IN VITRO METABOLISM OF DIARYLPYRAZOLES, A NOVEL GROUP OF CANNABINOID RECEPTOR LIGANDS

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ABSTRACT:

Diarylp yra zoles are a group of 1,5-diphenylpyrazole analogs of which several have been found to exhibit antagonist properties toward the cannabinoid receptors, SR141716A [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide], the first reported antagonist, is a highly potent and selective CB1 receptor ligand that prevents or reverses CB1-mediated effects. Other analogs, such as AM251 [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] and AM281 [1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-(morpholinyl)-1H-pyrazole-3-carboxamide], have also shown high binding affinities to the central cannabinoid receptor and behave as antagonists/inverse agonists. There has been no report on the metabolism of any of the diarylp yra zoles, and it is unknown whether their metabolites retain any receptor binding properties. We report a study of the in vitro metabolisms of three diarylp yra zole analogs, SR141716A, AM251, and AM281, in rat liver microsomes. The metabolic profile was obtained using high-performance liquid chromatography with UV and mass spectrometry detectors. All identified metabolites are characterized by structural modifications on the terminal group of the 3-substituent. Thus, three pairs of isomeric metabolites were identified from the microsomal incubation of SR141716A; these metabolites are products of hydroxylation, hydroxylation followed by dehydration, and a combination of the two. For AM251, only four metabolic products were detected, with two resulting from monohydroxylation of the piperidine ring and the other two being products of dehydration of the first pair of metabolites. For AM281, in which the terminal group of the 3-substituent is a morpholine ring, dehydration of the first two metabolites yielded a single third metabolite due to only one possible position for the carbon-carbon double bond on the morpholine ring.

The discovery of the cannabinoid receptors CB1 (Matsuda et al., 1990) and CB2 (Munro et al., 1993) represents a significant milestone in cannabinoid research. CB1 is the central receptor found in brain and neuronal cells and mediates such physiological responses as analgesia and catalepsy. The peripheral receptor, CB2, is primarily expressed in spleen and immune cells. The role of CB1 in mediating physiological effects has not been fully determined, but it is believed that CB2 may be involved in cannabinoid-mediated immune responses (Bouaboula et al., 1999; Portier et al., 1999). A large variety of compounds have been found to bind to the receptors, including natural and synthetic cannabinoids (Razdan, 1986; Keinowitz et al., 2000), amine alkyl indoles (Bell et al., 1991; Shim et al., 1998), endogenous ligands, and diarylp yra zoles (Compton et al., 1993; Wiley et al., 2001).

The first synthetic compound reported to have antagonist/inverse agonist properties toward the cannabinoid receptor is SR141716A (Rinaldi-Carmona et al., 1994), a diarylp yra zole analog (Fig. 1). SR141716A exhibits strong (low nanomolar) affinity for CB1, but rather low affinity (approaching micromolar) for CB2 (Rinaldi-Carmona et al., 1994; Showalter et al., 1996; Felder et al., 1995, 1998). Two less potent CB1-selective analogs, AM251 and AM281 (Fig. 1), have also been reported (Gatley et al., 1997, 1998; Lan et al., 1999; Cosenza et al., 2000). It is believed that the antagonistic activity of the diarylp yra zoles is attributable to the structural properties of 1- and 5-substituents, whereas the 3-substituent appears to be involved in agonism and receptor activation (Wiley et al., 2001). Thus, even small modifications on the 1- and 5-substituents result in decreased affinity and loss of antagonism, indicating the essential role of the area in conferring receptor recognition and antagonist activity to the diarylp yra zole compounds. On the other hand, replacement of the 3-substituent of the pyrazole ring with functional groups such as ethers and alkyl amides generally leads to dramatic decreases in CB2 binding affinities (Lan et al., 1999). For 3-substituent analogs that show significant receptor affinity, partial agonist properties have been observed (Wiley et al., 2001).

Structural modifications to the diarylp yra zoles as a result of metabolism, however, have not been reported. In light of the sensitivity of the receptor recognition and antagonistic efficacy of diarylp yra zole...
zoles to structural variations on the 1-, 3-, and 5-substituents (Fig. 1), it can be hypothesized that potential metabolites of these ligands may lose much of their receptor binding affinity and/or antagonist properties. It is also possible that some of the metabolic products may retain a significant amount of antagonism of the parent compounds if structural modifications occur at less crucial sites. Thus, knowledge of the metabolic transformation of the diarylpyrazoles is required to evaluate the role of metabolites in the overall efficacy of the antagonists. The current study investigates the in vitro metabolism of SR141716A, AM251, and AM281 using rat liver microsomes. High-performance liquid chromatography (HPLC) was used for the separation of complex metabolic mixtures, and tandem mass spectrometry (MS/MS) along with high-resolution NMR spectroscopy techniques were used in the structural elucidation of various metabolic products of the diarylpyrazole analogs.

Materials and Methods

Materials. AM281 and AM251 were purchased from Tocris Cookson Inc. (Ellisville, MO). HPLC-grade solvents (acetonitrile, methanol, and water) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were obtained from Fisher Scientific Co. (Pittsburgh, PA). Rat liver microsomes were purchased from BD Gentest (Woburn, MA) and stored at -20°C prior to use.

Synthesis of SR141716A. Synthesis of SR141716A was carried out following procedures reported by Dutta et al. (1994). Briefly, bromination of 4-chloropropiophenone gave the bromoketone intermediate. Treatment of the anion of ethyl acetocetate with the bromoketone furnished the acetocetate derivative. The sodium salt of the acetocetate derivative was allowed to react with a solution of 2,4-dichlorodiazonium chloride, followed by base hydrolysis to give the pyrazole carboxylic acid, which was then treated with 1-aminopyridine and triethylamine to give the target compound. The product was purified by flash chromatography and crystallized from methanol. Structural confirmation of the synthetic SR141716A was done by 1H and 13C NMR spectroscopy (see Tables 1–3) and HPLC-MS/MS (Fig. 2).

Microsomal Incubations. Stock solutions of SR141716A, AM251, and AM281 were prepared in dimethyl sulfoxide, at a concentration of 20 mM. Rat liver microsomes containing 1.8 mg/ml protein were preincubated at 37°C for 3 min. The 0.2-ml incubation aliquots contained 75 mM potassium phosphate (pH 7.4), 17 mM magnesium chloride, 7 mM NADP+, 17 mM glucose 6-phosphate, and 1.2 units/ml glucose-6-phosphate dehydrogenase. To the incubation aliquots was added 0.1 of the SR141716A, AM281, or AM251 stock solution. Incubation times ranged from 0.5 to 2 h. Incubations were halted by placing the vials in an ice bath, followed by adding an equal volume of methanol (0.2 ml). The quenched incubation mixtures were stored at -20°C until analysis. Before HPLC separation, microsomal proteins were precipitated by centrifugation (10,000g, 15 min) at room temperature, and the supernatant was evaporated with a stream of nitrogen at 37°C to 0.2 ml. The residual solution was applied to 6-ml Supelco (Bellefonte, PA) CN solid-phase extraction columns pretreated with water (four times, 4 ml) and methanol (four times, 4 ml). The columns were washed with HPLC-grade water (twice, 3 ml) and eluted with methanol (three times, 3 ml); the eluents were again concentrated by a nitrogen stream at 37°C to 1.0 ml. For semipreparative purposes, a total of five 10-ml larger-scale incubations were carried out. In each 10-ml incubation mixture were 18 mg of protein, 75 mM potassium phosphate buffer (pH 7.4), 17 mM magnesium chloride, 7 mM NADP+, 17 mM glucose 6-phosphate, and 1.2 units/ml glucose-6-phosphate dehydrogenase and 50 μl of 20 mM SR141716A.

Control Incubations. Control incubations were performed under identical conditions with heat-inactivated microsomes (heated to 100°C for 10 min). In addition, incubations in the absence of NADPH or in the absence of microsomes were carried out. To eliminate matrix interferences arising from microsomes and buffer components, blank incubations were performed in which all elements were present except the drug compounds.

HPLC-UV Analysis. Initial analysis of the incubation products was performed by a Shimadzu (Columbia, MD) HPLC system equipped with a UV-visible SPD-10ADVP detector. A 2.1 × 150 mm, 4-μm pore size Phenomenex (Torrance CA) ODS HPLC column was used for separation. Mobile phase flow rate was set at 0.3 ml/min, with gradient elution starting at 10% acetonitrile and 90% water for 5 min, followed by a linear increase to 50% acetonitrile in 14 min, a linear change to 100% acetonitrile in 5 min, and a final linear change back to 10% acetonitrile in 5 min. Injection volume was 10 μl. Eluted components were detected by the UV detector (Amax, 270 nm). To ensure reproducibility, at least three injections were performed for each incubation aliquot. No significant qualitative or quantitative differences were found between runs (CV% = 3.5).

Semipreparative HPLC. Separation of the metabolites was carried out on a Phenomenex ODS HPLC column (10.0 × 250 mm; 4-μm pore size) coupled to a Phenomenex ODS guard precolumn (10 × 50 mm, 4 μm). A model 7125 Rhodyne manual injector with 500-μl loop volume (Rhodyne LLC, Rohnert Park, CA) was used for sample introduction. Mobile phase flow rate was set at 5.5 ml/min, with gradient elution starting at 10% acetonitrile and 90% water for 5 min, followed by a linear increase of acetonitrile to 50% in 15 min, and a linear change to 100% acetonitrile in 7 min. Eluent absorbance was monitored at 270 nm using a variable wavelength detector. Samples corresponding to the metabolite were pooled and dried under vacuum and used for NMR analysis.

LC/MS and LC/MS/MS Analysis. A Phenomenex ODS HPLC column (2.1 × 150 mm; 4-μm pore size) coupled to a Supelco C18 guard column (2 × 18 mm, 5 μm) was used for separation. A Shimadzu LC-MS-2010 was used for initial screening of possible metabolic products generated from the microsomal incubations by obtaining the mass spectra of all chromatographic peaks. HPLC mobile phase flow rate was set at 0.20 ml/min, with gradient elution starting at 10% acetonitrile and 90% water for 5 min, followed by a linear increase of acetonitrile composition to 100% in 16 min. MS/MS experiments were performed on a Quattro II triple quadrupole tandem mass spectrometer equipped with an electrospray source (Micromass; Waters, Milford, MA). The electrospray needle potential was set at 3.46 kV, and the orifice potential was set at 70 V for MS scans and 62 to 67 V for MS/MS measurements. In MS/MS experiments the collision-induced dissociation (CID) of selected precursor ions took place in the central hexapole collision cell using argon as collision gas.
The collision energies ranged from 17 to 20 eV. The ion source was held at 250°C.

**NMR Spectroscopy.** NMR spectra were recorded at 400 MHz or 500 MHz on a Varian Unity-400 or Varian Unity-500 spectrometer (Varian Inc., Palo Alto, CA). Data were processed on a SUN-5 computer using Varian VNMR software version 6.1B. Sample was dissolved in 0.5 ml of chloroform-d$_1$ (99.8 atom % $^2$H) for NMR analysis. Chemical shifts are reported on the $^1$H scale by assigning the 7.26 (1H) and 77.0 (13C) for chloroform.

**Results**

SR141716A ($a$). Detailed LC/MS/MS and NMR spectroscopic analyses of the parent compound SR141716A ($a$) were carried out to facilitate identification of the structures of its metabolites. The full-scan mass spectrum of $a$ showed a prominent protonated molecule (MH$^+$) at $m/z$ 463 Da which, upon CID, yielded a base peak at $m/z$ 363 (Fig. 2). This fragment ion was proposed to arise from the exclusion of the aminopiperidine moiety. Further fragmentation of the ion at $m/z$ 363 yielded at least three product ions at $m/z$ 299, $m/z$ 282, and $m/z$ 164. The fragment ions observed at $m/z$ 99, $m/z$ 84, and $m/z$ 55 were proposed to be related to the aminopiperidine moiety. The $^1$H NMR chemical shifts as well as the respective coupling patterns of SR141716A and its metabolite $M_a4$ are summarized in Table 1. All proton assignments were assisted by COSY experiments (Table 2). The $^{13}$C NMR chemical shifts of SR141716A are presented in Table 3. The $^{13}$C NMR assignments were assisted by distortionless enhancement by polarization transfer, heteronuclear multiple quantum coherence spectroscopy, and heteronuclear multiple-bond correlation spectroscopy.

The broad single peak at around $\delta$ 7.60 ($^1$H) can be assigned to the NH-proton at $W$. The aromatic region of the $^1$H NMR spectrum of SR141716A indicated four protons having an AA/BB system, with

**TABLE 1**

$^1$H NMR assignments for SR141716A ($a$) and its metabolite $M_a4$ (reference: TMS)

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be-s, broad singlet; d, doublet; dd, doublet of doublets; J, coupling constant; m, multiplet; s, singlet.
multiple-bond correlation spectroscopy; DEPT, distortionless enhancement by polarization

SR141716A (in CDCl₃) COSY 

aM/H9254

HMQC, heteronuclear multiple quantum coherence spectroscopy; HMBC, heteronuclear spin coupling observed to the indicated resonance.

Proton chemical shift

NO, not observed with the acquisition parameters used in the experiment.

COSY, correlation spectroscopy; homonuclear spin coupling observed to the indicated resonance.

\[ \begin{array}{cccccccccccc}
\text{SR141716A (a)} & \text{A} & \text{B} & \text{C} & \text{D} & \text{E} & \text{F} & \text{G} & \text{H} & \text{I} & \text{J} & \text{K} & \text{L} \\
\text{(in CDCl₃)} & \text{COSY}^b & \text{δ}_a & 2.86 & 1.75 & 1.43 & 2.36 & 7.05 & 7.24 & 7.29 & 7.31 & 7.34 & 7.43 & 7.63 \\
\text{Mo4} & \text{δ}_a & 3.11 & 4.26 & 1.75 & 1.93 & 1.50 & 2.27 & 6.98 & 7.18 & 7.21 & 7.23 & 7.34 & 7.92 \\
\text{(in CDCl₃)} & \text{COSY}^b & 1.75 & 1.93 & 3.11 & 2.08 & 1.62 & NO & 7.18 & 6.98 & 7.23 & 7.34 & 7.21 & NO \\
\end{array} \]

\(^a\) Proton chemical shift δ in ppm relative to methanol or chloroform.

\(^b\) COSY, correlation spectroscopy; homonuclear spin coupling observed to the indicated resonance.

\[ \begin{array}{cccccccccccc}
\text{1H-1H COSY assignment for SR141716A (a) and its metabolite Mo4} \\
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\[ \begin{array}{cccccccccccc}
\text{TABLE 2} \\
\text{13C Assignment for SR141716A (a)} \\
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\[ \begin{array}{cccccccccccc}
\text{TABLE 3} \\
\text{13C Assignment for SR141716A (a)} \\
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\text{Assignment of the aliphatic region (between δ 1.40 and δ 2.90) is based upon the following. Integration of the singlet at δ 2.36 indicates the presence of three protons, linking the single peak to the methyl protons (J) on the pyrazole ring. The signals from the 10 protons on the piperidine moiety are accounted for by peaks at δ 1.40 (2H), δ 1.75 (4H), and δ 2.86 (4H). The protons at δ 1.40 and δ 2.86 all show correlation peaks with the protons at δ 1.75. Clearly, the four protons at δ 2.86 are the two CH₂ groups (A and E) next to the nitrogen atom, the two protons at δ 1.40 belong to the CH₂ group (D), and the other four at δ 1.75 are the two CH₂ groups (B and C).} \\
\end{array} \]

\[ \begin{array}{cccccccccccc}
\text{SR141716A Metabolites. Shown in Fig. 3 are the total ion chromatogram (TIC) obtained from an incubation product mixture and the selected ion chromatogram for m/z 477, 479, and 461 that were extracted from a single run. The selected ion chromatogram for m/z 477 is shown in Fig. 3A, in which two peaks are observed at retention times of 17.63 min and 18.25 min, respectively. These two metabolites, designated as Mo1 and Mo2, show an isobaric MH⁺ ion at m/z 477, 14 amu higher than the [M + H]⁺ ion of SR141716A and 16 amu higher than the protonated Mo5 and Mo6 (see also discussions on Mo5 and Mo6), suggesting that Mo1 and Mo2 are the products of hydroxylation of the corresponding Mo5 or Mo6. The product ion} \\
\end{array} \]

- HMQC, heteronuclear multiple quantum coherence spectroscopy; HMBC, heteronuclear multiple-bond correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer.
spectra of $M_a1$ and $M_a2$ are identical, and the spectrum is shown in Fig. 4. A comparison with the product ion spectrum of SR141716A and its proposed fragmentation pathways (Fig. 2) indicates that the fragment ion at $m/z$ 98 is related to the SR141716A fragment ion at $m/z$ 84. All other identical fragment ions provide evidence that the only site of metabolic oxidation occurring in $M_a1$ and $M_a2$ is the piperidine moiety. The proposed fragmentation pathways are also given in Fig. 4, based on the tandem mass spectral details.

Figure 3B is the selected ion chromatogram for $m/z$ 479 showing two metabolites ($M_a3$ and $M_a4$) eluting at 18.29 min and 19.20 min, respectively. Both metabolites have the same molecular weight of 478, indicating an increase of 16 mass units, which is consistent with addition of one oxygen atom to the structure. The product ion spectrum of the protonated $M_a3$ and $M_a4$ is given in Fig. 5. Compared with the fragment ions of $a$ in Fig. 2, a group of fragment ions is found to be identical, including ones at $m/z$ 363, $m/z$ 299, $m/z$ 282, and $m/z$ 164. The two fragment ions that differ from those of $a$ are 461 (loss of H$_2$O from $m/z$ 479) and $m/z$ 82 (2 amu lower than the fragment ion at $m/z$ 84 in that of $a$), revealing structural modifications on the piperidine ring via hydroxylation.

The major metabolite ($M_a4$) at 19.20 min was successfully isolated in sufficient amount for further structural analysis by NMR. Compared with the parent compound $a$, the $^1$H NMR spectrum of $M_a4$ in CDCl$_3$ showed only minor variations in proton chemical shift in the dichlorophenyl, chlorophenyl, and methyl substituents of the pyrazole ring, but significant changes in chemical shift were observed in the region corresponding to the piperidine ring (Table 1). The piperidine ring is therefore the site of oxidation. Four possible structures can be envisioned, the tertiary N-oxide ($i$), or a hydroxylated structure ($ii$, $iii$, or $iv$), as illustrated in Scheme 1.

The tertiary N-oxide $i$ would be expected to have 10 proton signals in the piperidine ring. In the $^1$H NMR spectrum of $M_a4$, only 9 proton signals were observed in this region (Table 1), indicating one of the 10 protons has been substituted, suggesting that $M_a4$ can be a hydroxylated structure ($ii$, $iii$, or $iv$). One proton of the piperidine ring shifted down-field significantly to /H9254 4.26, indicative of a hydroxyl group formed on one of the five carbons (A, B, C, D, or E), thus eliminating structure $i$ as a possibility. The second possible structure, $ii$, in which carbon B is the site of hydroxylation, would require the remaining proton on carbon B to have correlation peaks with four other protons (two on carbon A and two on carbon C). The third possible structure, $iii$, in which carbon C has the hydroxyl substituent, would also require the remaining proton on carbon C to have correlation peaks with four other protons (two on carbon B and two on carbon D). In the $^1$H-1H COSY spectrum of $M_a4$ (Table 2), the remaining proton on the hydroxylated carbon yields only correlation peaks with two protons,
thus eliminating ii and iii as possible structures. Overall, the NMR data point to the fourth structure (iv) as the structure of Ma4, with hydroxylation occurring on carbon E. The proton on carbon E (δ 4.26) gives correlation peaks with the two protons on carbon D (δ 1.93 and δ 2.08). Additional evidence includes correlation peaks between the two protons on C and B and the two protons on D, and those between the two protons on B and the two protons on A (Table 2). However, the stereochemistry of the hydroxyl group cannot be ascertained with the present NMR data.

Figure 3C represents two other metabolites, designated as Ma5 and Ma6, that have the same molecular weight of 460. The protonated molecule at m/z 461 is 2 amu lower than the [M + H]+ ion of SR141716A. A comparison of the product ion spectrum of Ma5 and Ma6 (Fig. 6) and SR141716A (Fig. 2) reveals that the fragment ions at m/z 97 and m/z 82 in Fig. 6 are also 2 amu lower than the fragment ion at m/z 99 and m/z 84, respectively, in Fig. 2, whereas all other fragment ions are identical. The above mass spectral information indicates that the piperidine ring of SR141716A has been dehydrogenated to yield Ma5 and Ma6. The proposed fragmentation pathways of Ma5 and Ma6 are given in Fig. 6. As discussed earlier, the fragment ion of the protonated Ma1 and Ma2 at m/z 98 corresponds to the fragment ion of the protonated Ma5 (or Ma6) at m/z 82. The mass difference of 16 and the fragmentation from m/z 98 to m/z 80 via loss of H2O suggests the involvement of a hydroxyl group on the dihydropiperidine moiety.

AM251 (b), The product ion spectrum of AM251 (b) was obtained before HPLC-MS/MS analysis of the incubation products. All tandem mass spectral data for AM251 and its metabolites are summarized in Table 4. The total ion chromatogram of an incubation product mixture and the selected ion chromatograms for the metabolites are shown in Fig. 7. The selected ion chromatogram for m/z 571 in Fig. 7A shows two peaks (M(b)1 and M(b)2) at retention times of 19.80 min and 19.88 min, respectively. The m/z value for the protonated M(b)1 and M(b)2 indicates an increase of 16 mass units compared with the parent compound, AM251, consistent with the addition of a single oxygen atom to the structure. The tandem mass spectra data (Table 4) show that M(b)1 and M(b)2 both share several identical fragment ions with AM251; i.e., F(b)1 to F(b)4 at m/z 455, 391, 328, 374, and 256, indicating no metabolic modification on these fragments. The two fragment ions, F(b)5 and F(b)6, are each at 16 mass units higher than their counterparts of the parent compound AM251. This information indicates that the piperidine ring of AM251 has been hydroxylated. The fragmentation of the ion at m/z 100 via a dehydration pathway is responsible for the ion observed at m/z 82, suggesting the involvement of a hydroxyl group on the dihydropiperidine moiety.

MB3 and MB4 are two isomeric metabolites observed at retention times of 19.96 min and 20.34 min, respectively. The two metabolites show a common [M + H]+ ion at m/z 553, 2 amu lower than the [M + H]+ ion of AM251. A comparison of their product ion spectrum and AM251 (Table 4) reveals that the fragment ion at m/z 82 for the metabolites is also 2 amu lower than the fragment ion at m/z 84 of AM251, whereas all other fragment ions are identical. The mass spectral evidence suggests that the piperidine ring of AM251 has been dehydrogenated to yield MB3 and MB4.

AM281 (c). The full-scan mass spectrum of the parent compound showed a prominent protonated peak at m/z 557 Da, which upon CID results in a product ion at m/z 455 (Table 5) via loss of the aminomorpholine moiety. Further fragmentation of the ion at m/z 455 yields peaks observed at m/z 328 and m/z 256. The fragment ions at m/z 101, m/z 86, and m/z 56 are related to the aminomorpholine moiety.

Shown in Fig. 8 are the TIC obtained from an incubation product mixture of AM281 and the selected ion chromatograms for m/z 573 and 555, all reconstructed from a single run. The selected ion chromatogram for m/z 573 (Fig. 8A) shows two peaks (Mc1 and Mc2) at retention times of 17.96 min and 18.38 min, respectively, indicating a
pair of isobaric metabolites. The increase of 16 mass units compared with the parent compound AM281 is consistent with the introduction of a hydroxyl group in replacing a hydrogen atom. LC/MS/MS data summarized in Table 5 show that these two metabolites yield, indicating no metabolic modification in these fragments of the molecule. Another fragment ion at \( m/z \) 555, 2 amu less than the protonated AM281, is apparently the result of losing a H2O molecule from the precursor ion at \( m/z \) 573. Further fragmentation of the ion at \( m/z \) 555 yields an ion of the morpholinyl moiety that is also 2 amu lower than the corresponding fragment ion of the protonated AM281. Clearly, hydroxylation has occurred on the morpholine ring.

The third metabolite, \( M_c3 \), is observed at a retention time of 19.21 min, which gives an \([M + H]^+\) ion at \( m/z \) 555, 2 amu lower than the \([M + H]^+\) ion of AM281. A comparison of the fragment ions of \( M_c3 \) and those of AM281 (Table 5) reveals that the fragment ion at \( m/z \) 84 of the metabolite is also 2 amu lower than the fragment ion at \( m/z \) 86 of AM281, with all other fragment ions being identical. Thus, the mass spectral evidence points to \( M_c3 \) as a metabolite containing a double bond on the morpholine ring.

**Discussion**

Based upon the chromatographic and mass spectrometric data on the metabolic products of the three diarylpyrazole compounds, the metabolic pathways for SR141716A, AM251, and AM281 are illustrated in Figs. 9, 10, and 11, respectively. All detected metabolites result from structural modifications on the terminal ring of the 3-substituent. For SR141716A, a total of six metabolites have been identified, which are designated as \( M_a1 \) through \( M_a6 \), in the order of increasing retention time during chromatographic elutions. Hydroxylation on the piperidine ring gives rise to the pair of isomeric metabolites \( M_a3 \) and \( M_a4 \) due to different possible sites of hydroxylation. However, with the isolation and purification of \( M_a4 \), the only metabolite obtained in pure form, it was possible to determine by NMR analysis that the hydroxyl group in \( M_a4 \) is located at the \( -H/9251 \) carbon on the piperidine ring. Dehydration of \( M_a3 \) and \( M_a4 \) leads to another pair of isomeric metabolites, \( M_a5 \) and \( M_a6 \), each representing one of the two possible positions of the double bond introduced on the piperidine ring. Although it is likely that \( M_a5 \) and \( M_a6 \) are preceded by \( M_a3 \) and \( M_a4 \), they may also be formed via direct dehydrogena-
tion of the piperidine ring (Sai et al., 2001). The next two metabolites, $M_a1$ and $M_a2$, may be formed via two possible routes. First, further oxidation of $M_a5$ and $M_a6$ on the piperidine ring introduces a hydroxyl group that can have several different sites of hydroxylation. However, it is also plausible that the monohydroxyl metabolites ($M_a3$ and $M_a4$) can be oxidized to first form dihydroxylated intermediates (Fig. 9), which, upon dehydration, leads to $M_a1$ and $M_a2$.

In AM251, the $p$-chlorophenyl group is replaced by a $p$-iodophenyl substituent, which is the only structural difference between it and SR141716A. It appears that such a structural difference may be responsible for the observation of only four metabolites for AM251 (Fig. 10), compared with six total for SR141716A. The metabolic pathways of AM251 involving hydroxylation on the piperidine ring followed by dehydration, however, are essentially the same as that proposed for SR141716A. Compared with AM 251, the third diarylpyrazole antagonist examined in this study, AM281, has an additional structural modification in that the piperidine ring is replaced by a morpholine group. As a result, only three metabolites of AM281 have been identified, of which two are an isomeric pair ($M_c1$ and $M_c2$) resulting from the monohydroxylation of the morpholine ring and the third, a product of dehydration of $M_c1$ and $M_c2$. It is noted that dehydration of both $M_c1$ and $M_c2$ leads to the same metabolite, $M_c3$, due to only one possible position for the carbon-carbon double bond (Fig. 11).

In conclusion, it has been demonstrated that three diarylpyrazole analogs that behave as cannabinoid receptor antagonists undergo unique metabolic pathways that are shared neither by traditional cannabinoids nor by synthetic agonists such as aminoalkylindoles. Unlike aminoalkylindoles, in which extensive hydroxylations were observed at various sites of the parent compounds (Zhang et al., 2002, 2004), the only site vulnerable to metabolic activities appears to be the terminal group of the 3-substituent on the pyrazole ring (Fig. 1), whereas no metabolic modification has been detected at other sites of the diarylpyrazoles. Given the sensitivity of antagonist properties to structural variations on the 3-substituent (Wiley et al., 2001; Lan et al., 1999), it will be of great interest to understand whether the metabolites still bind to the receptors with considerable affinity and, furthermore, whether they behave as antagonists or agonists after

### Table 5

<table>
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<th></th>
<th>$M$</th>
<th>$F_{c1}$</th>
<th>$F_{c1-127}$</th>
<th>$F_{c6}$</th>
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<td>328</td>
<td>86</td>
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<td>101</td>
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<td>455</td>
<td>328</td>
<td>84</td>
<td>555</td>
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<tr>
<td>$M_c3$</td>
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Fig. 8. HPLC-MS chromatograms of AM281(c) metabolites from rat microsomal incubation. A, SIM chromatogram of $m/z$ 573; B, SIM chromatogram of $m/z$ 555; C, SIM chromatogram of $m/z$ 557; and D, TIC.

FIG. 10. Proposed metabolic pathways of b (AM251).

FIG. 11. Proposed metabolic pathways of c (AM281).
structural modifications on the 3-substituents. With one major metabolite of SR141716A (Ma4) isolated in pure form, further studies are now possible to determine the receptor binding affinities and physiological efficacies of the metabolite for both CB1 and CB2.

References


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