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In vitro metabolic products of RWJ-34130, an antiarrythmic agent, in rat liver preparations

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Abstract

The in vitro metabolism of RWJ-34130, an antiarrhythmic agent, was conducted using rat hepatic $9000 \times g$ supernatant (S9) and microsomes in an NADPH-generating system, and the rat liver perfusion. The 100 and 20 μg ml^{-1} concentrations of RWJ-34130 aqueous solution were used for microsomal incubation and liver perfusion, respectively. Unchanged RWJ-34130 (~ 77–78% of the sample in both S9 and microsomes) plus a major metabolite, RWJ-34130 sulfoxide (20% of the sample in both S9 and microsomes) were profiled, isolated and identified from both hepatic S9 and microsomal incubates (60 min) using HPLC and mass spectrometry (MS), and by comparison to a synthetic RWJ-34130 sulfoxide, which was synthesized by reacting RWJ-34130 with MCPBA (meta-chloroperoxy benzoic acid). No unchanged RWJ-34130 was detected in the 3 h liver perfusate, however, 1-phenyl-2-oxo-pyrrolidine was profiled, isolated and identified as a major hydrolyzed metabolite of liver perfusate. RWJ-34130 is not extensively metabolized in vitro in rat hepatic S9 and microsomes. All HPLC metabolic profiles of hepatic S9 and microsomal samples (30 min, 60 min) were qualitatively and nearly quantitatively identical.

Keywords: RWJ-34130; Rat; In vitro hepatic metabolism; Metabolite identification; Mass spectrometry; HPLC

1. Introduction

Cardiac-slowing amidines containing the thioindole group are potential antianginal agents, and the chemistry, pharmacology, and structure–activity relationships of the 3-thioindolamidine series has been reported [1–3]. RWJ-34130, 3-[2-(1-phenyl-2-pyrrolidinylideneamino)ethylthio] indole is a new compound in the 3-thioindolamidine series possessing antiarrhythmic activity in the dog [2,3]. The objective of this study was to investigate the in vitro metabolism of RWJ-34130 using rat liver preparations and perfusion techniques [4–7]. The preliminary results of this work have been reported [8]. This paper describes the formation, profiling, isolation and identification of unchanged RWJ-34130 and two metabolites using HPLC, mass spectroscopy (MS) and synthetic techniques.

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2. Experimental section

2.1. Materials

RWJ-34130, 3-[2-(1-phenyl-2-pyrrolidinylideneamino)ethylthio] indole and four putative metabolites, RWJ-33180, RWJ-33180 methyl ester, RWJ-33200 and 1-phenyl-2-oxo-pyrrolidine were synthesized at The R.W. Johnson Pharmaceutical Research Institute, Spring House, PA [2,3]. Glucose-6-phosphate, NADP (nicotinamide-adenine dinucleotide phosphate), HEPES, and Trizma® base [tris(hydroxymethyl)aminomethane] were obtained from the Sigma Chemical Company, St. Louis, MO.

2.2. Hepatic 9000 × g (S9) fraction/microsomal incubations

Male CR Wistar rats (Charles River Breeding Laboratories, Inc., Newfield, NJ) weighing 200–250 g were sacrificed. Their livers were removed and homogenized in three volumes of cold 0.05 M Tris HCl buffer (pH 7.5) containing 1.15% potassium chloride. The homogenate was centrifuged at 9000 × *g* in a refrigerated (2°C) Sorvall RC-5B centrifuge (Dupont, Newtown, CT). Incubation mixtures were then prepared using 1 ml of this supernatant (containing 250 mg wet weight equivalent of liver, about 7.85 mg of microsomal protein), 0.5 mM NADP, 5 mM glucose-6-phosphate, 5 mM magnesium chloride and RWJ-34130 aqueous solution [100 μg (300 μM) ml^{-1}] in 5 ml of Tris/KCl. The samples were incubated at 37°C for 30 and 60 min in room air. The reaction was terminated by placing the flasks on ice. Controls were incubated with no enzyme added.

Microsomal suspensions were prepared by taking a measured volume of 9000 × *g* supernatant and centrifuging again at 105 000 × *g* (Type 40 rotor) for 1 h and then discarding the supernatant. The pellet was gently resuspended in cold 0.1 M HEPES/1.15% KCl buffer up to a volume 1/2 that of the original 9000 × *g* supernatant (approximately 12–16 mg of microsomal protein per ml with this method). Incubation mixtures were then prepared and carried out using the same method described for the 9000 × *g* mixtures, substituting 1 ml of the microsomal suspension for the supernatant.

2.3. Rat liver perfusion

The perfusion system and procedure described by Miller et al. were used with some modifications [9]. The perfusate was prepared in Krebs Henseleit buffer containing 0.9% NaCl, 4.5% Fraction V BSA, lactate (2.1 mM), pyruvate (0.3 mM), D-Glucose (10 mM) (the preceeding from Sigma Chemical Co.) and RWJ-34130 [20 μg (60 μM) ml^{-1}].

A rat (250–300 g) was anesthetized with sodium pentobarbital and the abdominal cavity was exposed. A cannula was inserted into the portal vein for the inflow of perfusate. The animal was eviscerated and the outflow cannula was inserted into the vena cava. The liver was carefully placed into the heated perfusion block while drug-free perfusate was circulated through the liver. Perfusion of RWJ-34130 (20 μg ml^{-1}) was initiated after the liver was perfused with filtered, oxygenated perfusate at 37°C for at least 5 min with no signs of deterioration and stable oxygen output. The perfusate was recirculated through the liver for 3 h at ~25–30 ml min^{-1}.

2.4. HPLC profiling and isolation of RWJ-34130 and metabolites

The HPLC used was a CRT-based gradient liquid chromatograph (Beckman Model 345) with UV detection (Model 165, UV 267 nm, Beckman Instrument Co., CA). LiChrosorb RP-2 MPLC guard and analytical columns (5 μm, 130 × 4.6 mm) were used for sample analysis at a flow rate of 2 ml min^{-1} for the mobile phase. The gradient elution was carried out from 0 to 100% B in 20 min with water (mobile phase A) and methanol (mobile phase B) both containing 0.02% ammonium acetate. HPLC profiling and analysis of RWJ-34130, four synthetically prepared putative metabolites, a synthetic mixture of RWJ-34130 sulfoxide and sulfone, rat hepatic 9000 × *g* fraction/microsomal incubated products, and rat liver perfusate products (untreated, Glusulase®-treated, and 5% HCl or 1 N NaOH hydrolyzed) were

conducted under the HPLC conditions decribed above. Metabolites obtained from the rat liver homogenate/microsomal incubation, perfusate, or reaction mixture were extracted individually with ethyl acetate. The extract was evaporated to dryness to yield a residue which was reconstituted in methanol and injected onto the HPLC. Metabolites and unchanged RWJ-34130 were individually collected from the effluents of HPLC and the solvent residues (post-evaporation) were analyzed by MS. An estimate of the relative percentages of unchanged RWJ-34130 and each metabolite in a given sample was made using the integrated peak intensity generated by the HPLC chromatogram.

2.5. Synthesis of RWJ-34130 sulfoxide and sulfone

RWJ-34130 sulfoxide, a major in vitro metabolite of RWJ-34130, was synthesized using RWJ-34130 (0.15 mM, 50 mg) in chloroform (20 ml) reacted with MCPBA (meta-chloroperoxybenzoic acid, 0.15 mM, 26 mg) at 0°C for 3 h [10]. The reaction mixture was allowed to return to room temperature, and extracted with 1 N aq NaOH solution (10 ml) to remove an excess amount of unreacted MCPBA and MCBA. The chloroform solution was washed with water, dried with anhydrous sodium sulfate, and evaporated to dryness

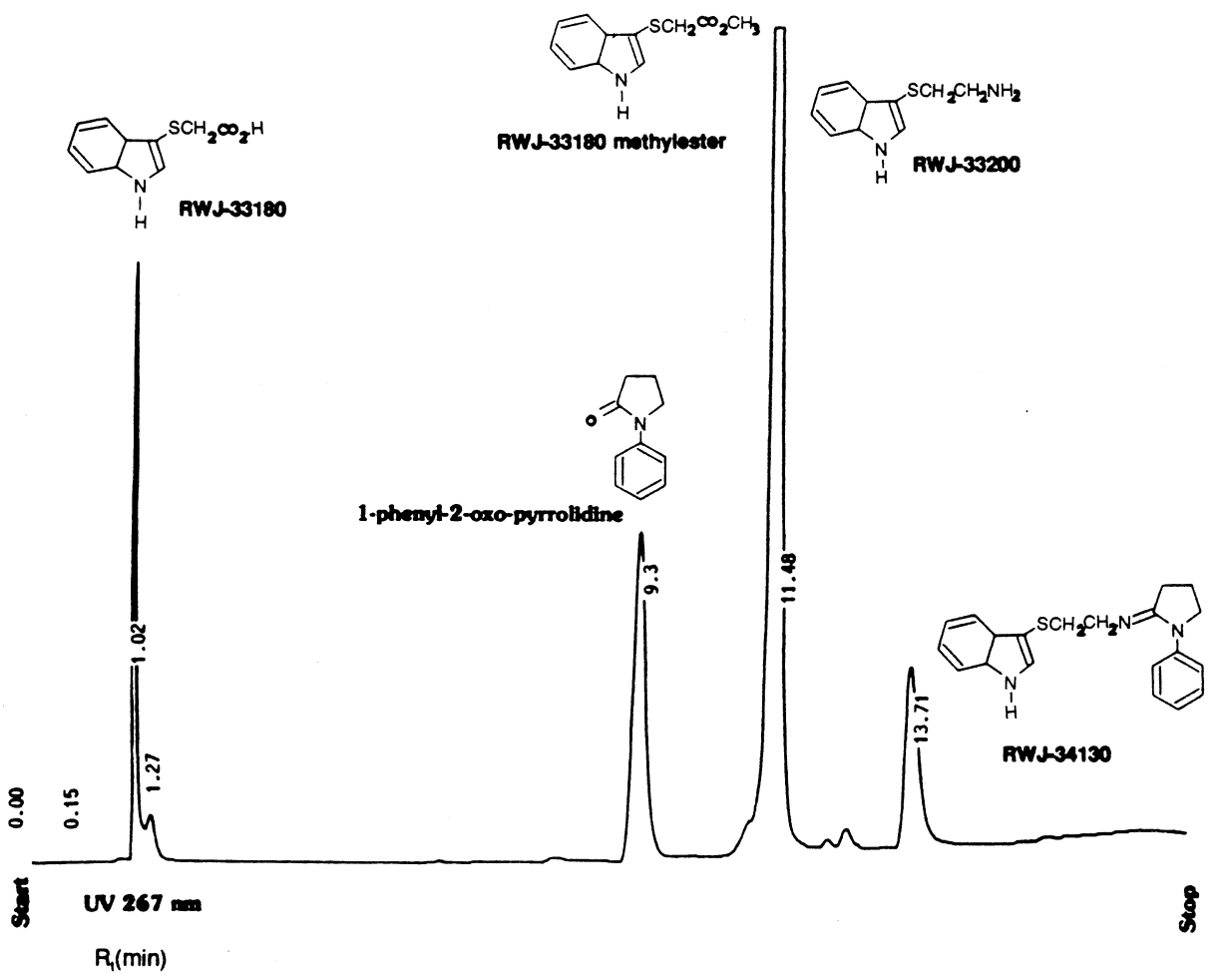


Fig. 1. Profiles of RWJ-34130 and four putative metabolites.

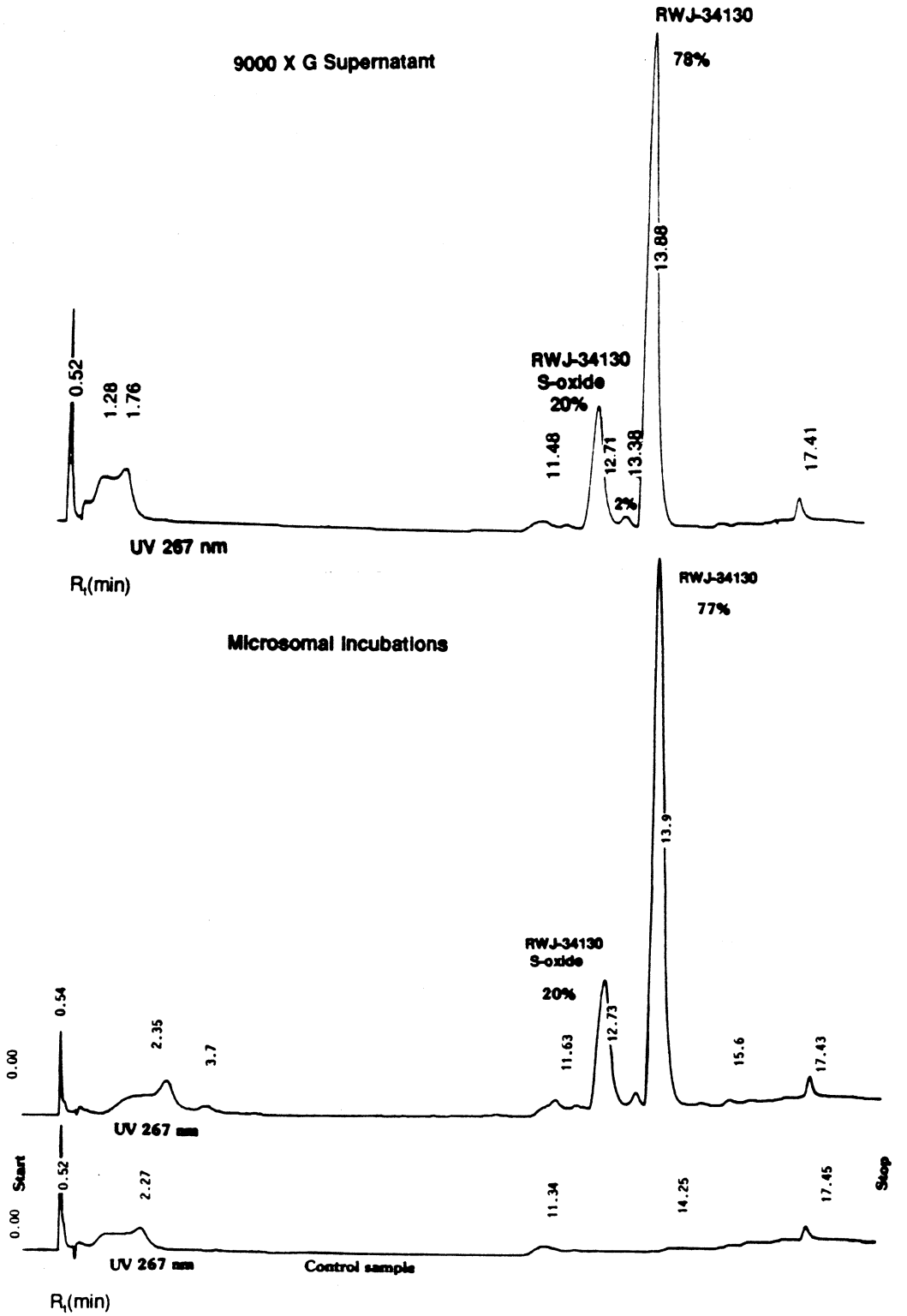


Fig. 2. Profiles of the rat liver 9000 × *g* supernatant and microsomal incubations of RWJ-34130.

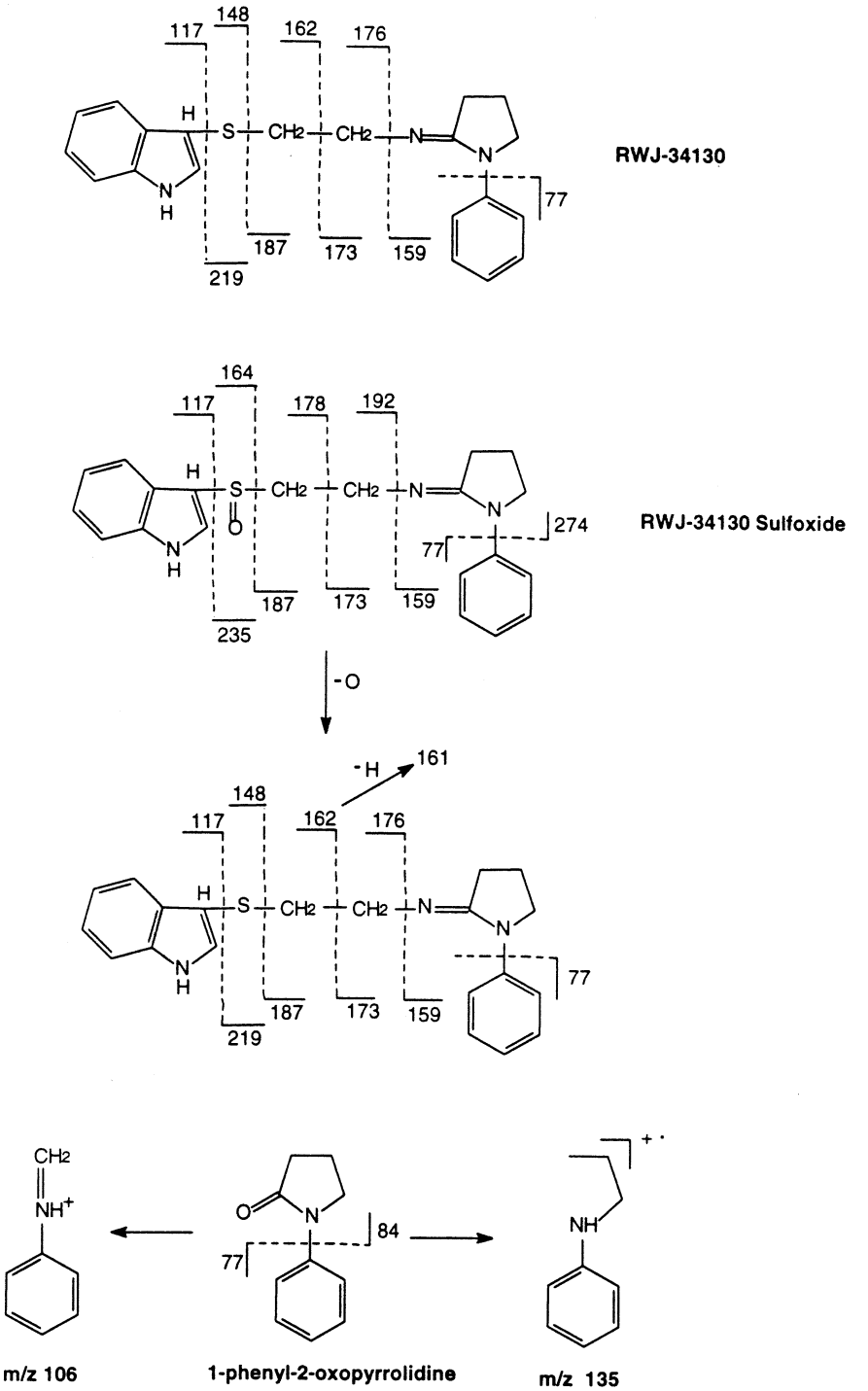
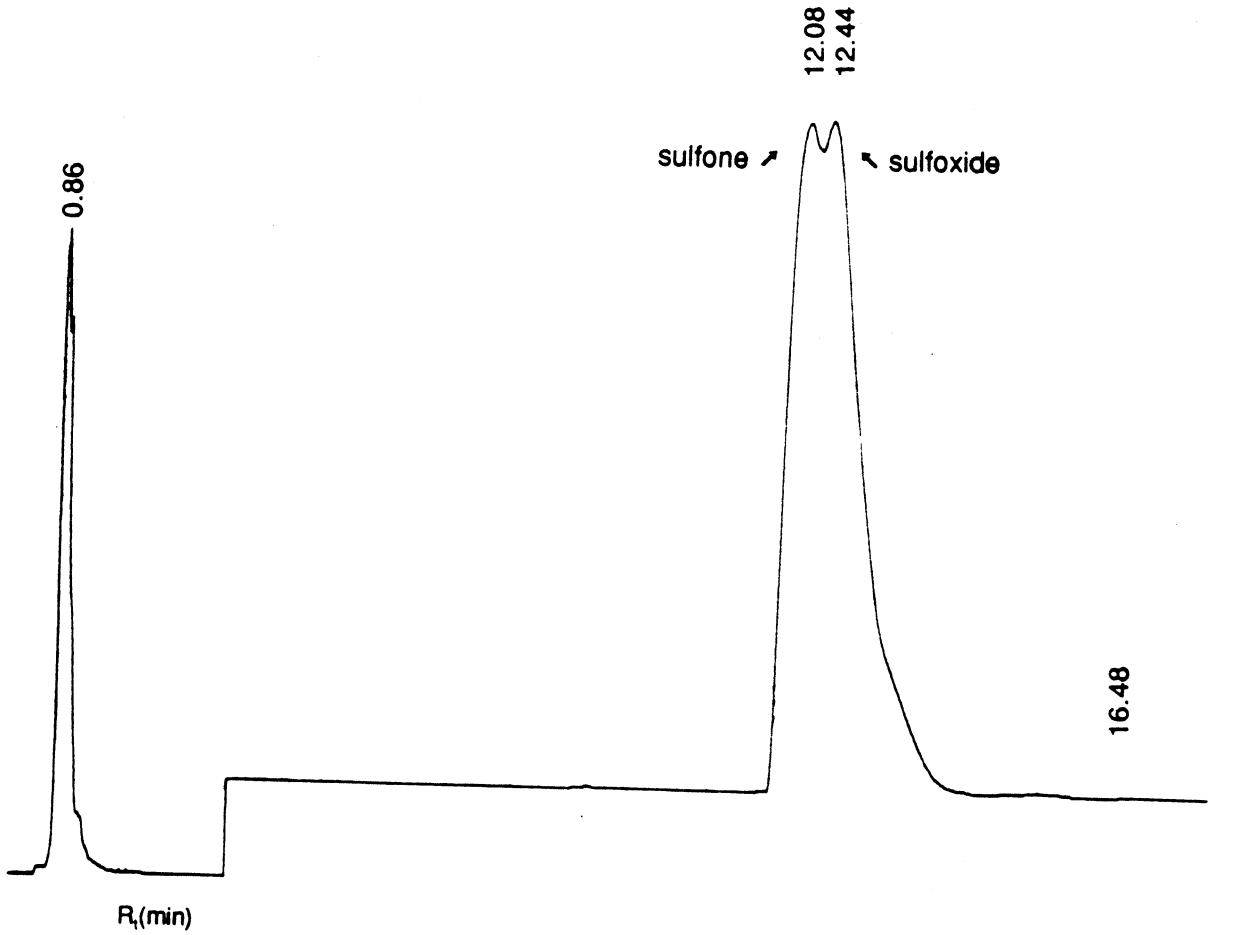


Fig. 3. Structures of RWJ-34130, its metabolites, and their MS fragment ions.



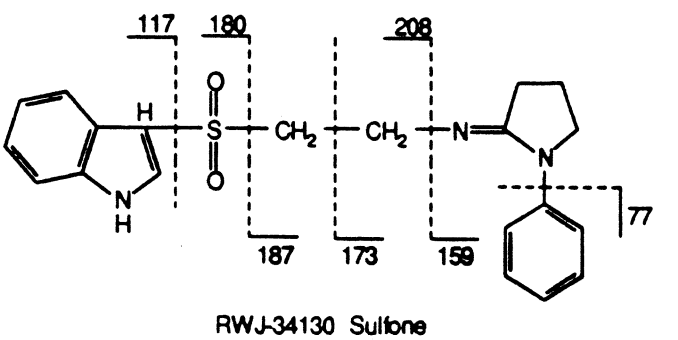


Fig. 4. HPLC chromatograms of the synthetic mixture of RWJ-34130 sulfoxide and sulfone and the MS data of sulfone.

to yield a residue (48 mg) which was reconstituted in methanol and injected onto the HPLC system.

2.6. Spectroscopic analysis

Electron impact and chemical ionization mass spectra were obtained on a Finnigan Model 9500-3300-6100 GC/MS/DS by direct inlet. The mass spectrometer was operated at an electron energy of 70 eV (EI) and 100 eV(CI), source temperature of 100°C, and reagent gas source pressure of 1000 μm for methane.

3. Results and discussion

HPLC profiles were obtained for RWJ-34130 and four putative metabolites (Fig. 1). Liver 9000 × *g* supernatant/microsomal samples (30 and 60 min) were profiled by HPLC and they were all qualitatively and nearly quantitatively identical. The profiles revealed unchanged RWJ-34130 (77.2% of the sample), one major metabolite, sulfoxide (20% of the sample), and one minor unknown metabolite (2.5% of the sample) with R_t of 13.8, 12.7, and 13.5 min, respectively (Fig. 2). Unchanged RWJ-34130 and the major sulfoxide metabolite were isolated by HPLC and analyzed by CI- and EI-MS.

The mass spectral analysis of unchanged RWJ-34130 gave an intense protonated molecular ion at *m/z* 336 (100%), along with two adduct molecular ions at *m/z* 364 ($MC_2H_5^+$, 13%) and 376 ($MC_3H_5^+$, 4%) in CI-MS and an apparent molecular ion at *m/z* 335 (20%) (Fig. 3). The prominent fragment ions at *m/z* 219 (4% CI), 187 (48% CI, 17% EI), 176 (6% CI), 173 (19% CI, 100% EI), 162 (6% CI), 161 (44% CI), 159 (6% CI, 8% EI), 148 (40% CI, 18% EI), 117 (11% CI, 15% EI), and 77 (45% EI) were consistent with the fragmentation pattern from those of authentic RWJ-34130.

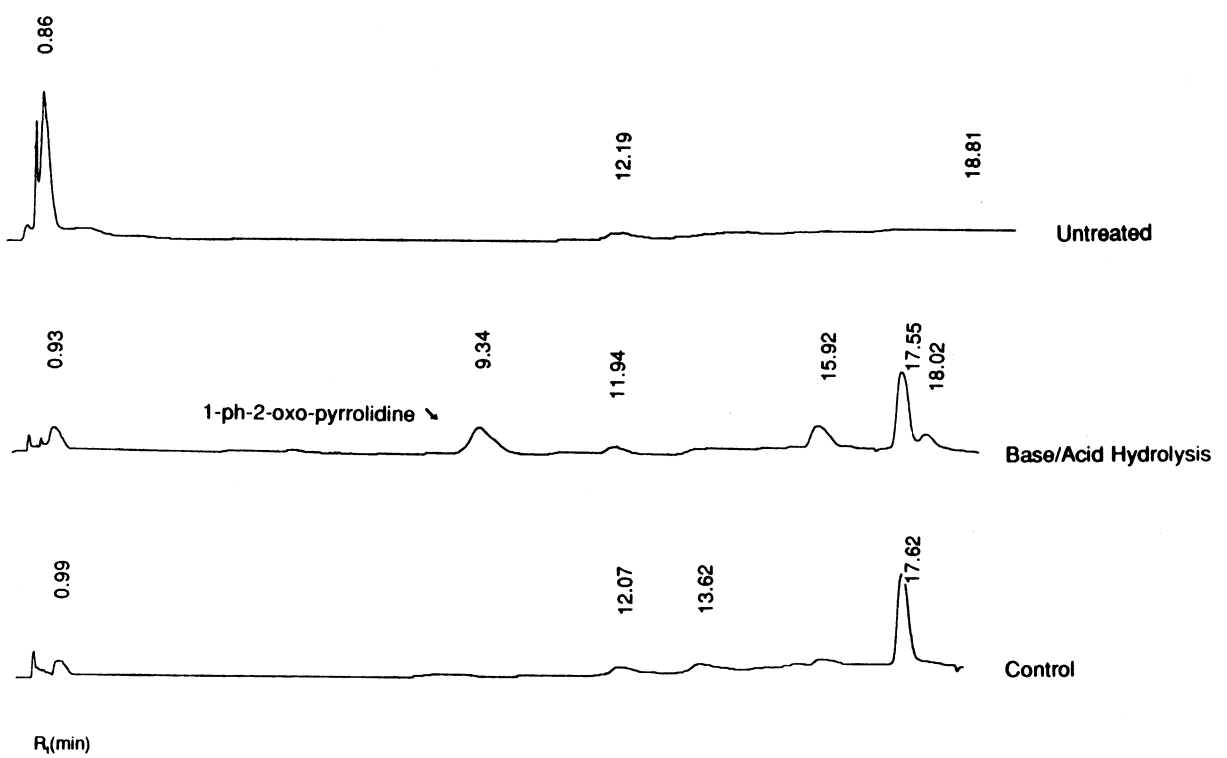


Fig. 5. HPLC profile of the rat liver perfusate.

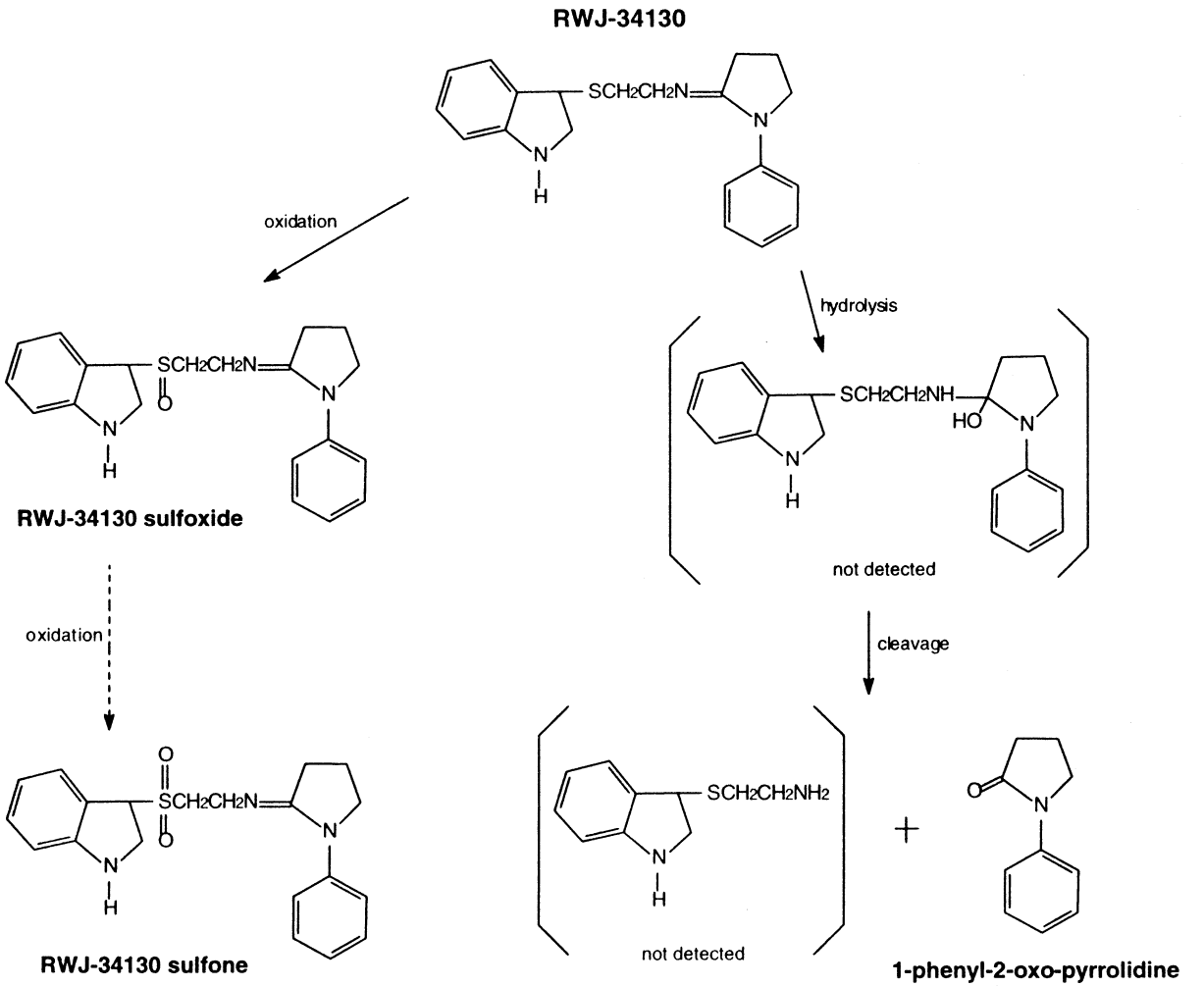


Fig. 6. In vitro metabolic pathways of RWJ-34130.

RWJ-34130 sulfoxide (20% of the sample) exhibited an apparent protonated molecular ion and a molecular ion at m/z 352 (10%) and 351 (14%) in CI-MS and EI-MS, respectively, which was in agreement with a formula of $C_{20}H_{21}ON_3S$, 16 amu higher than RWJ-34130 (Fig. 3). Significant fragment ions at m/z 336 (MH^+-oxygen, 12% CI), 335 (M^+-oxygen, 6% EI), 235 (5% CI), 187 (72% CI, 28% EI), 173 (10% CI, 100% EI), 161 (100% CI, 5% EI), 159 (30% CI, 55% EI), 148 (43% CI, 21% EI), 118 (32% CI, 11% EI), 117 (25% CI, 18% EI), and 77 (76% CI, 38% EI), indicated the formation of RWJ-34130 sulfoxide (Fig. 3). Final structural confirmation was obtained using synthetic sample for comparison (HPLC, CI- and EI-MS).

The synthetic mixture (50:50) of RWJ-34130 sulfoxide (R_t 12.4 m) and sulfone (R_t 12.1 m), obtained from the MCPBA oxidation of RWJ-34130, was isolated by HPLC and analyzed by MS (Fig. 4). The MS data of synthetic sulfoxide were identical to those of isolated sulfoxide metabolite (Fig. 3). The MS analysis of synthetic sulfone provided the following data: m/z 396 ($MC_2H_5^+$, 4% CI), 368 (MH^+, 18% CI), 367 (M^+, 11% EI), 352 (MH^+-oxygen, 2% CI), 351 (M^+-oxygen, 5% EI), 336 (MH^+-2 oxygens, 4% CI), 335 (M^+-2 oxygens, 4% EI), 208 (33% CI,

3% EI), 207 (15% EI), 187 (65% CI, 56% EI), 180 (15% EI), 173 (10% CI, 74% EI), 161 (100% CI), 159 (70% CI, 100% EI), 117 (58% CI, 58% EI), 77 (12% CI, 95% EI), which characterized the structure of sulfone, a putative metabolite of RWJ-34130 (Fig. 4).

The HCl or NaOH hydrolyzed metabolite of liver perfusate isolated from the HPLC collection (Fig. 5) was analyzed by MS to show three adduct molecular ions at m/z 202 ($MC_3H_5^+$, 5%), 190 ($MC_2H_5^+$, 15%) and 162 (MH^+, 100%) in CI and an intense molecular ion at m/z 161 (37%) in EI (Fig. 3). Informative fragment ions at m/z 135 (8% EI), 106 (4% CI, 100% EI), 84 (4% EI) and 77 (23% EI) were indicative of 1-phenyl-2-oxo-pyrrolidine. The product was finally identified by HPLC and MS comparison with an authentic sample.

In conclusion, metabolism of RWJ-34130 in the rat liver preparations appeared to occur via oxidation at the sulfur atom to form sulfoxide (Fig. 6) [11,12]. RWJ-34130 sulfoxide metabolite (R_t 12.7 min on HPLC chromatogram) was identified on the basis of HPLC and MS data by comparison to those of a synthetic sample. Cimetidine is metabolized to form cimetidine sulfoxide, which is an example of S-oxidation type of metabolic pathway [12,13]. Further oxidation of the sulfoxide could result in the formation of a sulfone and S,N-dioxide, although they were not detected in these preparations [13]. No unchanged RWJ-34130 was detected (HPLC) in liver perfusate and may be bound to the perfused rat liver. Polar metabolites in the perfusate were not hydrolyzed by Glusulase® and remain uncharacterized. However, a HCl/NaOH hydrolyzed metabolite, 1-phenyl-2-oxo-pyrrolidine was formed via cleavage of RWJ-34130. Aminoethylthioindole (RWJ-33200), another cleavage product, was not detected by HPLC or MS data (Fig. 1).

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