IN VITRO METABOLISM OF \( R(+)\)\text{-}[2,3-DIHYDRO-5-METHYL-3-[(MORPHOLINYL)METHYL]PYRROLO [1,2,3-DE]1,4-BENZOAZINYL]-(1-NAPHTHALEYL) METHANONE MESYLATE, A CANNABINOID RECEPTOR AGONIST

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ABSTRACT:

\( R(+)\)\text{-}[2,3-Dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate (WIN55212-2) is a potent cannabinoid receptor agonist that has been found to exhibit anticonvulsant activity and to inhibit brain cyclooxygenase. The metabolism of WIN55212-2 has not been reported, and it is unknown whether its metabolites retain any agonist properties. In this study, in vivo metabolism of WIN55212-2 in rat liver microsome was investigated. The metabolic profile was obtained using high-performance liquid chromatography (HPLC) with UV and mass spectrometry detectors. The HPLC chromatogram revealed two major and at least six minor metabolites derived from the parent compound ([M + H]\(^+\) = m/z 427). The two major metabolites (structural isomers at m/z 461), constituting 60 to 75% of the total metabolites, were each identified as dihydrodihol metabolites resulting from the arene oxide pathway. The minor metabolites were all detected as protonated molecules, three of which appeared at m/z 477, corresponding to structural isomers of trihydroxylated parent compound; another two appeared at m/z 443, representing monohydroxylated isomers; and another was observed at m/z 425, and was assigned as a dehydrogenation product. These structural assignments are based on HPLC/tandem mass spectrometry and NMR analysis. Metabolic pathways have been proposed to account for the various metabolites observed. Two major metabolites have been isolated in pure form, allowing future receptor binding studies to be conducted.

Aminoalkylindoles (AAIs\(^2\)) are a structurally distinct group of cannabinoid receptor agonists that were originally developed from pravadoline (Ward et al., 1990; Bell et al., 1991; D’Ambra et al., 1992; Kuster et al., 1992; Eissenstat et al., 1995). AAIs have been found to displace potent cannabinoid ligands such as CP-55940 in competitive binding to the CB1 cannabinoid receptor (Ward et al., 1990). The ability of AAIs to inhibit cerebella adenylyl cyclase activity and neurally stimulated contractions in the mouse vas deferens preparation, and lower intraocular pressure, suggests a receptor-mediated mechanism of action (Pacheco et al., 1990; D’Ambra et al., 1992; Song and Slowey, 1998). A widely used prototype of AAIs is \( R(+)\)\text{-}[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate (WIN55212-2), a structurally constrained analog. WIN55212-2 has been used as a radiolabeled probe for CB1 binding assays (Kuster et al., 1992).

Whereas the physiological effects of WIN55212-2 via CB1 receptor mediation have been studied extensively (Aceto et al., 2001; Bridges et al., 2001; Gardiner et al., 2001; Simoneau et al., 2001), to our knowledge, there has been very little research on the metabolism of WIN55212-2 and other aminoalkylindole analogs, either in vitro or in vivo. Because of the remarkable structural difference between aminoalkylindoles and other cannabinoid ligands, it is hypothesized that AAIs could undergo biotransformations that also differ significantly from the metabolic patterns of the classical and nonclassical cannabinoids. Furthermore, some of the metabolic products of WIN55212-2 may retain cannabimimetic activities of their parent compound, thereby contributing to the overall physiological efficacy of WIN55212-2. However, no metabolites of WIN55212-2 have been identified or isolated to date, and their potential biological activities remain unknown. Thus, the current study was undertaken to investigate the metabolic fate of WIN55212-2 using liver microsome preparations. The purpose of the study also included the isolation and purification of major WIN55212-2 metabolites for detailed structural characterization as well as for future cannabinoid receptor binding studies. Previous metabolic studies have focused on naturally occurring cannabinoids and structurally similar synthetic derivatives. The...
low polarity of the cannabinoids makes them amenable to gas chromatographic analysis and the metabolites, upon derivatization, have also been qualitatively determined by gas chromatography-mass spectrometry (Harvey and Paton, 1984; Harvey, 1999). The highly polar nature of WIN55212-2 and its metabolites does not allow direct analysis by gas chromatography, and derivatization can be tedious and technically difficult. In this study, high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) was used for separation and identification of metabolic products. In addition, where possible, NMR spectroscopy was employed to provide complementary structural information.

**Materials and Methods**

**Materials.** WIN55212-2 was purchased from Sigma/RBI (Natick, MA). HPLC-grade solvents (acetonitrile, methanol, and water) and deuterated solvents (CDCl$_3$ and CD$_3$OD) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Rat liver microsomes were purchased from BD Gentest Corporation (Woburn, MA) and stored at $-80^\circ$C prior to use.

**Microsomal Incubations.** One microliter of a 20 mM WIN55212-2 stock solution, prepared in dimethyl sulfoxide, was added as substrate to individual incubation aliquots. Liver microsomes (protein concentration at 1.5 mg/ml) were preincubated at 37°C for 3 min. The 0.2-ml incubation solution consisted of 75 mM potassium phosphate (pH 7.4), 17 mM magnesium chloride, 7 mM NADP$^+$, 17 mM glucose 6-phosphate, and 1.2 units of glucose-6-phosphate dehydrogenase. Reactions were initiated by the addition of WIN-55212–2 (final concentration 100 nM). Incubation times ranged from 1 to 4 h. Incubations were halted by placing the incubation vials in an ice bath, with addition of an equal volume of methanol (0.2 ml) and stored at $-20^\circ$C until analysis. Prior to HPLC analysis, microsomal proteins were precipitated by centrifugation at room temperature, and the methanol was evaporated with a stream of nitrogen at 37°C. The residual solution was applied to a Shimadzu QP8000 HPLC-MS system equipped with a parallel UV-Vis SPD-10AVP detector (Shimadzu Instruments Co. Columbia, MD). A 4.6 × 150 mm, 5-μm Supelco C$_8$ HPLC column (Supelco Corp., Bellefonte, PA), coupled to a Supelco C$_{18}$ guard column (4.0 × 18 mm, 5 μm) was used for separation. Mobile phase flowrate was set at 1.0 ml/min, with gradient elution starting at 10% acetonitrile and 90% water for 5 min, followed by a linear increase to 60% acetonitrile in 20 min, and a linear change to 100% acetonitrile in 5 min, and a final linear change back to 10% acetonitrile in 5 min. Eluted components were detected by the UV detector ($\lambda_{	ext{max}}$, 330 nm). To ensure reproducibility, at least three injections were performed for each incubation aliquot. No significant qualitative or quantitative differences were found between runs.

**Semipreparative HPLC separation.** Semipreparative HPLC separation of the metabolites was carried out on a Phenomenex (Torrance, CA) 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 Rheodyne manual injector with 500-μl loop volume (Rheodyne Inc., Cotati, CA) was used for sample introduction. Mobile phase flowrate 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 composition to 50% in 15 min and a linear change to 100% acetonitrile in 5 min. Eluent absorbance was monitored at 330 nm using a variable wavelength detector. Incubation products were centrifuged to precipitate proteins, and the supernatant was subjected to HPLC separation.
Three peaks were collected corresponding to metabolites M4 (m/z 461), M5 (m/z 461), and the unchanged WIN55212-2 (m/z 427), respectively. Samples corresponding to each metabolite were pooled and dried under vacuum and used for NMR analyses.

**LC/MS and LC/MS/MS Analyses.** 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. Mobile phase flowrate was set at 0.1 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 20 min. MS/MS experiments were performed on a Quattro II triple quadrupole tandem mass spectrometer equipped with an electrospray ionization (ESI) source (Micromass Inc., Beverly, MA). The ESI “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 examining collision-induced dissociation of selected precursors taking place in the central hexapole collision cell, argon was used as the collision gas at collision energies between 17 to 20 eV and collision-induced dissociation pressure 1.9 to 10 mbar. The ion source was held at 250°C.

**NMR Spectroscopy.** 1H NMR spectra were recorded at 400 MHz on a Varian Unity-400 spectrometer (Varian Medical Systems, Palo Alto, CA). Data were processed on a SUN-5 computer using Varian VNMR software version 6.1B. Each metabolite was dissolved in 0.5 ml methanol-d4 (99.9 atom % 2 H) or chloroform-d1 (99.8 atom % 2 H) for 1H NMR analysis. Chemical shifts are reported on the δ scale (parts per million) by assigning the residual solvent peak to 3.35 ppm for methanol and 7.26 ppm for chloroform, respectively. Typical data acquisition parameters were as follows: data size, 32,000; sweep width, 8125 Hz; filter width, 8945 Hz; acquisition time, 2.38 s; flip angle, 90°; relaxation delay, 1 s; temperature, 298 K. Two-dimensional COSY experiments were performed on the purified metabolites to improve signal assignment of the complex aromatic region. The parameters for the COSY experiments were as follows. The number of scans per increment was 16, the spectral width was 3881.32 Hz, and 1024 increments were performed in the F1 dimension. The free induction decays were collected into 1-kilobyte computer data points. The relaxation delay between successive pulses was 1.5, with no zero-filling in F2. Unshifted sinebell windows were applied before transformation.

**Results**

WIN55212-2 was subjected to enzymatic breakdown using rat liver microsomes as outlined in the experimental section. Rat liver microsomes were chosen for their convenience and availability, and they are considered to engage in metabolic processes conserved in a broad spectrum of mammalian species.

**HPLC-UV.** Shown in Fig. 1 is the HPLC chromatogram of the products of microsomal incubation employing UV absorbance detection. The parent compound, WIN55212-2 elutes at 29.1 min. Preceding peaks observed in the chromatogram that are absent in control incubation products and the standard solution of WIN55212-2 are considered possible metabolites formed from microsomal incubation. Thus, peaks at 14.8, 16.0, 16.5, 19.3, 19.9, 24.6, 25.0, and 25.4 min are possible metabolic products. Peak areas of the 19.3 and 19.9 min metabolites constitute 60 to 75% of the total metabolite peak areas. It is noted that two chromatographic peaks (31.8 and 44.8 min) that elute after the parent compound are also present in control/blank samples, indicating that they are likely substances introduced by the NADPH-regenerating solutions. However, no degradation product of WIN55212-2 was found in any control incubations.

**HPLC-MS/MS and NMR Spectra of WIN55212-2.** To assist in the identification of metabolites that usually contain diagnostic fragment ions that are related to those of the parent compound, the product ion spectrum of the protonated parent compound, WIN55212-2, was first acquired as shown in Fig. 2. In the conventional ESI mass spectrum, intact protonated WIN55212-2 (FW 426) was observed at m/z 427 as the dominant peak. Subsequently, this ion was isolated by the first quadrupole (Q1) as the precursor for collision induced dis-
associations. The fragment ions generated as a result of collision with Ar gas in the radio frequency only hexapole (Q3) collision cell were then mass analyzed by the third quadrupole (Q3). The three characteristic gas in the radio frequency only hexapole (Q2) collision cell were then

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The ¹H NMR chemical shifts and the respective coupling patterns of WIN55212-2 and its metabolites M4, M5 obtained in the present investigation are summarized in Table 1. The numbering scheme for the protons on the WIN55212-2 structure is given in Fig. 2. All proton assignments were derived from one dimensional and COSY experiments. Of the 10 aromatic protons in the mole-
cule (δ 6.4–8.0 ppm), two protons (δ 6.43 and δ 6.64 ppm) show correlation peaks with the proton at δ 6.83 ppm, and these three protons show no correlation peaks with any other protons in the molecule. The δ values and the correlation pattern indicate that these are the three aromatic protons on the indole ring and are assigned as follows: δ 6.83 ppm for proton I, δ 6.43 ppm for proton

![Diagram of molecular structure and fragmentation pathway](image)
H, and δ 6.64 ppm for proton J. The remaining seven aromatic proton signals are assigned to the naphthalene ring. The COSY experiments show that the two protons at δ 7.58 and δ 7.99 ppm give rise to correlation peaks with the proton at δ 7.54 ppm, and the three protons have no correlation peaks with the other four protons in this region. Thus, δ 7.58, δ 7.54, and δ 7.99 ppm are the signals from aromatic protons A, B, and C. For the last four aromatic protons, correlation peaks are observed between the two protons at δ 7.93 and δ 7.44 ppm and the proton at δ 7.51 ppm, and between the proton at δ 7.44 ppm and the proton at δ 8.08 ppm. These  

![Diagram](image_url)

**Fig. 5.** Product ion spectra obtained by collision induced dissociation of the protonated ion (m/z 461) of M4 (A) and M5 (B) and their proposed fragmentation pathways.

**Table 2**

|            | A | B | C | D | E | F | G | H | I | J | K | K' | L | M | M' | N | O | P | Q | R |

a Proton chemical shift δ in ppm relative to methanol or chloroform.
b COSY, correlation spectroscopy; homonuclear spin coupling observed to the indicated resonance.
c N.O., not observed with the acquisition parameters used in the experiment.
Fig. 6. Product ion spectra of the protonated M6(A) and M7(B) ions (m/z 443) and their proposed fragmentation pathways.

Fig. 7. MS/MS spectrum of the protonated M8 (m/z 425) obtained by collision-induced dissociation and its proposed fragmentation pathway.
correlations allow the assignments of the last four protons as follows: δ 7.93 ppm for proton $D$, δ 7.44 ppm for proton $F$, δ 7.51 ppm for proton $E$, and δ 8.08 ppm for proton $G$. All aromatic protons show coupling patterns that are consistent with the above proton assignments ($J = 8$ and 1.2 Hz).

In the aliphatic region (between $\delta$ 2.5 and $\delta$ 4.8 ppm), integration of the singlet at $\delta$ 2.59 ppm indicates the presence of three protons, linking the single peak to the methyl protons (R) on the indole ring. The signals from the eight protons on the morpholine moiety are accounted for by protons at $\delta$ 2.57 ppm and at $\delta$ 3.73 ppm, which show correlation peaks with each other. The four protons at $\delta$ 2.57 ppm are most likely the two CH$_2$ (N and Q) next to the nitrogen atom, the other four at $\delta$ 3.73 ppm being the two CH$_2$ (O and P) next to the oxygen. The proton at $\delta$ 2.76 ppm is coupled with the proton at $\delta$ 2.50 ppm, both of which are also coupled to the proton at $\delta$ 4.67 ppm. Thus, $\delta$ 2.76 and $\delta$ 2.50 ppm proton peaks are those of the CH$_2$ (M) between indole and morpholine rings, and $\delta$ 4.67 ppm is assigned to the proton at the L position. Clearly, the splitting of the CH$_2$ (M) protons into a doublet reflects the chemical nonequivalence of the two protons caused by the neighboring chiral carbon of L. The remaining two aliphatic signals at $\delta$ 4.84 (IH) and $\delta$ 4.22 ppm (1H) are coupled to each other with a coupling constant of 11.6 Hz (clearly a geminal coupling), and they may be assigned to the two protons on CH$_2$ (K) bonded to an oxygen atom.

**HPLC-MS/MS Spectra of WIN55212-2 Metabolites.** Shown in Fig. 3E are the total ion chromatogram obtained from an incubation product mixture and the selected ion chromatograms for $m/z$ 425, 443, 461, and 477 all reconstructed from a single run. The rationale for the structural characterization of each proposed metabolite is given below in the order of increasing retention time.

The selected ion chromatogram for $m/z$ 477 is shown in Fig. 3A, in which three peaks are observed at retention times of 14.4, 15.9, and 17.0 min, respectively. The well resolved peaks indicate three metabolites, designated as M1, M2, and M3 that have the same molecular weight but differ in positions of hydroxylation. These metabolites show a common $[M + H]^+$ ion at $m/z$ 477, 50 amu higher than the $[M + H]^+$ ion of WIN55212-2. It is proposed that M1, M2, and M3 are likely the products of trihydroxylation of WIN55212-2. The product ion spectra of M1, M2, and M3 are identical, and the spectrum is shown in Fig. 4. A comparison with the product ion spectrum of WIN55212-2 and its proposed fragmentation pathways (Fig. 2) suggests that the fragment ions at $m/z$ 171 and 143 are related to the WIN55212-2 fragment ions at $m/z$ 155 and 127, respectively. The mass difference of 16 suggests the involvement of a hydroxyl group on the naphthyl moiety. The fragment ion at $m/z$ 189 indicates the presence of two hydroxyl groups on the moiety a. Finally, the fragment ion at $m/z$ 100 provides evidence that M1, M2, and M3 all have an unaltered moiety c, leaving the third hydroxyl group at moiety b. The results discussed above led us to propose the fragmentation pathways as illustrated in Fig. 4.

Figure 3B is the selected ion chromatogram extracted for the ion at $m/z$ 461, in which two distinct chromatographic peaks are observed, suggesting two isomeric metabolites (assigned as M4 and M5) with hydroxylation occurring at different positions. The daughter ion spectra for the protonated ions of M4 and M5 are shown in Fig. 5, in which identical fragment ions with varying relative abundances are
The protonated M4 and M5 ions, both at m/z 461, are 34 amu higher than the protonated WIN55212-2; thus, dihydrodiol products differing in sites of hydroxylation are proposed for M4 and M5. Again, fragment ions at m/z 189, 171, 143, and 115 suggest that the two hydroxyl groups are within moiety a. Also illustrated in Fig. 5 are the proposed fragmentation mechanisms for M4 and M5.

The structural elucidation of M4 and M5 is also assisted by NMR studies. From the 1H NMR spectra of M4 and M5 (Table 1), integration of the aromatic (and alkene, once dihydrodiol products are formed) region give only 8 proton signals (as compared with 10 found in WIN55212-2), indicating that hydroxylations have taken place in this region. Whereas the chemical shifts of protons H, I, and J remain in the aromatic region, two new signals emerge in the aliphatic region. A comparison with the NMR spectrum of WIN55212-2 shows that all original aliphatic protons are found intact in both M4 and M5, providing further evidence that the two metabolites have the intact moiety c, consistent with the mass spectral findings (i.e., presence of the fragment ion at m/z 100 in the product ion mass spectra of M4 and M5).

In the NMR spectra of M4, proton signals at δ 5.92 and δ 6.57 are coupled to each other with a coupling constant of 10 Hz (Table 2). The newly emerged aliphatic signals at δ 4.87 (1H, J = 10.4 Hz) and δ 4.56 (1H, J = 10.4 Hz) are also coupled to each other whereas the δ 4.56 proton shows additional coupling with proton signals at δ 5.92 and δ 6.57. These observations strongly suggest that dihydroxylation has taken place on two adjacent naphthyl carbons resulting in some loss in aromaticity. Moreover, the remaining two alkene protons (δ 5.92 and δ 6.57) should also be ortho to one another and adjacent to one of the new aliphatic signals (δ 4.56). Thus, the possible hydroxylation sites are either D and E or F and G. Because all chemical shifts of the aromatic protons on the indole ring of M4 remain unchanged compared with those of WIN55212-2, the only possible dihydroxylation sites are D and E. Had the hydroxylations taken place on F and G, the chemical shift of the aromatic proton H on the indole ring would have shifted to a lower field due to the effect of the OH group on the G carbon (the two bonds connecting the naphthalene ring, carboxyl group, and indole ring can both rotate freely).

As summarized in Table 2, the 1H NMR spectrum of M5 also contains two newly emerged aliphatic signals at δ 4.99 ppm (1H, J = 10.8 Hz) and δ 4.67 ppm (1H, J = 10.8 Hz) coupled to each other. The proton at δ 4.67 is also coupled to a single alkene proton signal at δ 6.22 ppm (J = 2.4 Hz). The coupling patterns suggest that, like in M4, the two hydroxylation sites are ortho to each other, and that unlike M4 only one alkene proton (δ 6.22 ppm) is observed, the coupling pattern of which indicates that it is adjacent to one of the new aliphatic proton signals (δ 4.67). Thus, the possible dihydroxylation positions are either A and B or B and C. In contrast to the case of M4, the chemical shift of the aromatic proton at H on the indole ring does show a shift to the lower field (from δ 6.43 to δ 7.30 ppm) as a result of interaction between the proton at H and the OH group at A. Based on the above information, the dihydroxylation positions are A and B for M5.

At least two metabolic products exhibit an [M + H]+ ion at m/z 443, identified as M6 and M7 (Fig. 3C). At 16 atomic mass units higher than the protonated ion of WIN55212-2, M6 and M7 are presumed to be two isomeric monohydroxylation products (OH substitution for H). As shown in Fig. 6B, the product-ion spectrum of [M7 + H]+ at m/z 443 yields fragment ions at m/z 171 and 143, which are analogous to fragment ions from WIN55212-2 at m/z 155 and 127, respectively, and indicate that monohydroxylation occurs on the naphthyl ring. It is confirmed by the observation of the intact moiety c at m/z 100. However, it is noted that the chromatographic peak repre-
taneous rearrangement to give a phenolic product (Daly et al., 1972). In the present study, it appears that M4 and M5 are not metabolic products from further hydroxylation of M7; rather, all three metabolites are proposed to originate from the common epoxide intermediates via either spontaneous rearrangement or the action of epoxide hydrolase.

At least three trihydroxylation metabolites, M1, M2, and M3 have been identified that are believed to be the products of further hydroxylation of M4 and M5. Whereas there are three peaks, the production ion spectra of which indicate that the third hydroxyl group must occur within moiety b containing the indole ring structure, it should be pointed out that there may be more than three isomeric trihydroxylated metabolites that couldn’t be resolved under the present HPLC conditions. Finally, these three metabolites are not considered to be products arising from further metabolism of the monohydroxylated M6 via an epoxide mechanism, because if that were the case, additional dihydroxylated metabolites ([M + H]+, m/z 459) should have been observed, resulting from spontaneous rearrangement of the epoxide intermediates of M6.

In summary, this study shows that WIN55212-2, a potent CB1 agonist that bears little structural similarity to classical cannabinoids, indeed undergoes distinct metabolic pathways. For example, the major microsomal metabolite of Δ1-tetrahydrocannabinol in rat is 7-hydroxy-Δ1-tetrahydrocannabinol, a product of alkyl hydroxylation (Harvey and Paton, 1984; Agurell et al., 1986). The major metabolic pathway of WIN55212-2, however, is predominantly via arene oxide formation to give dihydrodiols. Such metabolic differences may have implications on the extent to which cannabimimetic properties are retained or removed in WIN55212-2 metabolites. With two major metabolites isolated in pure form, further physiological studies are now possible to determine their agonistic activities.

References