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Isolation and identification of the major urinary metabolite of 4-(4-fluorophenoxy)benzaldehyde semicarbazone after oral dosing to rats

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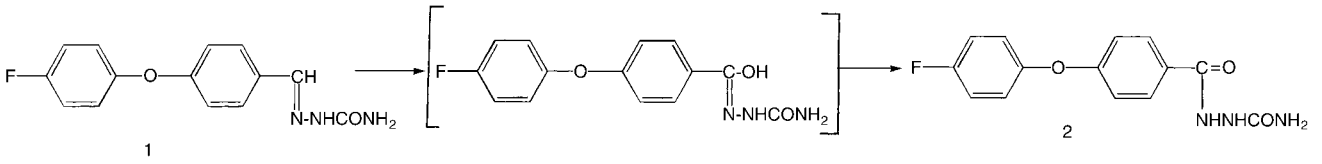
4-(4-Fluorophenoxy)benzaldehyde semicarbazone (**1**) is a novel anticonvulsant affording excellent protection in the rat oral maximal electroshock (MES) screen as well as having an apparent protection index of over 300. The metabolism of this compound was studied by examining the urine or rats dosed orally with 50 mg/kg of **1** which revealed that most of the drug was converted into one metabolite **2**. The structure of **2** was shown by mass spectrometry to be 1-[4-(4-fluorophenoxy)benzoyl]semicarbazide which was confirmed by an independent synthesis. Compound **2** was bereft of activity in the rat oral MES screen when nine times the ED_{50} dose of **1** was administered. This datum provided strong evidence that the anticonvulsant activity of **1** and related compounds is due to the intact molecules and is not produced by breakdown products *in vivo*.

1. Introduction

Recently the synthesis of a number of aryloxyaryl semicarbazones possessing excellent anticonvulsant properties has been described [1]. After intraperitoneal injection into mice, protection was afforded in the maximal electroshock (MES) and subcutaneous pentylenetetrazole (scPTZ) screens while ataxia was also demonstrated. The average protection index (PI ie TD_{50}/ED_{50} where TD_{50} is the dose required to cause ataxia in 50% of the animals and ED_{50} is the dose which protects against seizures in 50% of the animals) of a number of these compounds was approximately 7. However when these compounds were administered orally to rats, the doses required to afford protection in the MES test were much lower than those in the mouse i.p. screen while less activity in the scPTZ test was demonstrated. An important observation was the fact that ataxia was virtually abolished and hence the compounds possessed very high PI figures in the MES test many of which were greater than 100. From this group of compounds, 4-(4-fluorophenoxy)benzaldehyde semicarbazone (**1**, Scheme 1), was chosen for preclinical development. This compound had an ED_{50} value in the rat oral MES screen of 1.59 mg/kg and no ataxia was observed over a 24 hour period at the maximum dose utilized namely 500 mg/kg [1]. Thus compound **1** had an apparent PI value of >315.

The remarkable protection of **1** in the MES screen after oral administration to rats led to the question being posed of whether the anticonvulsant activity noted was due to the compound per se or to a metabolite. This study reports the isolation, identification and anticonvulsant evaluation of the major metabolite from **1**.

Scheme 1



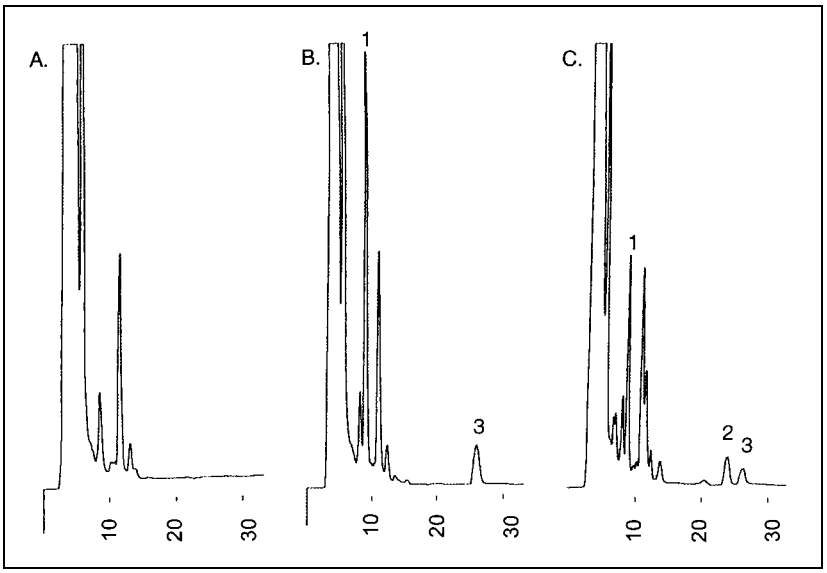


Fig. 1:
Chromatograms of extracts from A) blank rat urine; B) blank rat urine spiked with compound **2** (peak 1) and unmetabolized **1** (peak 3); C) urine from rat dosed with 50 mg/kg of **1**. The time scale is in minutes

2. Investigations and results

Rats were given an oral dose of 50 mg/kg of **1** and the urine from these animals was collected over an eighteen hour period. HPLC analysis of extracted samples was undertaken and chromatograms of blank urine were compared with those obtained from dosed rats (Fig. 1). Additional peaks were found in the urine from dosed animals with retention times of 8.8 (peak 1), 24.0 (peak 2) and 26.0 min (peak 3); the latter peak corresponded to that of the parent drug. The total drug-related peak areas for peaks 1–3 were 71, 18 and 11%, respectively. Mobile phase collections from each of the two metabolite peaks provided only enough material for mass spectrometric analysis of peak 1. The 60ev EI spectrum revealed a molecular ion at m/z 289. The base peak was at m/z 215 and the remaining fragment ions had intensities which were 20% or less of the base peak. Accurate mass measurements were made at m/z 272, 246 and 215 and these results are presented in the Table. The fast atom bombardment mass spectrum of the principal metabolite is portrayed in Fig. 2 which indicated a protonated molecular ion of m/z 290 whose intensity was 47% of the base peak at m/z 215 (Scheme 2). The evidence indicated that the structure was 1-[4-(4-fluorophenoxy)benzoyl]semicarbazide (**2**) which was confirmed by an independent synthesis of this compound. The synthetic amide coeluted with the major metabolite on an HPLC column. Examination of the synthetic amide **2** in the rat oral MES screen revealed that it afforded no protection using doses up to and including 15 mg/kg.

3. Discussion

The structure of the principal metabolite was identified by MS. The molecular ion was obtained from a FAB spectrum and an accurate mass measurement revealed its elemental composition. The loss of ammonia and isocyanic acid from the parent molecule giving rise to daughter ions having m/z values of 272 and 206, respectively, were proposed whose structures are given in Scheme 2 along with the putative structure of the base peak m/z 215.

An independent synthesis of the metabolite confirmed the assignment which had been made by MS. Details of the preparation of **2** will be presented elsewhere.[3] Briefly the synthesis of the metabolite was accomplished by oxidizing 4-(4-fluorophenoxy)benzaldehyde [1] with potassium permanganate to form the corresponding carboxylic acid which was treated with 1,1′-carbonyldiimidazole and the resultant intermediate was reacted with semicarbazide to give **2**.

A proposed mechanism whereby **1** is converted into **2** *in vivo* is given in Scheme 1. Hydroxylation of a methine or methylene group which is attached to a nitrogen atom occurs with many drugs and xenobiotics [2]. Thus in the case of **1**, its conversion into the corresponding iminoalcohol is postulated. However such compounds would be expected to undergo tautomerism yielding the more stable keto tautomer [3] and hence formation of **2** from the intermediate iminoalcohol would likely occur readily.

In conclusion, this study revealed that the acylsemicarbazide **2** is the principal urinary metabolite isolated after oral dosage of the anticonvulsant semicarbazone **1** to rats. The metabolite **2** is inactive in the rat oral MES screen at a dose nine times the ED_{50} of the parent compound **1**. Previously the hypothesis was advanced representing the alignment of **1** and related analogues at a binding site [1] in which the assumption was made that the administered compounds were responsible for the anticonvulsant activity. The data presented herein support this viewpoint since in the case of a representative compound **1**, strong evidence has been provided that bioactivity resides in the intact molecule and not in compounds formed *in vivo*.

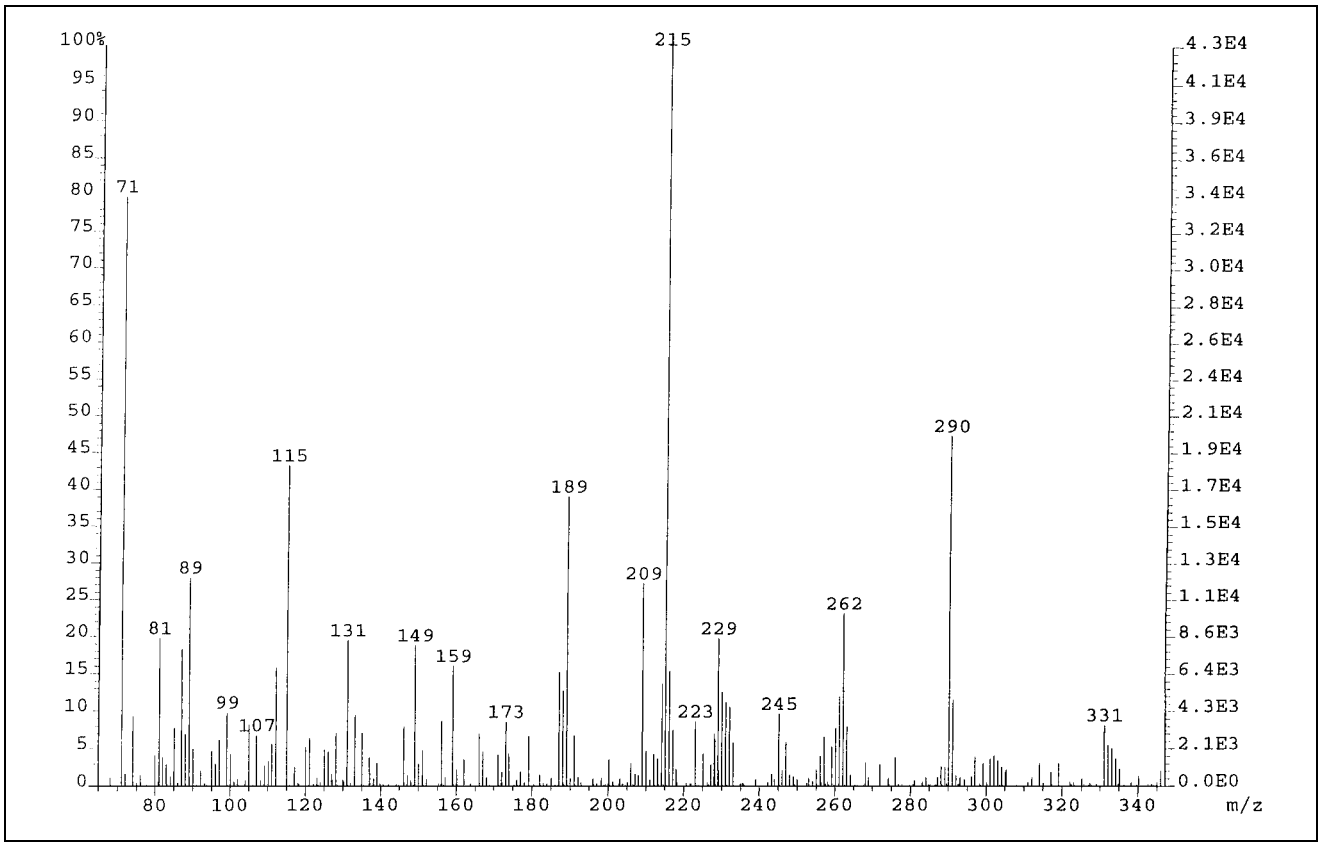
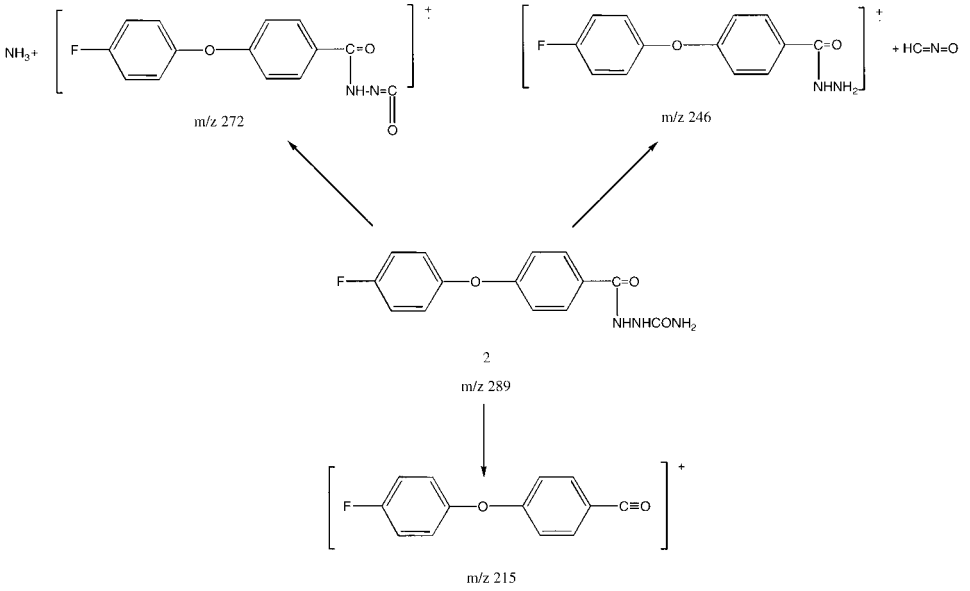


Fig. 2: Fast atom bombardment mass spectrum of **2** obtained from rat urine

Scheme 2



4. Experimental

4.1. Materials

All of the solvents were HPLC grade (BDH Inc., Toronto, Canada). Compound **1** was synthesized by a method described previously [1] and compound **2** was prepared by CoCensys Inc., details of which will be reported elsewhere.

4.2. Animal methods

The use of animals in this study was in accord with the Canadian Council on Animal Care and was approved by the University Committee on Animal Care and Supply, University of Saskatchewan.
A 50 mg/kg dose of **1** was suspended in an aqueous solution of methylcellulose (0.5%, 3 ml) and administered orally to three male Sprague-Dawley rats (250–350 g, Charles River). The urine was collected for 18 h in metabolism cages and 1 ml samples were transferred to borosilicate glass culture tubes (16 × 100 mm) closed with PTFE-lines caps. The animals were fasted but water was available during the collection period.

4.3. HPLC of the urine extracts

The metabolites and parent drug were separated by HPLC using the following conditions. The column employed was a Nova-Pak C_{18} Radial Pak cartridge column (Waters, Milford, MA) measuring 8 mm × 10 cm. The particle size was 4 μM. The mobile phase consisted of water/methanol/tetrahydrofuran (5 : 4 : 1) pumped at a flow rate of 1.0 ml/min. The instrumentation comprised a Waters M-45 pump, a Rheodyne 7125 manual injector fitted with a 500 μl loop, a Waters 490 UV Detector (Millipore-Waters, Mississauga, ON, Canada) and a Shimadzu CR601 Chromatopac integrator (Mandel, Guelph, ON, Canada). The analytes were detected by UV absorption at 290 nm.
Immediately after collection, samples of urine (1 ml) were extracted with ethyl acetate (5 ml) and the mixture was vortexed for 10 min followed by centrifugation at 1200 × g for 10 min. The organic layer was separated and evaporated under a stream of nitrogen at 40 °C. The residue was reconstituted in 100 μl of the mobile phase.

4.4. Identification of compound 2

Peak 1 at 8.8 min was collected and several samples were pooled. The resultant solution was lyophilized and stored at −20 °C until examined by mass spectrometry. A 60eV positive ion electron impact spectrum of **2** was obtained in the College of Pharmacy and Nutrition, University of Saskatchewan, using a Fisons 705Q hybrid mass spectrometer linked directly to a PDP11-250J data system. The accelerating voltage was 6000 volts with a source temperature of 200 °C. The sample was placed on the solids probe and directly inserted into the instrument. The probe was heated to 160 °C. Accurate mass measurements were made using perfluorokeresene as a reference standard and an instrument resolution of 5000. The positive ion continuous flow fast atom bombardment spectrum was obtained at the Plant Biotechnology Institute, Saskatoon. Compound **2** was dissolved in a solution of acetonitrile/water (1 : 1) which contained glycerol (2% v/v) and trifluoroacetic acid (0.1% v/v) and introduced by loop injection into a VG Analytical 70-250 SEQ hybrid mass spectrometer equipped for continuous flow secondary ion mass spectrometry analysis.

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[3] Wang, Y.; Cai, S-X.: unpublished data

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Table: Composition of certain ions from compound 2 obtained by mass spectrometry

Peak (m/z)	Accurate mass measurement (m/z)	Composition	Calculated mass measurement (m/z)
290	290.0937	$C_{14}H_{13}FN_3O_3^{+}$ [a]	290.0941
272	272.0598	$C_{14}H_9FN_2O_3^{+\cdot}$	272.0597
246	246.0801	$C_{13}H_{11}FN_2O_2^{+}$	246.0805
215	215.0513	$C_{13}H_8FO_2^{+}$	215.0508

[a] This is the protonated molecular ion obtained from the FAB spectrum. The remaining ions were obtained from the E1 spectrum