Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 6, pp. 771–775, 1994

0731–7085/94 $7.00 + 0.00

0731–7085(93)E0035-L

Characterization and identification of the metabolites of fenoctimine using *in vitro* drug metabolizing systems

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Abstract: Fenoctimine sulphate (4-(diphenylmethyl)-1-[(octylimino)methyl]piperidine sulphate) and one of its metabolites, 1-formyl-4-(diphenylmethyl) piperidine (RWJ-34321), were incubated with a rat liver post-mitochondrial supernatant preparation and an NADPH generating system. The metabolites, 7-hydroxyoctyl fenoctimine and 7-oxoocytl fenoctimine were identified as *in vitro* oxidative metabolites of fenoctimine on the basis of mass spectrometry and thin layer chromatography in comparison to authentic samples. RWJ-34321, a third metabolite, was confirmed as a hydrolyzed product of fenoctimine on the same basis. In separate incubations with RWJ-34321, one metabolite (4-(diphenylmethyl)piperidine), was identified as an *in vitro* metabolite of RWJ-34321 by mass spectrometry and thin layer chromatography. Thus, the *in vitro* metabolism of fenoctimine by rat liver homogenates resulted in the oxidation of the aliphatic chain at the seven carbon, initially to an alcohol and then to a ketone. The metabolism of RWJ-34321 resulted in decarbonylation of the formyl carbon.

Keywords: *Fenoctimine; rat;* in vitro *hepatic metabolism; metabolite identification; MS; TLC.*

Introduction

Fenoctimine sulphate is a novel compound possessing gastric anti-secretory activity in the rat, dog, pig, guinea pig, and human [1–3]. Preliminary results on the disposition and metabolism of fenoctimine in mouse, rat, rabbit, dog and human have been reported and indicated that it was extensively metabolized by most species [4–7]. This paper describes the investigation of the nature of the metabolites of fenoctimine using *in vitro* drug metabolizing systems.

Materials and Methods

Chemicals

^{14}C-Fenoctimine sulphate (0.457 μCi mg^{-1}), 4-(diphenylmethyl)-1-[(octylimino)methyl]-piperidine sulphate, was synthesized with the ^{14}C-label at the formyl position as shown in Fig. 1. It was prepared from the octylamine and 1-^{14}C-formyl-4-(diphenylmethyl)piperidine (L.E. Weaner, personal communication and unpublished results). The radiochemical and chemical purities of this material were determined by radio-TLC to be >97%. ^{14}C-RWJ-34321 (Metabolite III, 1-formyl-4-(diphenylmethyl)piperidine) was synthesized with the ^{14}C-label at the benzhydryl position (5.47 μCi mg^{-1}) by condensing diphenyl-^{14}C-methane with 4-piperidone and then with acetic-formic anhydride, and determined to be >99% radiochemically and chemically pure (L.E. Weaner, personal communication and unpublished results).

RWJ-34978 (Metabolite I, 4-(diphenylmethyl)-1-{[(7-hydroxyoctyl)imino]methyl}-piperidine), RWJ-34955 (Metabolite II, 4-(diphenylmethyl)-1-{[(7-oxooctyl)imino]-methyl}piperidine), RWJ-34321 (Metabolite III, 1-formyl-4-(diphenylmethyl)piperidine), and RWJ-43724 (Metabolite IV, 4-(diphenylmethyl)piperidine) were used as reference samples for TLC and MS (Fig. 1) and their purities were >98% [L.E. Weaner, personal communication and unpublished results, 8]. Metabolite II was obtained by reacting 1-formyl-4-(diphenylmethyl)piperidine with 2-methyl-1,3-dioxolane-2-hexanamine in the presence of phosgene [8]. Reduction of Metabolite II with sodium borohydride yielded

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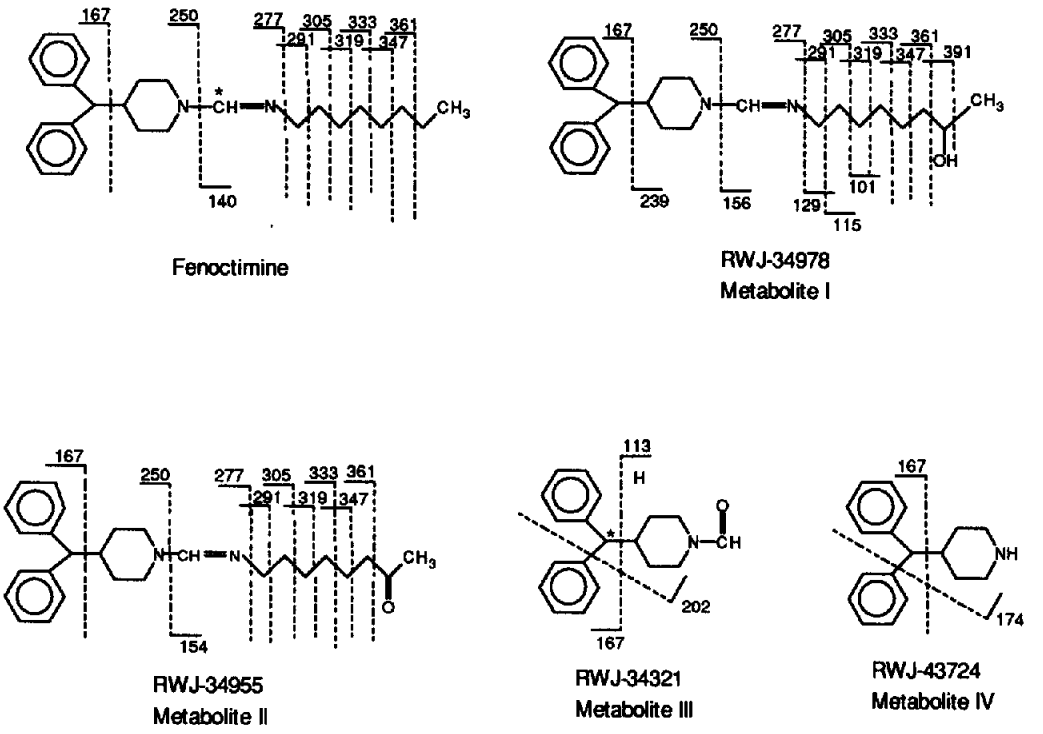

Figure 1
Structures of fenoctimine, its metabolites, and their MS fragment ions.

Metabolite I [8]. 4-(Diphenylmethyl)piperidine (Metabolite IV) purchased from Aldrich Chemical Co., Milwaukee, WI was converted to 1-formyl-4-(diphenylmethyl)piperidine (Metabolite III) by reacting with acetic-formic anhydride (L.E. Weaner, personal communication and unpublished results).

Glucose-6-phosphate, NADP (nicotinamide-adenine dinucleotide phosphate) and Trizma® base [tris(hydroxymethyl)aminomethane] were obtained from Sigma (St Louis, MO). Biofluor™ (New England Nuclear, Boston, MA) was used as a scintillation solution.

Hepatic fraction incubations obtained at 9000 g

Male CR Wistar rats (Charles River Breeding Laboratories, Newfield, NJ) weighing 175–225 g were killed, their livers removed and homogenized using a Willems Polytron® (Brinkmann Instruments, Westbury, NY), 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*** at 2°C in a refrigerated 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 0.1 mM drug (added as 20 μl of a PEG 200 solution of fenoctimine sulphate or as 25 μl of a methanolic solution of RWJ-34321) in 5 ml of Tris/KCl. The samples were incubated at 37°C for 30 min in an air atmosphere. The reaction was terminated by placing the flasks on ice. Fenoctimine and RWJ-34321 controls were run with no enzyme added.

Extraction and isolation procedure for the fenoctimine hepatic metabolites from rat

Sixty millilitres of the ^{14}C-fenoctimine sulphate incubation mixture were acidified to pH 3 by dropwise addition of 6 N sulphuric acid, and extracted with two volumes of petroleum ether. The organic and aqueous layers were separated. The organic layer (containing 7% of the radioactivity) was separated and discarded, and the aqueous fraction was neutralized with 1 N sodium hydroxide. The aqueous mixture was then extracted twice with approximately five volumes of ethylene dichloride. The combined ethylene dichloride extracts (485 ml) were evaporated to dryness under reduced pressure to yield 0.92 mg equiv-

alents by radioassay of drug and metabolites. The residue was resuspended in 0.5 ml of ethylene dichloride and 0.1 ml portions of the concentrate were applied as bands on each of five 20 × 20 cm silica gel GF plates (250 micron, Analtech, Newark, DE). Each plate was chromatographed twice in a solvent system of isopropanol–hexane (90:10, v/v; system A). The biotransformation profile of the sample remained qualitatively the same before and after extraction.

The five TLC plates were radioscanned and visualized under a short-wavelength UV light, and four radioactive zones (R_f = 0.8, 0.5, 0.4 and 0.3) were removed and extracted with ethyl acetate–methanol (80:20, v/v). The four extracted samples were evaporated to dryness to yield residues which were submitted for mass spectral (EI and CI) analysis.

Extraction and isolation procedure for RWJ-34321 metabolites

Twenty-five millilitres of the ^{14}C-RWJ-34321 incubation mixture were adsorbed onto Amberlite® XAD-2 resin (2.5 g) and extracted with methanol (100 ml), which then contained approximately 80% of the total radioactivity. The extract was evaporated to dryness, resuspended in methanol and chromatographed on a 5 × 20 cm silica gel GF plate (250 micron). The plate was developed in a solvent system of chloroform/methanol/water (60:38:2, v/v/v; system B). The plate was visualized under UV light and radioscanned (Berthold Model 600 or Berthold Model LD 260, Varian Instruments). Two intense radioactive zones, R_f 0.5–0.6 and 0.8–0.9, were removed individually and extracted in the same manner described previously. Aliquots of the extracts were evaporated to dryness and analysed by CI-MS.

Spectroscopic analysis

Electron impact mass spectra were obtained on an Hitachi Perkin–Elmer RMU-6 Spectrometer by direct inlet at 70 eV. 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 100 eV (CI only), source temperature of 100°C, and reagent gas source pressure of 1 torr for methane.

Total radioactivity determination

Total radioactivity in each incubation or extraction mixture was determined by directly counting aliquots of each sample in 10 ml of Biofluor™ in a refrigerated liquid scintillation spectrometer (Searle Analytic 81).

Results and Discussion

Fenoctimine metabolism in vitro

The solvent extraction of the incubation mixture and thin layer chromatographic separation (system A) of the extract residue resulted in the isolation of three products. The major component (47% of the sample radioactivity) was at R_f 0.5 on TLC in solvent system A. This product exhibited an intense protonated parent ion with two adduct ions in CI-MS at *m/z* 391 (MH^+, 100%), 419 ($MC_2H_5^+$) and 431 ($MC_3H_5^+$), which was consistent with the formula of $C_{27}H_{38}N_2$. The EI-MS (Fig. 1) revealed a molecular ion at *m/z* 390 (6%), fragment ions at *m/z* 361 (6%), 347 (19%), 333 (3%), 319 (2%), 305 (7%), 291 (9%), 277 (4%), 250 (80%), 167 (37%) and 140 (100%), which were consistent with the fragmentation pattern of fenoctimine. Identification was confirmed by direct comparison (TLC, CI and EI-MS) with authentic fenoctimine [8].

Metabolite I (22% of the sample radioactivity), 7-hydroxyoctyl fenoctimine, showed an R_f of 0.4 on TLC in solvent system A. The CI mass spectrum indicated an intense protonated molecular ion, together with two adduct ions at *m/z* 407 (MH^+, 100%), 435 ($MC_2H_5^+$, 20%) and 447 ($MC_3H_5^+$, 5%), which were in agreement with the formula of $C_{27}H_{38}N_2O$, 16 amu higher than fenoctimine. In addition, a rather intense ion in CI-MS at *m/z* 389 (MH^+-H_2O, 25%) was observed, resulting from the dehydration of an alcohol on the molecule. The EI-MS (Fig. 1) also revealed a molecular ion at *m/z* 406 ($C_{27}H_{38}N_2O$, 5%) and important fragment ions at *m/z* 391 (10%), 361 (19%), 347 (36%), 333 (6%), 319 (6%), 305 (13%), 291 (16%), 277 (6%), 250 (90%), 167 (29%) and 156 (100%), which were indicative of an alcohol metabolite of fenoctimine. Direct comparison (TLC, CI and EI-MS) with a synthesized product (RWJ-34978) confirmed this assignment [9].

Metabolite II (18% of the sample radioactivity), 7-oxooctyl fenoctimine, showed an R_f of 0.3 on TLC in solvent system A. The CI-MS of the product exhibited an intense protonated molecular ion and two adduct ions at *m/z* 405 (MH^+, 67%), 433 ($MC_2H_5^+$, 18%), and 445

($MC_3H_5^+$, 9%), which correspond to a formula of $C_{27}H_{36}N_2O$, 14 amu higher than fenoctimine. The EI-MS (Fig. 1) contained an apparent molecular ion at *m/z* 404 ($C_{27}H_{36}N_2O$, 7%) and significant fragment ions at *m/z* 389 (1%), 361 (7%), 347 (43%), 333 (2%), 319 (1%), 305 (5%), 291 (2%), 277 (1%), 250 (21%), 167 (100%) and 154 (14%), indicating a ketone group on the side chain of the molecule. Final confirmation was obtained using synthetic material (RWJ-34955) for comparison (TLC, CI and EI-MS) [9].

A minor metabolite (Metabolite III, 6% of the sample radioactivity) with R_f 0.8 was also isolated during the course of the work-up process. It gave an intense protonated parent ion with two adduct ions in CI-MS at m/z 280 (MH^+, 100%), 308 ($MC_2H_5^+$, 12%) and 320 ($MC_3H_5^+$, 4%), consistent with the formula of $C_{19}H_{21}NO$. The EI-MS of this product also indicated a rather intense parent ion at *m/z* 279 ($C_{19}H_{21}NO$, 13%) and three prominent ions at *m/z* 202 (12%), 167 (100%), and 113 (22%) (Fig. 1). Direct comparison of TLC and CI/EI-MS of the metabolic/hydrolysed product with those of RWJ-34321 confirmed the identity of the product as 1-formyl-4-(diphenylmethyl)-piperidine, presumably arising from the hydrolysis of fenoctimine, Metabolite I, or Metabolite II.

RWJ-34321 (Metabolite III) in vitro *metabolism*

The extraction and thin-layer chromatographic separation (solvent system B) of the RWJ-34321 incubation mixture resulted in the isolation of two products. One component (38% of the sample radioactivity) was confirmed by TLC and CI-MS to be unmetabolized RWJ-34321 (R_f 0.8–0.9). The second product, 4-(diphenylmethyl)piperidine (Metabolite IV, 53% of the sample radioactivity), had an R_f of 0.6 by TLC using the solvent system previously indicated. CI-MS revealed an intense protonated parent ion and two adduct ions at *m/z* 252 (MH^+, 100%), 280 ($MC_2H_5^+$, 15%) and 292 ($MC_3H_5^+$, 5%), which were consistent with the formula $C_{18}H_{21}N$ (Fig. 1). The important fragment ions at *m/z* 174 (97%) and 167 (21%) were in agreement with the structure proposed. Final confirmation of both RWJ-34321 and 4-(diphenyl-methyl)piperidine, (RWJ-43724), was obtained using synthetic material for comparison by both TLC and CI-MS [8, 9].

Conclusion

Fenoctimine metabolites were formed by the successive oxidation of the penultimate carbon

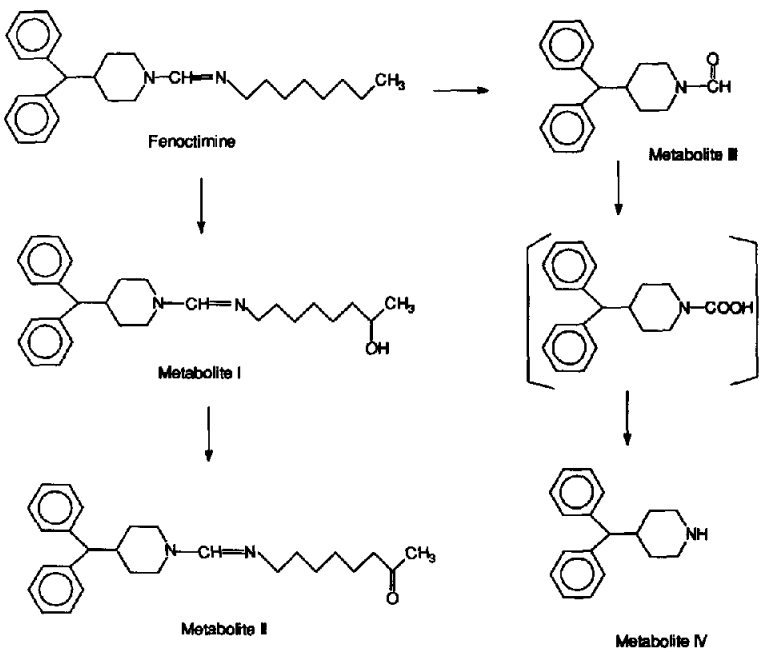

Figure 2
Proposed biotransformation pathways for fenoctimine and RWJ-34321 (Metabolite III) in rat liver 9000 *g* homogenate.

of the aliphatic chain, forming first the alcohol and then the ketone. Similar metabolic pathways for the oxidation of the penultimate carbon of aliphatic chain compounds have been documented [10, 11]. Because fenoctimine readily undergoes hydrolysis to RWJ-34321 (Metabolite III) *in vivo*, it was of interest to also evaluate the metabolism of RWJ-34321 in the *in vitro* system. A proposed mechanism for the formation of 4-(diphenylmethyl)piperidine (Metabolite IV) from RWJ-34321 involves oxidation at the formyl carbon to form a carbamic acid derivative followed by nonenzymatic decarboxylation. The proposed biotransformation pathway for fenoctimine in an *in vitro* drug metabolizing system is summarized in Fig. 2.

Acknowledgements — We would like to thank Dr Larry E. Weaner for synthesizing ^{14}C-fenoctimine and ^{14}C-RWJ-34321, Dr M.K. Scott for synthesizing fenoctimine metabolites, Mr James Kalbron and Mr Martin Mutter for the MS analysis of fenoctimine metabolites, and Drs Adrienne R. Takacs, Anthony J. Streeter and Bobbe L. Ferraiolo for reviewing the manuscript.

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[*Received for review 5 October 1993; revised manuscript received 13 December 1993*]