 2 amu could be explained by the formation of oxo-piperidinyl group via further oxidation of M4. MS/MS analysis of protonated molecular ion (*m/z* 479) provided prominent as well as informative daughter ions at *m/z* 447, 437, 324, 259, 137, and 119 (100%), along with an intense protonated molecular ion (Figs. 1 and 2).

Metabolite 7 was detected in trace amounts in the 60-min incubate and showed a protonated molecular ion at *m/z* 421, which exhibited important daughter ions at *m/z* 230, 190, 175, 119 (100%) and 91 in MS/MS spectrum (Fig. 1). The MS data assigned M7 as oxo-piperidinyl-*O*-desisopropyl-RWJ-46344.

Metabolite 8 was profiled as a trace metabolite (< 1%) in the 60-min incubate. The Q1 scan MS and MS/MS showed a protonated molecular ion at *m/z* 463 and daughter ions at *m/z* 421, 350, 308, 232, 218, 190, 119 (100%), 91 and 70 (Figs. 1 and 2). The structure of M8, oxo-piperidinyl-RWJ-46344, was tentatively assigned by means of MS/MS data.

Metabolite 9 was identified as a minor metabolic product, which revealed an apparent molecular ion at *m/z* 437 (Fig. 1, 2). MS/MS analysis of m/z 437 displayed the diagnostic daughter ions at m/z 294, 218, 192, 119 (100%) and 91 (Fig. 1). Metabolite 9 was tentatively elucidated as OH-phenyl-oxo-piperidinyl-*O*-desisopropyl-RWJ-46344 on the basis of MS data.

Metabolite 10 was found as a minor metabolite which exhibited a protonated molecular ion at *m/z* 447, 2 amu less than parent compound. The MS/MS spectrum of the metabolite provided important fragment ions at *m/z* 405, 285, 259, 230 and 119 (100%) (Figs. 1 and 2). These data were consistent with the structure of dehydro-RWJ-46344 tentatively assigned for M10.

Metabolite 11 was profiled as a major metabolite (~24%) in 60-min incubate (Fig. 1, Table 1). The Q1 scan MS of the metabolite showed an intense protonated molecular ion at *m/z* 220 (Fig. 1) and the MS/MS spectrum displayed the prominent daughter ions at *m/z* 178, 161, 133, 84 (100%) and 56 (Figs. 1 and 3).

Metabolite 12 was profiled as a minor metabolic component (~3%) (Table 1), which gave an apparent protonated molecular ion at *m/z* 248 and diagnostic daughter ions at *m/z* 152, 135 (100%) and 111 from the MS/MS/MS analysis (Figs. 1 and 2). These data tentatively assigned M12 to be an alcohol metabolite.

4. Conclusion

The in vitro metabolism of RWJ-46344 was conducted in the rat hepatic S9 fraction. Unchanged RWJ-46344 plus 12 metabolites were profiled, quantified, characterized, and tentatively identified by means of MS data. API ionspray-MS and MS/MS exhibited apparent protonated molecular ions, and prominent, as well as important, fragment daughter ions for the structural elucidation of RWJ-46344, its metabolites and methyl derivatives. Formation of these metabolites in the rat hepatic S9 can be explained by six pathways, 1, *O*-dealkylation; 2, piperidinyl oxidation; 3, *N*-debenzylation; 4, phenyl hydroxylation; 5, dehydration; and 6, reduction. Pathways 1 to 3 appeared to be the most important pathways, forming major and moderate metabolites, *O*-desisopropyl-RWJ-46344 (M3, ~13%), its hydroxylated metabolite (M5), hydroxy-piperidinyl-RWJ-46344 (M1, ~5%), and a phenylpiperidine metabolite (M11, ~24%) (Table 1). Eight minor and/or trace metabolites (M2, M4, M6, M7–10, M12; each < 2%) were formed via a combination of six steps. The proposed in vitro metabolic

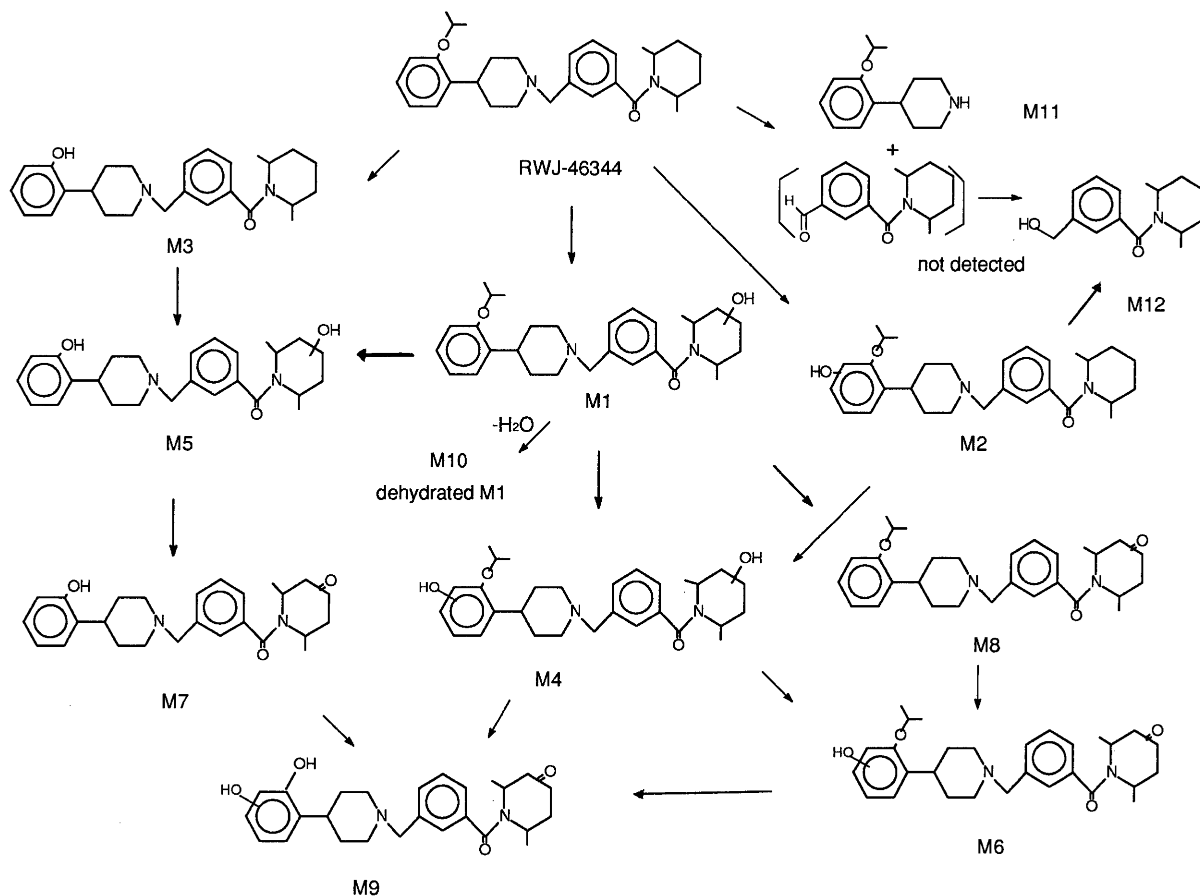


Fig. 4. Proposed in vitro metabolic pathways for RWJ-46344 in the rat.

pathways for RWJ-46344 in rat hepatic S9 fraction are depicted in Fig. 4. In conclusion, our results indicated that RWJ-46344 is substantially metabolized in rat hepatic S9 fraction.

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