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Evaluation of the excretion and metabolism of the new analgesic agent RWJ-22757 in male and female CR Wistar rats

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Abstract

The excretion and metabolism of (±)-*trans*-3-(2-bromophenyl)octahydroindolizine hydrochloride (RWJ-22757) have been investigated in male and female CR Wistar rats. Radiolabeled [^{14}C] RWJ-22757 was administered orally to each of the rats as a single 60 mg/kg suspension dose. Plasma (0–48 h), urine (0–168 h) and fecal (0–168 h) samples were collected and analyzed. There were no significant gender differences observed in the data. The estimated elimination half-life of the total radioactivity from plasma was 19 h while the estimated elimination half-life of RWJ-22757 was 15 h. Recoveries of total radioactivity in urine and feces were 58.4 ± 5.8 and 42.4 ± 6.3%, respectively. RWJ-22757 and a total of 11 metabolites were isolated in rat plasma, urine, and fecal extracts. The structures of four of these metabolites were tentatively identified. Unchanged RWJ-22757 accounted for < 4% of the dose in plasma and urine and 28% in feces; thus, indicating the drug was extensively metabolized and either not absorbed well or biliary excreted. Identified metabolites accounted for > 80% of the total radioactivity contained in the samples. The following pathways were used to describe the formation of the metabolites identified in rats: octahydroindolizine ring oxidation, phenyl hydroxylation, octahydroindolizine ring oxidation followed by ring opening to a carboxylic acid function and octahydroindolizine ring oxidation followed by ring opening and *N*-methylation.

Keywords: Excretion; Metabolism; Pharmacokinetics; (±)-*trans*-3-(2-Bromophenyl)octahydroindolizine hydrochloride; RWJ-22757

1. Introduction

(±)-*trans*-3-(2-Bromophenyl)octahydroindolizine hydrochloride (RWJ-22757) has been shown to produce significant antinociception in rodents via a non-opioid mechanism [1]. RWJ-22757 ap-

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pears to have a predominantly supraspinal site of action and the antinociception is mediated by activation of descending inhibitory systems (i.e. adrenergic and serotonergic systems) [2]. It has also been suggested that RWJ-22757 has no genotoxic risk to rodents [3]. Preliminary results on the metabolism of RWJ-22757 in rats have been reported [4]; however, no structural information was disclosed. The support of ADME modeling requires reliable data to validate computational methods. Thus, the present work describes the excretion and metabolism of [^{14}C] RWJ-22757 in male and female rats. We report the isolation of RWJ-22757 and a total of 11 metabolites in rats using column chromatography, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), mass spectrometry (MS), and derivatization techniques. The structures of four of these metabolites were tentatively identified.

2. Experimental

2.1. Materials

[^{14}C] RWJ-22757 and RWJ-22757 [5] were synthesized at the R.W. Johnson Pharmaceutical Research Institute (Spring House, PA, USA) and radiochemical and unlabeled purity were shown to be $>98\%$ by TLC and HPLC (Fig. 1). The specific activity of [^{14}C] RWJ-22757 was 12.0 μCi/mg. Using a water bath sonicator to ensure a uniform suspension, a suspension was prepared by combining 360 mg of [^{14}C] RWJ-22757, 360 mg of RWJ-22757, and 120 ml of water. The final concentration of the suspension was 6 mg/ml at 6 μCi/mg (or 36 μCi/ml). This concentration was selected to ensure that the rats received less than 100 μCi when dosed at 60 mg/kg. Glusulase®, a mixture of arylsulfatase and β-glucuronidase (1:4 v/v) from *Helix pomatia*, was obtained from Endo Laboratories Inc. (Wilmington, DE, USA). Diazald® (diazomethane reagent) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Biofluor®, used as a scintillation solution, was from New England Nuclear (Boston, MA, USA). HPLC grade solvents were from the Fisher Scientific Co. (Fair Lawn, NJ, USA) and glass-distilled solvents were purchased from Burdick and Jackson Laboratories Inc. (Muskegon, MI, USA). Other reagent chemicals were from commercial sources and used without further purification.

2.2. Rat study

Twenty-four male and 24 female CR Wistar rats (Charles River Breeding Labs, Kingston, NY, USA) weighing between 200 and 250 g were used in this study. Rats were housed one rat per cage in a rodent room where the lighting was on a 12 h light/12 h dark cycle. The rodent room was maintained between 18 and 26 °C and 30–70%

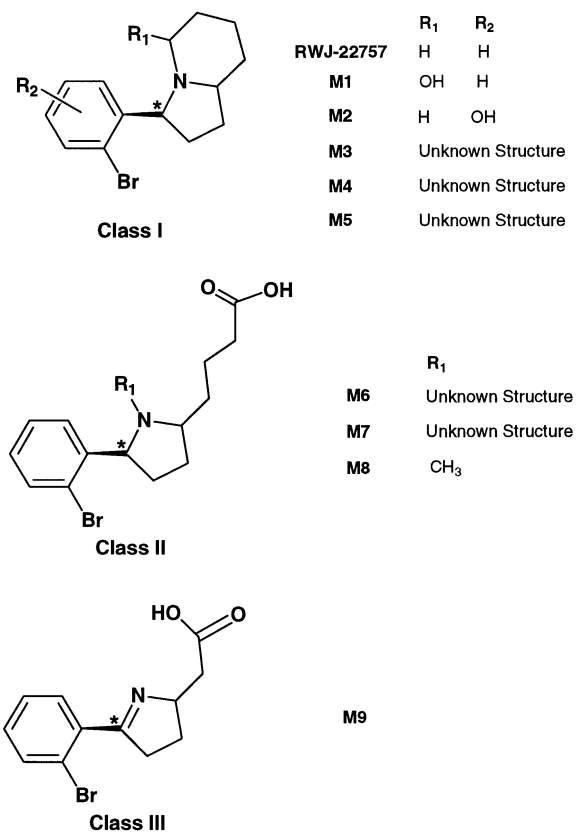


Fig. 1. Structures of RWJ-22757 and its metabolites identified in rats (M1, M2, M8, M9). The * indicates the position of the ^{14}C label.

humidity. Rats were fed laboratory rodent diet # 5001 (Purina Mills, St. Louis, MO, USA). The rats were fasted 18 h prior to dosing. Their normal feeding schedule was resumed 4 h after dosing. Water was available ad lib. at all times during the study. Depending on their weigh, the rats each received between 2 and 2.5 ml of RWJ-22757 (6 mg/ml or 36 μCi/ml) via a gavage needle. Thus, the rats received a single dose of 60 mg/kg of RWJ-22757. Rats were randomly assigned to two sample collection groups. The blood collection group contained 20 rats of each gender while the excretion group contained four rats of each gender. Four rats of each gender were used at each of the five time points. At pre-selected times after dosing, blood samples (ca. 8 ml) were collected by cardiac puncture into heparinized Vacutainers® (Becton-Dickinson & Co., Franklin Lakes, NJ, USA) after the rats were anesthetized with carbon dioxide gas. The blood samples were centrifuged for 15 min at $700 \times g$ at 15 °C to obtain plasma. Plasma samples were stored in glass screw-cap test tubes at about −20 °C until analysis. Urine and fecal sample extracts were collected in cooled plastic bottles at specified intervals for 7 days following dose administration. Urine and feces samples were stored at about −20 °C until purification and analysis.

2.3. Analytical

Thin-layer radiochemical analyses were performed using 5×20 cm silica gel GF plates (250 μm gel thickness; Analtech Inc., Newark, DE, USA). TLC plates were analyzed using a Radiochromatogram Imaging System (BID100) (Bioscan Inc., Washington, DC, USA). The HPLC used was a gradient liquid chromatograph (Beckman Instruments, Fullerton, CA, USA) connected to a radioactivity flow detector (IN/US Service Corp., Fairfield, NJ, USA). LiChrosorb RP-18 MPLC guard and analytical columns (10 μm, 130×4.6 mm i.d.) were used for sample analysis (Brownlee Labs. Inc., Santa Clara, CA, USA). Electron ionization (EI) mass spectra were obtained on a VG7070E (VG Micromass, Manchester, UK) where the operating conditions were as follows: source temperature at 200 °C; ionization potential at 70 eV; multiplier voltage at 1.8 keV; electrometer setting at 10^{-7} A/V; mass ranges 18–1000 Da. Direct Chemical ionization (DCI) mass spectra were obtained utilizing a VG7070E (VG Micromass, Manchester, UK) where the operating conditions for the spectrometer were as follows: source temperature at 200 °C; ionization potential at 70 eV; multiplier voltage at 1.8 keV; electrometer setting at 10^{-7} A/V; reagent gas was methane; mass ranges 100–1000 Da. Proton (^{1}H) NMR spectra were obtained on a Bruker AM-400 Fourier transform spectrometer equipped with an Aspect 3000 data system. Typical ^{1}H NMR spectra were obtained using a pulse width of 3 μs (30°); a 1 s recycling delay; 100 transients processed with a 0.1 Hz exponential weighting function and a spectral width of 8064 Hz over 32,768 data points, resulting in a digital resolution of 0.5 Hz per point. The experimental temperature used for NMR data collection was 24 ± 1 °C. The estimated amount of metabolites was between 50 and 100 μg with a purity between 70 and 90%. The compounds were dissolved in deuterated acetonitrile-d_3 in 5 mm tubes. The chemical shifts (δ) measured in parts-per-million (ppm) were referenced internally to trichloromethane.

2.4. Radioactivity determination

Duplicate aliquots (0.1 ml) of plasma and triplicate aliquots of urine and fecal extracts (0.1–1.0 ml) were assayed for total radioactivity in 10 ml of Biofluor® by liquid scintillation spectrometry. Plasma, urine, and fecal extract pools were prepared for metabolite isolation and identification. The total radioactivity contained in each sample pool, before and after purification, was also determined by direct liquid scintillation counting. Feces residue (300 mg per aliquot) was analyzed in triplicate by combustion. [^{14}C]-labeled carbon dioxide formed was trapped with a PerafluorV/Carbosorb-CO_2 (3:2, v/v) mixture and analyzed by a Beckman 3801 Liquid Scintillation Counting System purchased from Beckman Instruments (Fullerton, CA, USA).

2.5. Chromatography

Selected samples were pooled and analyzed by TLC and HPLC for biotransformation profiling. Prior to chromatographic analysis, it was necessary to purify most of the samples. The plasma (1–48 h) samples were extracted with ethyl acetate, evaporated to dryness and reconstituted with methanol. The radioactivity profiles of the plasma samples were comparable before and after purification. The percentage of radioactivity recovered following purification of plasma samples was 86%. The urine samples (0–48 h) were purified using two methods. First, the same procedure described for the plasma samples was used and the percentage of radioactivity recovered following purification was 60%. Second, the urine samples were hydrolyzed with 1% Glusulase® in 1 M acetate buffer, extracted with ethyl acetate, evaporated to dryness and reconstituted with methanol. The percentage of radioactivity recovered following purification of urine samples using this method was 61%. The rat fecal extract samples (0–48 h) were evaporated to dryness and reconstituted with methanol. The percentage of radioactivity recovered following purification was 100%.

TLC analysis of the samples was performed using the following basic solvent system: methylene chloride/methanol/ammonium hydroxide (90/10/1, v/v/v) and a neutral solvent system: methylene chloride/methanol (95/5, v/v). Each TLC plate was developed to a height of 14 cm above the spot or band where the sample was originally applied. Each sample was co-chromatographed with RWJ-22757 as a reference compound, which was visualized with short wavelength ultraviolet (UV) light after each plate was air-dried. Each plate was also analyzed utilizing a RTLC scanner radiochromatogram imaging system (Radiomatic Instrument and Chemical Co., Tampa, FL, USA).

HPLC analysis of each sample pool (50–100 μl injected) was performed using a gradient elution method at 254 nm using the following conditions. Solvent A was water containing 0.5% triethylamine (TEA) buffered with phosphoric acid to pH 3.3, while solvent B was acetonitrile containing the same percentage of buffered TEA. The gradient program was carried out from 0 to 30% B in 20 min, 30 to 80% B in 10 min, and 80 to 100% B in 4 min at a flow rate of 2 ml/min. All [^{14}C] tracings were obtained using an on-line radioactivity flow detector. The limit of detection for the parent in plasma was approximately 5 ng-equiv./ml.

2.6. Metabolite isolation and identification

Following sample purification, individual metabolites were isolated using column chromatography and HPLC peak collection methods. Unresolved column fractionation residues or HPLC metabolite peaks were further separated using TLC zone scraping methods. The TLC and HPLC procedures were identical to those outlined above. All metabolites were characterized by either electron ionization (EI) or desorption chemical ionization using methane as the reagent gas (DCI-CH_4) mass spectrometry. Proton (^{1}H) NMR spectra were obtained for some of the metabolites. Specifically, the TLC R_f values were obtained for RWJ-22757 and all isolated metabolites. The HPLC R_t values were obtained for RWJ-22757 and all isolated metabolites except for M3, M4 and M11. NMR data was collected for RWJ-22757 and M1, M2, M8 and M9. MS (EI) data was collected for RWJ-22757 and all isolated metabolites, while MS (DCI) data was only collected for M1, M2, M4, M8, M9 and M10. M5–M10 were derivatized using ethereal diazomethane to form methyl ethers or esters. These derivatives (M5–M10) were further analyzed using MS while NMR data was only collected for M8 and M9.

2.7. Pharmacokinetic analysis

The plasma concentration versus time data of the total radioactivity for RWJ-26899 was analyzed by a noncompartmental method [6]. The highest observed plasma concentration and its corresponding sampling time were defined as C_{max} and T_{max}, respectively. The area under the curve (AUC) from time zero to the last sampling time point was calculated by the trapezoidal method. The terminal half-life ($t_{1/2}$) was estimated by a

Table 1
Summary of the plasma concentrations and pharmacokinetic parameters of total radioactivity (μg-equiv./ml) and unchanged [^{14}C] RWJ-22757 in male and female CR Wistar rats after a single oral solution dose of [^{14}C] RWJ-22757 (60 mg/kg)

Time (h)	Males ($N=4$)	Females ($N=4$)	Mean ± SD[a] ($N=8$)
Total radioactivity			
1.0	8.9 ± 3.2	6.8 ± 2.0	7.8 ± 2.7
3.0	10.7 ± 4.4	11.5 ± 4.8	11.1 ± 4.3
8.0	14.7 ± 5.2	15.2 ± 4.4	15.0 ± 4.5
24.0	11.0 ± 4.5	15.1 ± 6.5	13.1 ± 5.6
48.0	4.2 ± 2.8	6.6 ± 4.0	5.4 ± 3.5
C_{max}[b] (μg-equiv./ml)	14.7 ± 5.2	15.2 ± 4.4	15.0 ± 4.5
T_{max} (h)[c]	8.0	8.0	8.0
AUC[d] (0–48 h) (μg-equiv.-h/ml)	475.5 ± 62.3	591.4 ± 71.1	535.0 ± 64.0
$t_{1/2}$ (h)[e]	17	20	19
Unchanged [^{14}C] RWJ-22757			
Time (h)	Males ($N=4$)	Females ($N=4$)	Mean ± SD[a] ($N=8$)
1.0	0.17 ± 0.05	1.1 ± 0.27	0.64 ± 0.52
3.0	0.16 ± 0.01	0.43 ± 0.01	0.30 ± 0.09
8.0	0.10 ± 0.06	0.17 ± 0.01	0.14 ± 0.05
24.0	0.09 ± 0.01	0.14 ± 0.03	0.11 ± 0.04
48.0	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
C_{max}[b] (μg-equiv./ml)	0.17 ± 0.05	1.1 ± 0.27	0.64 ± 0.52
T_{max} (h)[c]	1.0	1.0	1.0
AUC[d] (0–48 h) (μg-equiv.-h/ml)	4.0 ± 1.2	8.2 ± 3.3	6.1 ± 2.2
$t_{1/2}$ (h)[e]	17	13	15

[a] SD denotes one standard deviation.
[b] Due to the large gap in the plasma data, the C_{max} values may have significantly more variability than indicated by the SD values.
[c] Due to the large gap in the plasma data, the T_{max} values are only estimations.
[d] Since $P>0.05$ between males and females, the difference between the means is not significant.
[e] Estimated elimination half-life based on last two time-points.

linear regression analysis of the final two data points of the terminal linear segment of the log plasma concentration versus time curve.

2.8. Statistics

Values are given as means ± standard deviations (SD). Statistical analysis of the experimental results was performed by comparing the means of male and female assay groups by the pooled *t*-test. Unpaired two-tail *P* values at a significance level of $P=0.05$ was used to reject the null hypothesis. The *F*-test was used for the comparison of the SD values (Graph Pad Prism® Version 2.01; Graph Pad Software Inc., San Diego, CA, USA).

3. Results and discussion

The total radioactivity and unchanged [^{14}C] RWJ-22757 was measured for each plasma sample obtained from the study (Table 1). No gender differences were found in the plasma data based on application of the *t*-test; therefore, mean values were calculated from the male and female rat studies. The mean C_{max} for the total radioactivity was 15.0 ± 4.5 μg-equiv./ml (%CV 30%), which occurred at a T_{max} of 8.0 h. The C_{max} for unchanged RWJ-22757 was 0.64 ± 0.52 μg-equiv./ml (%CV 81%) which occurred at a T_{max} of 1.0 h. Due to the large gap in the plasma data, the C_{max} values may have significantly more variability

than indicated by the SD values. The T_{max} values are only estimations due to the same reason. The mean $AUC_{0-48\ h}$ of total radioactivity in rat plasma was 535.0 ± 64.0 μg-equiv.-h/ml (%CV 12%) while the mean $AUC_{0-48\ h}$ of RWJ-22757 was 6.1 ± 2.2 μg-equiv.-h/ml (%CV 35%). The estimated elimination half-life of the total radioactivity was 19 h while the estimated elimination half-life of RWJ-22757 was estimated to be 15 h. These estimated half-lives are based only on the last two plasma time points. The recovery of total radioactivity in urine (0–168 h) and feces (0–168 h) was determined for mass balance considerations (Table 2). Again, no statistically significant gender differences in the cumulative urinary and fecal excretion were identified based on application of the *t*-test. The cumulative results were 58.4 ± 5.8 and 42.4 ± 6.3% of the dose excreted in the urine and the feces, respectively.

Unchanged RWJ-22757 and a total of 11 metabolites (M1–M11) were isolated and four metabolites (M1, M2, M8 and M9) were tentatively identified. The structures of RWJ-22757 and these four metabolite structures are presented in Fig. 1. The TLC R_f values, HPLC R_t values, proton (^{1}H) NMR chemical shifts (ppm) and MS fragments (m/z) for RWJ-22757 and the isolated metabolites (M1, M2, M8, M9) are presented in Table 3. Due to lack of NMR data, M3–M7, M10 and M11 were not structurally identified although their MS data is given in Table 3. The percent of administered dose excreted in urine and fecal extract and the percent of sample represented by each metabolite in plasma, urine and fecal extract pools are summarized in (Table 4). For plasma pools, the numbers are expressed as percent of total radioactivity in the sample, since the percent of dose values cannot be calculated. The percent values were obtained by peak area integration of radiochromatograms. In general, these values had a coefficient of variation and accuracy within 15% if the values were greater than 5% of the sample. Therefore, metabolites accounting for less than 5% of the radioactivity in a sample are represented by a plus. The total percent of radioactivity in each sample pool represented by identified metabolites are also listed at the bottom of Table 4. Approximately 95% of the radioactivity in plasma extracts, approximately 70% in the urine extracts and approximately 73% in the fecal extracts has been accounted for by identified metabolites.

Table 2
Percent of total radioactive dose recovered in urine and feces in male and female CR Wistar rats after a single oral solution dose of [^{14}C] labeled RWJ-26899 (60 mg/kg)

Time (days)	Males ($N=4$)	Females ($N=4$)	Mean ± SD[a] ($N=8$)
Urine			
1	34.0 ± 7.3	30.1 ± 5.4	32.0 ± 6.3
2	16.0 ± 2.6	23.4 ± 7.5	19.7 ± 6.5
3	3.3 ± 0.7	4.6 ± 2.0	3.9 ± 1.6
4	1.1 ± 0.3	1.7 ± 0.2	1.4 ± 0.4
5–7	1.3 ± 0.5	1.5 ± 0.7	1.4 ± 0.5
0–7	55.6 ± 4.9	61.2 ± 5.8	58.4 ± 5.8
Feces			
1[b]	18.8 ± 5.1	8.6 ± 3.4	13.7 ± 6.8
2	19.8 ± 4.5	22.9 ± 8.0	21.3 ± 6.3
3	4.7 ± 1.1	4.6 ± 2.9	4.7 ± 2.0
4	1.5 ± 0.5	1.5 ± 0.3	1.5 ± 0.4
5–7	1.3 ± 0.5	1.2 ± 0.3	1.2 ± 0.4
0–7	46.0 ± 5.4	38.7 ± 5.3	42.4 ± 6.3
Total Recovery	101.6 ± 0.8	99.9 ± 2.0	100.8 ± 1.7

[a] SD denotes one standard deviation.

[b] The difference between males and females for this time point was significant at $P<0.05$. All other time points are not significant at $P>0.05$.

RWJ-22757 metabolites can be categorized into three classes (Fig. 1). Class I metabolites (M1 and M2) are RWJ-22757 analogs, class II metabolite (M8) is 2-pyrrolidinepropanoic acid analogs and class III metabolite (M9) is 2-*H*-pyrrole-2-acetic acid analogs. Based on mass spectrometry electron ionization (i.e. MS (EI)) fragmentation data (Fig. 2), the remaining metabolites were assigned to these families; however, their structures could not be assigned due to lack of NMR data. The M11 metabolite could not be assigned to any of these families.

Unchanged RWJ-22757 was isolated from plasma and feces and detected in urine by TLC and HPLC Table 4. It was identified on the basis of TLC, HPLC, NMR and MS data Table 3 in comparison with those data obtained from authentic RWJ-22757 [5]. The MS (EI) analysis of

Table 3
TLC R_f values, HPLC R_t values, proton (^{1}H) NMR chemical shifts and MS fragments of RWJ-22757 and its metabolites from rat samples

Metabolite	TLC[a] R_f value (min)	HPLC R_t value (min)	^{1}H NMR[b] chemical shifts (δ) ppm	MS [EI and DCI (CH_4)] m/z (% relative intensity)
RWJ-22757	0.90–0.96	16.2–16.8	7.5–7.6 (d, H), 7.4–7.5 (m, H), 7.2–7.3 (d, H), 7.0–7.1 (m, H), 3.6 (t, H, Ar–C*H*), 2.8 (m, H, N–C*H*), 2.3 (m, H, N–CH_2), 2.1 (m, H, N–CH_2), 1.7–1.9 (m, 4H), and 1.2–1.6 (m, 6H)	279 [M^+, (Br^{79}), 14%, EI], 278 [$M-H^+$, (Br^{79}), 18%, EI], 250 [M^+, (Br^{79}), $-C_2H_5$, 14%, EI] and 124 [100%, EI]
M1	0.46–0.54	12.3–12.8	7.5–7.6 (d, H), 7.4–7.5 (m, H), 7.2–7.3 (d, H), 7.0–7.1 (m, H), 3.8 (m, H, HO–C*H*–N), 3.7 (t, H, Ar–C*H*), 3.0 (m, H, N–C*H*), and 1.2–1.9 (m, 10H)	295 [M^+, (Br^{79}), 3%, EI], 294 [$M-H^+$, (Br^{79}), 20%, EI], 296 [$M+H^+$, (Br^{79}), 20%, EI], 266 [(Br^{79}), 10%, EI] and 140 [100%, EI]. 296 [MH^+ (Br^{79}), 70%, DCI], 294 [$M-H^+$ (Br^{79}), 40%, DCI], 278 [MH^+ (Br^{79}) $-H_2O$, 80%, DCI] and 140 [100%, DCI]
M2	0.22–0.28	10.2–11.0	7.5–7.6 (d, H), 7.0–7.1 (s, H), 6.8–6.9 (d, H), 3.6 (t, H, Ar–C*H*), 2.8 (m, H, N–C*H*), 2.3 (m, H, N–CH_2), 2.1 (m, H, N–CH_2), and 1.2–1.9 (m, 10H)	295 [M^+, (Br^{79}), 60%, EI], 266 [M^+ (Br^{79}), 10%] and 124 [100%, EI]. 296 [MH^+ (Br^{79}), 60%, DCI], 294 [M–H^+ (Br^{79}), 40%, DCI], and 124 [100%, DCI]. Diazomethane treated: 309 [M^+, (Br^{79}), 40%, EI,], and 124 [100%, EI]
M3	0.61–0.67	ND[c]		295 [M^+, (Br^{79}), 40%, EI] and 140 [100%, EI]
M4	0.41–0.47	ND		295 [M^+, (Br^{79}), 40%, EI], 294 [$M-H^+$, (Br^{79}), 30%, EI], 278 [MH^+, (Br^{79}), $-H_2O$, 10%, EI] and 140 [100%, EI]. 296 [MH^+, (Br^{79}), 40%, DCI], 294 [$M-H^+$, (Br^{79}), 30%, DCI], 278 [MH^+, (Br^{79}), $-H_2O$, 20%, DCI] and 140 [100%, DCI]
M5	0.15–0.21	8.0–9.0		Methyl derivative: 325 [M^+, (Br^{79}), 90%, EI,], 324 [$M-H^+$, (Br^{79}), 100%, EI], 308 [MH^+, (Br^{79}), $-H_2O$, 10%, EI] and 140 [100%, EI]
M6	0.00–0.05	20.8–21.4		Methyl derivative: 325 [M^+, (Br^{79}), 20%, EI], 294 [(Br^{79}), 10%, EI], 252 [(Br^{79}), 10%, EI], 238 [(Br^{79}), 40%, EI], and 170 [25%, EI]
M7	0.00–0.05	18.5–19.1		Methyl derivative: 355 [M^+, (Br^{79}), 20%, EI], 354 [M^+-H, (Br^{79}), 20%, EI], 324 [(Br^{79}), 100%, EI], 282 [(Br^{79}), 15%, EI], 268 [(Br^{79}), 15%, EI], and 170 [25%, EI]
M8	0.00–0.05	22.7–23.3	7.5–7.6 (d, H), 7.4–7.3 (m, H), 7.2–7.3 (d, H), 7.0–7.1 (m, H), 3.7 (s, 3H, O–CH_3), and 3.6 (s, 3H, N–CH_3)	Methyl derivative: 339 [M^+, (Br^{79}), 50%, EI], 308 [(Br^{79}), 30%, EI], 266 [(Br^{79}), 40%, EI], 252 [(Br^{79}), 50%, EI], and 238 [(Br^{79}), 40%, EI]. 340 [MH^+, (Br^{79}), 40%, DCI], and 308 [(Br^{79}), 100%, DCI]
M9	0.00–0.05	14.4–15.0	7.5–7.6 (d, H), 7.4–7.3 (m, H), 7.2–7.3 (d, H), 7.0–7.1 (m, H), 4.6 (m, H, N–C*H*), 3.7 (s, 3H, O–CH_3) and 2.0–1.1 (m, 6H)	Methyl derivative: 295 [M^+, (Br^{79}), 45%, EI], 236 [(Br^{79}), 100%, EI], 222 [(Br^{79}), 30%, EI], and 208 [(Br^{79}), 50%, EI]. 296 [MH^+, (Br^{79}), 40%, DCI]
M10	0.00–0.1	15.1–15.7		Methyl derivative: 309 [M^+, (Br^{79}), 35%, EI], and 310 [MH^+, (Br^{79}), 45%, DCI]
M11	0.82–0.88	ND[b]		289 [M^+, (Br^{79}), 35%, EI]

[a] Basic solvent system was used.
[b] s, singlet; d, doublet; t, triplet; q, quartet; and m, multiplet.
[c] ND denotes not detected.

Table 4
Percent of total ^{14}C recovered and percent of dose represented by RWJ-22757 and its metabolites in each sample

Pooled Sample	Plasma[a] (0–24 h)	Urine[b] (0–48 h)	Fecal extract[c](0–48 h)
(%Dose) sample content		52.1	13.9
RWJ-22757	+	+	28
M1	+	5	5
M2		+	5
M3			6
M4			+
M5		+	
M6		+	
M7		5	
M8		5	13
M9	77	10	13
M10		+	
M11	+		
(% Sample) total identified	90	47	75

Males and females samples were pooled. The symbol + represents $<5\%$ of total ^{14}C in sample.
[a] The recovery of RA following sample preparation was 86%. The missing RA may be due to volatile metabolites.
[b] The recovery of RA following sample preparation was 60–61%. The missing RA may be due to volatile metabolites.
[c] The recovery of RA following sample preparation was 100%.

unchanged RWJ-22757 provided a molecular cation at m/z 279 (14% relative intensity) with a characteristic bromine isotope pattern. The MS (EI) spectra also contained an informative fragment cation at m/z 124 (100%) which did not contain any bromine atoms. This fragment is interpreted in Fig. 2 and can be used to approximately locate oxidation sites for the other metabolites. The ^1H NMR spectrum of isolated RWJ-22757 and the reference compound are shown in Fig. 3. Based on coupling constants and chemical shift arguments the spectrum was assigned. The characteristic chemical shifts (ppm) between δ 7.0–7.6 (m, 4H) are the aromatic protons 3′–6′. The triplet resonance at δ 3.6 (t, H), which integrated for one proton, was assigned to position 3 (phenyl–C*H*–N proton). The multiplet resonance at δ 2.8 (m, H), which integrated for one proton, was assigned to position 8a (N–C*H*–CH_2 proton). The multiplet resonances at δ 2.3 and 2.1 (m, H), which integrated for two protons, were assigned to position 5 (N–*CH_2* protons). The resonances between δ 1.2 and 1.9 (m, 10H) were assigned to the aliphatic ring protons 1, 2 and 6–8. Fecal extracts contained 28% of the sample of RWJ-22757, which either indicated that the drug was not well absorbed or was biliary excreted. However, a large number of metabolites in significant amounts Table 4 were detected in the fecal extract and thus, indicates that a biliary excretion pathway is present.

Metabolite M1 was isolated from urine and fecal extracts. The identification of M1 was performed on the basis of the MS and NMR data. The MS (EI) spectrum of M1 is shown in Fig. 4. The MS (EI) analysis of the isolated metabolite gave a molecular cation (M^+) at m/z 295, a protonated molecular cation ($M^+ + 1$) at m/z 296 and a ($M^+ - 1$) at m/z 294 all with characteristic bromine isotope patterns. This cation was 16 m/z greater than the molecular cation of RWJ-22757 (i.e. m/z 279). A prominent cation at m/z 140 (i.e. $124 + 16$) was observed in the MS (EI) and MS (DCI) data and suggested M1 was mono-oxygenated on the octahydrondolizine ring of RWJ-22757 (Figs. 1 and 2). ^1H NMR data revealed identical resonance patterns of aromatic protons as those of RWJ-22757. The position of the hy-

droxy group on the octahydrondolizine moiety was tentatively assigned to the five position based on fact that the N–CH_2 protons were not present in the ^{1}H NMR spectrum of M1. A new resonance at δ 3.8 ppm was present in the NMR spectrum of M1 that was assigned as the N–C*H*–OH proton. The MS and ^{1}H NMR data suggested M1 was 3-(2′-bromophenyl)octahydroindolizin-5-ol.

Metabolite M2 was detected in urine and fecal extracts Table 4. The MS (EI) spectrum of the isolated metabolite contained an intense molecular cation at m/z 295 (i.e. 16 amu greater than RWJ-22757) with a characteristic bromine isotope pattern. The protonated molecular cation (MH^+) at m/z 296 was also observed in the MS (DCI) data. A prominent cation at m/z 124 was observed in the MS (EI) and MS (DCI) data and suggested M2 was mono-oxygenated on the phenyl ring of RWJ-22757 (Figs. 1 and 2). M2 was further derivatized to a mono-methyl ether using diazomethane. The MS (EI) spectrum of this derivatized metabolite contained a molecular cation at m/z 309 (i.e. 295 + CH_2) with a characteristic bromine isotope pattern and a prominent cation at m/z 124. The position of the hydroxy group on the aromatic ring was tentatively assigned to either the 4′ or 5′ position based on the fact that only three aromatic protons were present in the NMR spectrum of M2 and that one of these protons was not coupled. The singlet resonance at δ 7.0–7.1 ppm and its chemical shift relative to the aromatic protons (i.e. ABX pat-

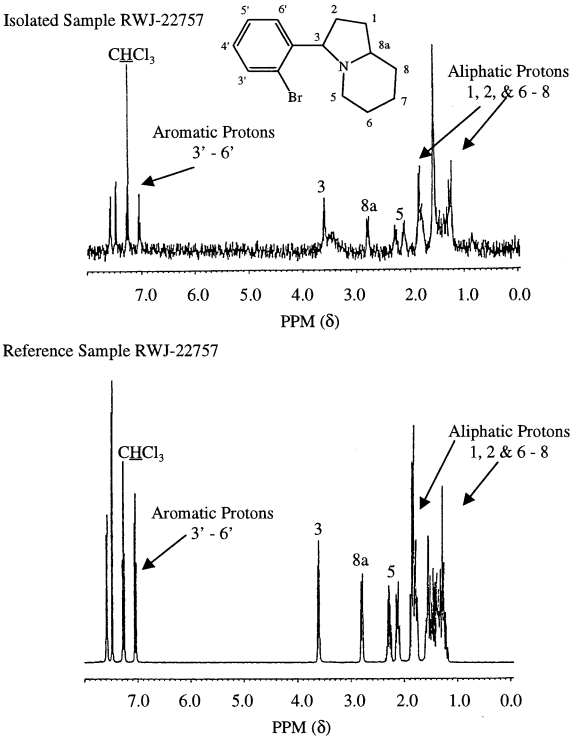


Fig. 3. ^{1}H NMR spectra of the isolated RWJ-22757 and the reference compound.

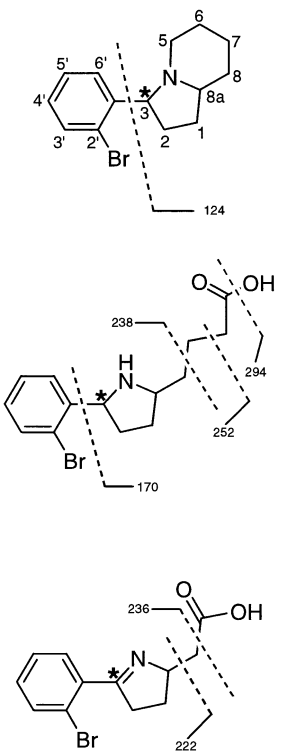


Fig. 2. Sites of MS fragment ions and numbering scheme in RWJ-22757, 2-pyrrolidinepropanoic acid and the 2-pyrrole-2-acetic acid analogs. The * indicates the position of the ^{14}C label.

tern) suggested an oxygen atom was present at the 4′ position on the aromatic ring; however, this could not be confirmed. The MS and ^{1}H NMR spectra suggested that M2 was 3′-bromo-4′-(octahydroindolizin-3-yl)phenol or 3′-bromo-5′-(octahydroindolizin-3-yl)phenol.

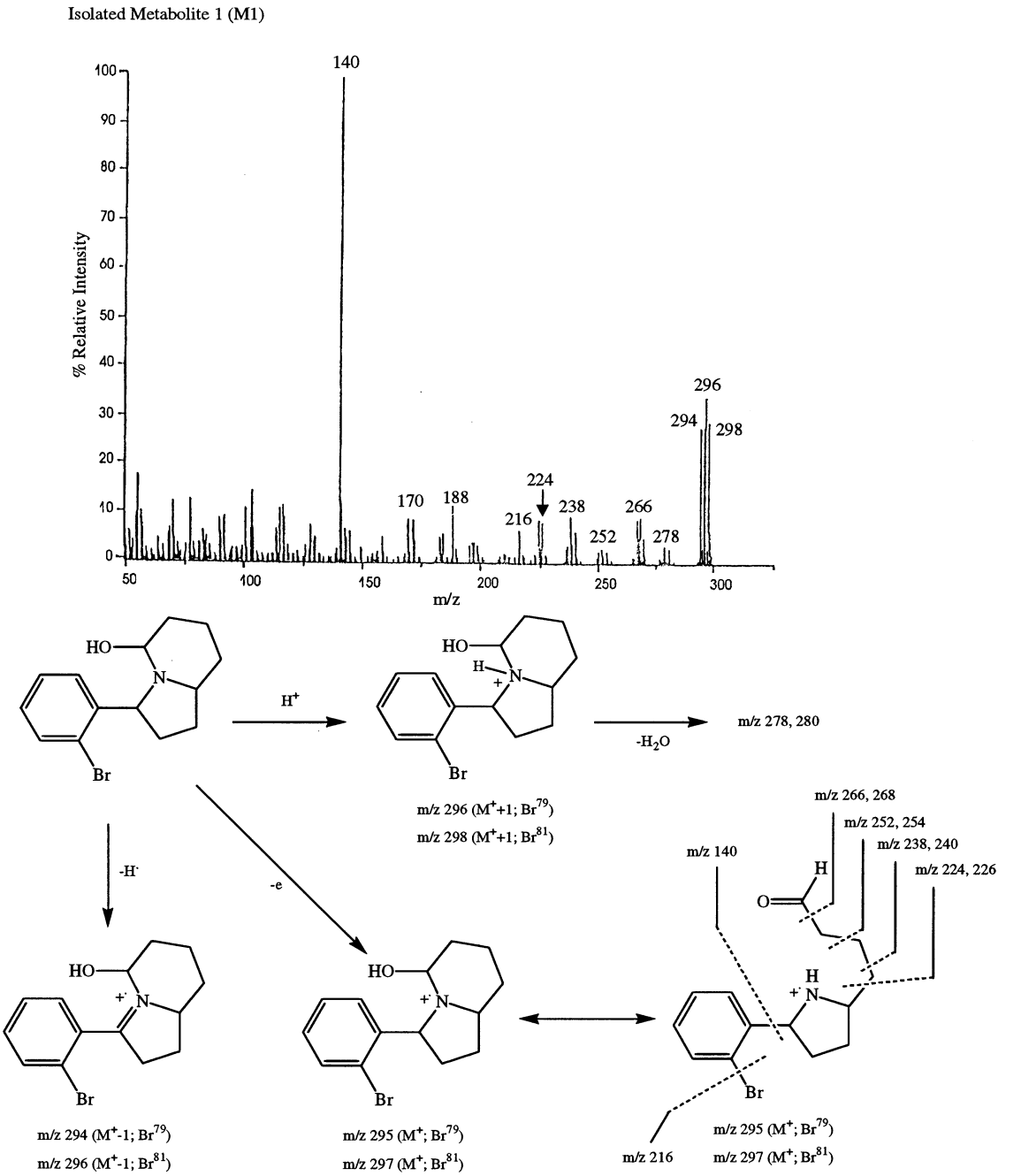


Fig. 4. Electron ionization mass spectrum of M1 with a proposed fragmentation scheme.

Metabolites M3 and M4 were isolated by column chromatography from fecal samples. The structures of M3 and M4 were tentatively elucidated on the basis of MS data only and the results from M1. The MS (EI) spectrum of both M3 and M4 showed a molecular cation at m/z 295 (i.e. RWJ-22757 plus 16 amu) with a characteristic bromine isotope pattern along with a fragment at m/z 140. These data along with the structural assignment of M1 suggested that M3 and M4 were mono-oxygenated at either the 1–3, 8a or 6–8 positions on the heterocyclic moiety of RWJ-22757 (Fig. 2). Further structural confirmation of these metabolites was terminated because of insufficient material for NMR analysis.

Metabolite M5 was isolated in small quantities from urine. After derivatizing the sample with diazomethane, MS tentatively identified M5. The MS (EI) data of the derivatized compound exhibited an intense molecular cation at m/z 325 (i.e. RWJ-22757 plus 46 amu) and a dehydrated cation at m/z 308 (i.e. MH^+–H_2O) both with a characteristic bromine isotope pattern. A prominent fragment cation observed at m/z 140 (i.e. 16 amu greater than m/z 124 in RWJ-22757), which did not contain any bromine atoms, suggested a hydroxyl group was attached at the heterocyclic rings of RWJ-22757 and a methoxy group was attached at the aromatic ring. Further structural confirmation of this metabolite was terminated because of insufficient material for NMR analysis.

Metabolite M6 was isolated in minor amounts from urine and was further derivatized to a methyl ester. The structure of M6 was tentatively elucidated on the basis of MS data only. The MS (EI) data of the derivatized compound exhibited a molecular cation at m/z 325 (i.e. RWJ-22757 plus 46 amu) with a characteristic bromine isotope pattern. Four prominent fragment cations with a characteristic bromine isotope pattern were observed at m/z 294 (i.e. M^+–OCH_3), 252, 238 and 170. These fragments suggested that the six-membered ring of the heterocyclic moiety had oxidized to form M1 a carbinolamine and further oxidation subsequently opened up M1 to form a carboxylic acid.

Metabolite M7 was observed in urine samples and was further derivatized as a methyl derivative. The MS (EI) data of the derivatized compound exhibited a molecular cation at m/z 355 (i.e. RWJ-22757 plus 76 amu) with a characteristic bromine isotope pattern. Four prominent fragment cations, each with a characteristic bromine isotope pattern, were observed at m/z 324 (i.e. M^+–OCH_3), 282 (i.e. M^+–$CH_2CO_2CH_3$), 268 (i.e. M^+–$CH_2CH_2CO_2CH_3$), and 170. These fragments suggested that the six-membered ring of the heterocyclic moiety had oxidized and opened up to a carboxylic acid (Fig. 2). Based on these results and the data from M6, the diazomethane treatment of M7 lead to the formation of a methyl phenyl ether and a methyl ester derivative (Figs. 1 and 2). The MS (EI) data tentatively indicated M7 to be a mono-oxygenated metabolite of M6 with a hydroxyl group on the phenyl ring.

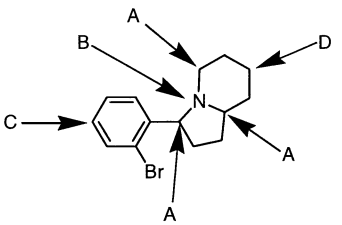
Metabolite M8 was obtained in significant amounts from urine and fecal extracts after HPLC separation and TLC purification. The sample was further derivatized to a methyl ester. The MS (EI) data of the derivatized compound exhibited a M^+ at m/z 339 (i.e. RWJ-22757 plus 60 amu) with a characteristic bromine isotope pattern. The MH^+ at m/z 340 was also observed in the MS (DCI) data. Three prominent fragment cations, each with a characteristic bromine isotope pattern, were observed at m/z 308 (i.e. M^+–OCH_3), 266 (i.e. M^+–$CH_2CO_2CH_3$), and 252 (i.e. M^+–$CH_2CH_2CO_2CH_3$). These fragments suggested that the six-membered ring of the heterocyclic moiety had oxidized and opened up to a methyl ester (or the carboxylic acid for the original metabolite). ^1H NMR data of the metabolite and the derivatized metabolite were compared. Both samples revealed identical resonance patterns of aromatic protons as those of RWJ-22757. However, the M8 showed a singlet methyl resonance at δ 3.6 while the derivatized metabolite showed two singlet methyl resonances at δ 3.7 and 3.6 ppm. These resonances were interpreted to arise from an *O*- and *N*-methyl group. Combining the MS (EI) and NMR data suggested M8 to be an *N*-methyl metabolite.

Metabolite M9 was isolated by a combination of chromatographies (column, HPLC, and TLC)

from plasma, urine and feces. The isolated sample was methylated using diazomethane and MS (EI) and ^{1}H NMR were used to analyze the product. The MS (EI) data of the derivatized compound exhibited a M^+ at m/z 295 (i.e. RWJ-22757 plus 16 amu) with a characteristic bromine isotope pattern. The MH^+ at m/z 296 was also observed in the MS (DCI) data. Two prominent fragment cations each with a characteristic bromine isotope pattern were observed at m/z 236 (i.e. $M^+ - CO_2CH_3$), and 222 (i.e. $M^+ - CH_2CO_2CH_3$). These fragments are interpreted in Fig. 2 and suggested that M9 was a 2-*H*-pyrrole-2-acetic acid analog. Comparing the ^{1}H NMR data of RWJ-22757, M9 and the derivatized metabolite revealed identical resonance patterns of aromatic protons and a methyl ester group. However, a methine proton at δ 4.6 was observed in the spectra of M9 and was interpreted to arise from a C=N–CH group. Combining these data suggested M9 to be 5-(2-bromophenyl)-3,4-dihydro-2-*H*-pyrrole-2-acetic acid (Fig. 1).

Metabolites M10 and M11 were found in trace amounts in urine and feces, respectively. The methyl ester derivative of M10 was prepared and analyzed. The MS (EI) data of the derivatized compound exhibited a M^+ at m/z 309 with a characteristic bromine isotope pattern. The MH^+ at m/z 310 was also observed in the MS (DCI) data of M10. The MS (EI) data of M11 exhibited a M^+ at m/z 289 with a characteristic bromine isotope pattern. No prominent fragment cations were observed for M10 and M11. Further structural refinement for M10 and M11 were terminated because of insufficient material for NMR analysis.

Four proposed pathways were used to describe the formation of the metabolites in rats: (A) octahydroindolizine ring oxidation; (B) octahydroindolizine ring opening followed by *N*-methylation; (C) phenyl hydroxylation; and (D) β-oxidation of the octahydroindolizine ring followed by ring opening. The proposed metabolic sites for the formation of metabolites are depicted in Fig. 5 and the biotransformations are shown in Fig. 1. The most dominant pathway of RWJ-22757 in rat appeared to be pathway (A) oxidation at the 5 position of the octahydroindolizine ring to form



Sites of Reaction	Metabolic Reaction	Rat
A	Octahydroindolizine ring oxidation	Major
B	Ring opening/N-methylation	Minor
C	Phenyl hydroxylation	Minor
D	β–Oxidation/ring opening	Major

Fig. 5. Sites of oxidation and corresponding metabolic reactions involving RWJ-22757 in rats.

3-(2-bromophenyl)octahydroindolizin-5-ol. This was followed by ring-opening oxidation followed by β-oxidation (M9) and *N*-methylation (M8). Metabolites M1 and M8 were major metabolites in urine and feces, whereas M9 was a major metabolite in plasma, urine and feces. It is speculated that Pathway (A) oxidation at 3 and 8a positions of the octahydroindolizine ring produced two minor mono-hydroxylated metabolites (M3 and M4). Pathway (C) formed M2. Pathway (A) in conjunction with pathway (C) produced metabolites M5 and M7. Pathway (A) followed by ring opening produced M6. Finally, no significant levels of Phase 2 metabolites were observed based on the lack of effects of Glusulase experiments.

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