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Determination of *p*-trifluoromethylphenol, a metabolite of fluoxetine, in tissues and body fluids using an electron-capture gas chromatographic procedure

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Abstract

An electron-capture gas chromatographic procedure was developed for the analysis of *p*-trifluoromethylphenol, an O-dealkylated metabolite of fluoxetine, in biological samples. A basic extraction of the biological sample was employed, followed by derivatization with pentafluorobenzenesulfonyl chloride. The internal standard, 2,4-dichlorophenol, was added to all samples used in the procedure to aid in quantitation. The practical limit of detection (signal-to-noise ratio>3) for *p*-trifluoromethylphenol was <5 ng/ml in human plasma samples, <10 ng/g of rat brain tissue, <25 ng/g of rat liver tissue and <25 ng/ml in human and rat urine samples. In the rat, the levels of free *p*-trifluoromethylphenol in the liver were 10-fold higher than those in the brain, and a substantial amount was excreted in the urine. Human urine samples contained levels of free *p*-trifluoromethylphenol approximately 30-fold higher than those found in human plasma samples. The procedure described is useful for the detection and quantitation of free *p*-trifluoromethylphenol in humans and rats treated with fluoxetine. © 1997 Elsevier Science B.V.

Keywords: *p*-Trifluoromethylphenol; Fluoxetine; Pentafluorobenzenesulfonyl chloride

1. Introduction

Fluoxetine (FLU, Prozac®; Fig. 1) is a selective serotonin (5-hydroxytryptamine, 5-HT) reuptake inhibitor that was approved for clinical use as an antidepressant by the Food and Drug Administration (FDA) in 1987 [1]. Despite the extensive use of FLU [2], it is thought that as much as 50% of its metabolism is still unaccounted for (estimated from reports by Lemberger et al. [3] and Bergstrom et al. [4]). This could prove to be very important because metabolites are often either active therapeutically or they may contribute to the side effect profile of the parent drug [5–7]. In addition, metabolites may compete with other exogenous substrates for catabolic enzymes and result in marked changes in tissue and body fluid levels of these other drugs and/or their metabolites [8].

The major route of metabolism for FLU identified to date is N-demethylation to norfluoxetine (NFLU; Fig. 1) [9,10]. It has also been proposed that FLU, and possibly also NFLU, can be O-dealkylated to form *p*-trifluoromethylphenol (TFMP; Fig. 1) [9–11] but, to the best of our knowledge, this metabolite has

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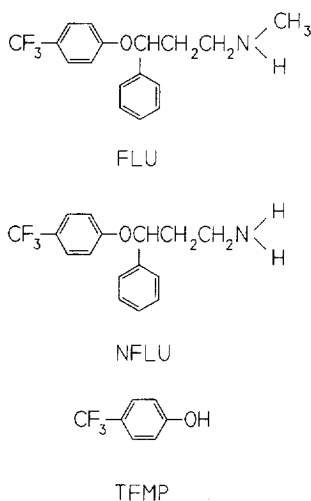

Fig. 1. Structures of fluoxetine, norfluoxetine and TFMP.

never been quantitated. In order to quantitate TFMP in biological fluids, an electron-capture gas chromatography (GC) procedure was developed which utilizes pentafluorobenzenesulfonyl chloride (PFBSC) as the derivatizing reagent. This reagent has previously been shown to be useful for the extractive derivatization of amine- and phenol-containing drugs [12–14], and for derivatization of tyrosyl peptides [15], nucleic acid pyrimidine bases [16], and proteins [12].

2. Experimental

2.1. Chemicals and reagents

Pure standards of FLU were provided by Lilly Research Labs. (Indianapolis, IN, USA) and TFMP was purchased from Aldrich (St. Louis, MO, USA). Reagents were purchased from various sources: glass-distilled toluene, ethyl acetate and acetonitrile from BDH (Toronto, Canada); potassium bicarbonate from Fisher Scientific (Edmonton, Canada); ammonium hydroxide from Fisher Scientific (Edmonton, Canada); the internal standard 2,4-dichlorophenol and the derivatizing reagent PFBSC from Aldrich.

2.2. Animals

Male Sprague–Dawley rats (Ellerslie Biosciences, Edmonton, Canada), 200–300 g, were housed in pairs in an environmentally controlled room using a 12 h light–12 h dark cycle. At the appropriate time animals were injected intraperitoneally (i.p.) with either FLU·HCl (10 mg/kg; dissolved in 20% DMSO in double-distilled water) or vehicle (20% DMSO in double-distilled water). Animals were placed in metabolic cages immediately after injection in order to collect 24-h urine samples. The volume of urine collected was measured and the samples were frozen at −20°C until the time of analysis. Following 10 drug-free days, rats were reinjected with the drugs and sacrificed by decapitation 5 h post-injection; brains and livers were removed and immediately frozen in dry ice-cooled isopentane or on dry ice, respectively.

2.3. Human samples

Patients diagnosed with major depressive disorder were asked to collect a 24-h urine sample 3 weeks after commencing treatment with FLU·HCl (Prozac; 10–20 mg, once daily). A blood sample (16 ml) was collected into vacutainers (with EDTA as the anticoagulant) and centrifuged at 1000 *g* for 10 min to separate the red cells from the plasma. Plasma and urine samples were collected from these subjects before FLU treatment began and again after 3 weeks of treatment. Urine was collected for the 24 h prior to the day of blood collection. The plasma and urine samples were frozen at −20°C until analyzed.

2.4. Sample preparation

Aliquots (1 ml) of human plasma and human and rat urine were used for the analytical procedure. Rat brain and liver tissue were weighed and homogenized in five volumes of ice-cold double-distilled water. An aliquot (2 ml) was removed from the homogenized sample and used in the analytical procedure. Appropriate calibration standards of TFMP were prepared along with the samples by diluting standard solutions of TFMP in control biological samples prepared from drug-naive rats or in plasma or urine from drug-naive individuals. The final calibration concentration ranges were as follows: 0, 50 ng, 100 ng, 250 ng, 500 ng, 1.0 μg and 2.0 μg TFMP per volume of human or rat urine; 0, 1 ng, 5 ng, 10 ng, 25 ng, 50 ng and 100 ng TFMP per volume of human plasma; and 0, 5 ng, 10 ng, 25 ng,

50 ng, 125 ng, 250 ng and 500 ng TFMP per volume of tissue homogenate. In all studies, the volumes of biological sample used in the calibration curves were the same as those used from the FLU-treated subjects. Calibration curves consisting of known, varying amounts of TFMP and a known, fixed amount of internal standard (2,4-dichlorophenol dissolved in distilled water) were included with each assay run.

2.5. Sample extraction

An aliquot (1 ml) of human plasma, human or rat urine (1 ml), or rat tissue homogenate (2 ml) was placed in a screw-cap culture tube (Fisher Scientific) and diluted, if required, to a final volume of 2 ml with distilled water. The internal standard (250 ng for rat liver and human urine extracts and 500 ng for rat brain homogenate) was added and the solution was thoroughly vortex-mixed. Following the procedure established for derivatization with PFBSC [14,17], the samples were then basified by adding excess potassium bicarbonate (400 mg) and briefly vortex-mixed. The mixture was then decanted into clean screw-cap culture tubes (160 mm×15 mm) to prevent the formation of an emulsion during shaking and to avoid carrying excess potassium bicarbonate through the procedure. Next, 4.5 ml of ethyl acetate containing acetonitrile (10%, v/v) and the derivatizing reagent, PFBSC (0.1%, v/v), was added to each sample. The two phases were mixed for 20 min in an Ika Vibrex VXR vortex-mixer (Janke and Kunkel, Staufen, Germany) and centrifuged for 2 min at 1000 *g* in a benchtop centrifuge (Sorvall GLC-2B general laboratory centrifuge, Du Pont, Wilmington, DE, USA). The upper ethyl acetate layer was retained and transferred to another screw-cap culture tube (160 mm×15 mm) and washed briefly by adding 600 μl distilled water, vortex-mixing for 5 s and centrifuging for 2 min at 1000 *g*. The upper organic layer was retained and transferred to a glass drying tube. The excess reagent was evaporated using a Savant Speed Vac SSI (Savant Instruments, Farmington, NT, USA) and the residue was reconstituted in 200 μl toluene. A final wash included adding 200 μl 1 *M* ammonium hydroxide to the samples, vortex-mixing for 5 s, transferring to 400 μl microfuge tubes and centrifuging for 10 s. The upper toluene layer was retained for GC analysis.

2.6. Gas chromatography

Samples were analysed using a chromatographic system consisting of a Hewlett-Packard (HP) 5880 GC equipped with a 15 mCi ^{63}Ni linear electron-capture detector (ECD) and a Hewlett-Packard Model HP 7673 A automatic sample injector. The chromatographic column was a narrow-bore fused-silica capillary column (25 m×0.32 mm I.D., 1.05 μm film of 5% phenylmethylsilicone as a stationary phase; Hewlett-Packard, Palo Alto, CA, USA). The carrier gas was ultra-pure helium (Praxair, Edmonton, Canada) at a flow-rate of 3 ml/min and the make-up gas was argon–methane (95:5; Praxair) at a flow-rate of 30 ml/min. The injector port and detector temperatures were 200°C and 325°C, respectively. The oven temperature was set initially at 80°C which was maintained for 0.5 min and then increased at a rate of 10°C/min to a final temperature of 270°C which was maintained for 10 min. A Hewlett-Packard 5880 A integrator was used to measure peak areas.

2.7. Gas chromatography–mass spectrometry

Confirmation of the chemical structures of the derivatives of TFMP and the internal standard, 2,4-dichlorophenol, was obtained by combined gas chromatography–mass spectrometry (GC–MS). The GC–MS system utilized a HP 5840A GC inlet coupled to an HP 5985A MS with dual EI/CI sources and an HP 7920 data system. The system also included an HP 2648A graphics terminal, an HP 9876A printer, HP 7920 disc drive (software) and HP 21 MX series E computer (hardware). Operating conditions for the MS were as follows: ion source temperature, 200°C; interface temperature, 275°C; column pressure, 34.5 kPa; accelerating voltage, 2200 eV; ionization voltage, 70 eV; scan speed, 100 u/s and dwell time, 200 ms. The column and temperature program that was utilized for the GC–ECD of TFMP was also utilized for GC–MS separation.

3. Results and discussion

The procedure described is useful for the detection and quantitation of TFMP, and the derivative that is

formed is stable. Typical chromatograms of derivatized extracts of brain and liver tissue homogenates obtained from drug-naive and FLU-treated rats are shown in Fig. 2. Similarly, Fig. 3 shows representative GC traces of derivatized extracts of patient urine obtained prior to drug treatment and following 3 weeks of daily treatment with FLU.

The chemical structures of the derivatives for TFMP and the internal standard were confirmed by combined GC–MS and are depicted in Figs. 4 and 5, respectively.

Calibration curves were obtained by analyzing standards prepared in parallel with the samples for each assay run. Regression analysis of the relationship between standard concentrations of TFMP and the chromatographic peak-area ratio of TFMP/internal standard yielded a linear relationship over the concentration range analyzed (1.0 ng to 2.0 μg of TFMP per volume of biological sample), with r^2 values of 0.996±0.001 (n=4) for rat urine samples, 0.994±0.001 (n=3) for rat brain samples, 0.996±0.002 (n=3) for rat liver samples, 0.997±0.0001 (n=3) for human urine samples and 0.985±0.006 (n=3) for human plasma samples. Calibration curves generated on different days were reproducible. The mean interassay coefficients of variation for TFMP were as follows: 7.4% (n=5) for 500 ng in extracts of rat urine, 9.7% (n=4) for 250 ng in samples of rat liver, 3.1% (n=4) for 50 ng in samples of rat brain, 6.9% (n=5) for 500 ng in extracts of human urine and 7.7% (n=4) for 25 ng in extracts of human plasma. The practical limit of

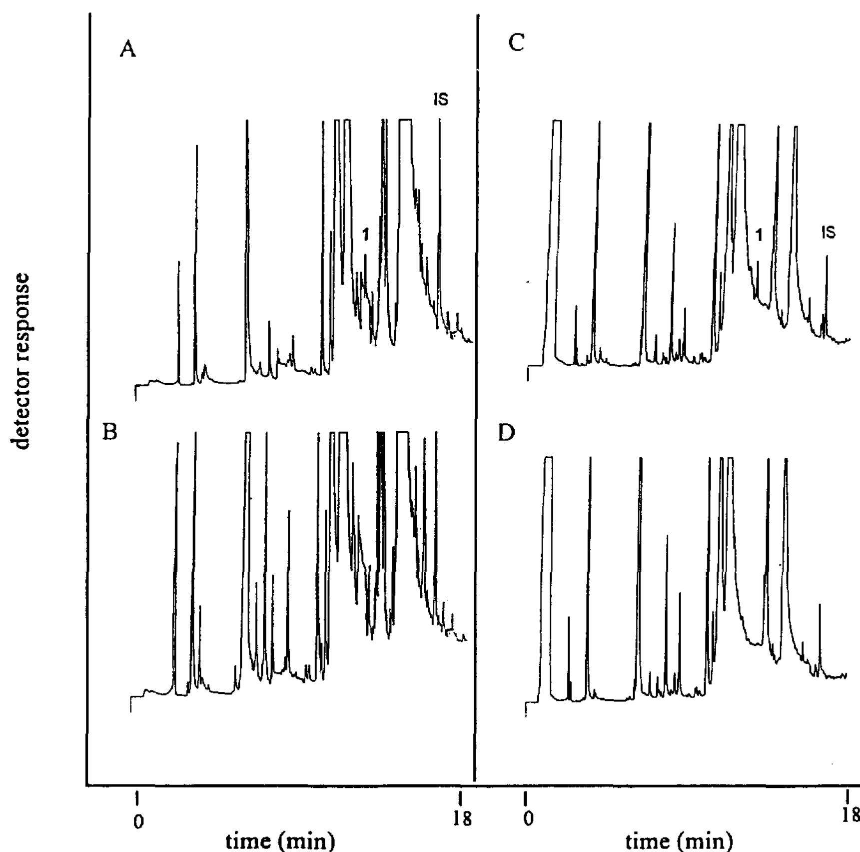

Fig. 2. Derivatized extracts of (A) brain and (C) liver from rats injected with fluoxetine (10 mg/kg) and sacrificed 5 h post-injection or of (B) brain and (D) liver from drug-naive rats injected with 20% DMSO in water. The GC peaks are identified as follows: 1=TFMP and I.S.=internal standard. The amount of I.S. added to brain samples (500 ng) was twice the amount of that added to liver samples (250 ng). The GC retention times of these peaks were 12.74 and 16.76 min, respectively.

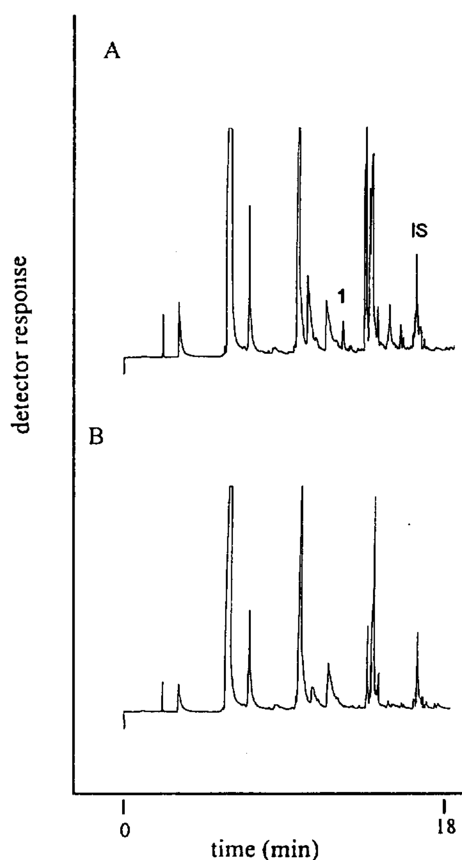

Fig. 3. Derivatized extracts of (A) urine obtained from depressed patients treated with FLU·HCl (Prozac; 10–20 mg once daily) for three weeks or (B) urine from drug-naive individuals. The GC peaks are identified as follows: 1=TFMP and I.S.=internal standard.

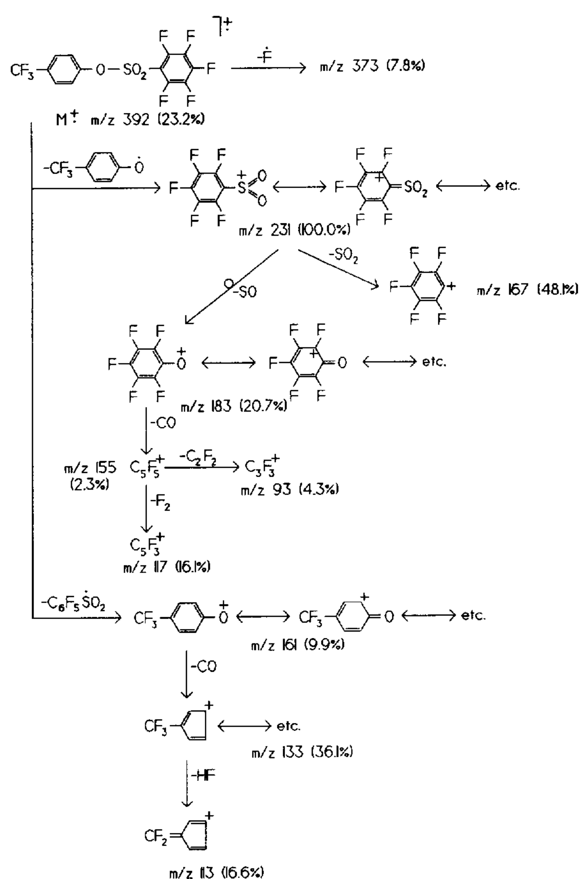

Fig. 4. Proposed electron impact mass spectrometric fragmentation pattern for the pentafluorobenzenesulfonyl derivative of TFMP. The % relative abundance values are shown in parentheses.

sensitivity (signal-to-noise ratio>3) for TFMP was <5 ng/ml (<25 pg "on-column") in human plasma samples, <10 ng/g of rat brain tissue, <25 ng/g of rat liver tissue and <25 ng/ml in human and rat urine extracts. The mean percent recovery of TFMP was 85.6±2.9% (n=5) from rat urine extracts, 89.4±3.6% (n=6) from rat liver samples, 86.2±1.9% (n=5) from rat brain samples, 86.3±3.9% (n=5) from human urine extracts and 87.5±4.6% (n=5) from human plasma extracts.

The mean free TFMP levels in brain and liver samples obtained from FLU-treated rats are shown in Table 1. Similarly, Table 1 shows 24 h urine levels of TFMP obtained from rats following a single injection of FLU.

Levels of free TFMP were also determined in plasma and urine from patients following 3 weeks of treatment of FLU·HCl (10–20 mg; once daily). The mean plasma levels of TFMP were determined to be 8.4±0.95 ng/ml plasma (Table 1). The mean urine levels of TFMP were determined to be 229±41 µg/24 h urine (Table 1). Preliminary results from our laboratory now indicate that TFMP may be extensively conjugated in human urine and studies are now underway to investigate this phenomenon in urine samples taken from humans and rats treated with FLU.

It will now be of interest to investigate whether one or more isozymes of cytochrome P450 (CYP) are involved in the formation of TFMP from FLU and its metabolite NFLU. Such a metabolic pathway

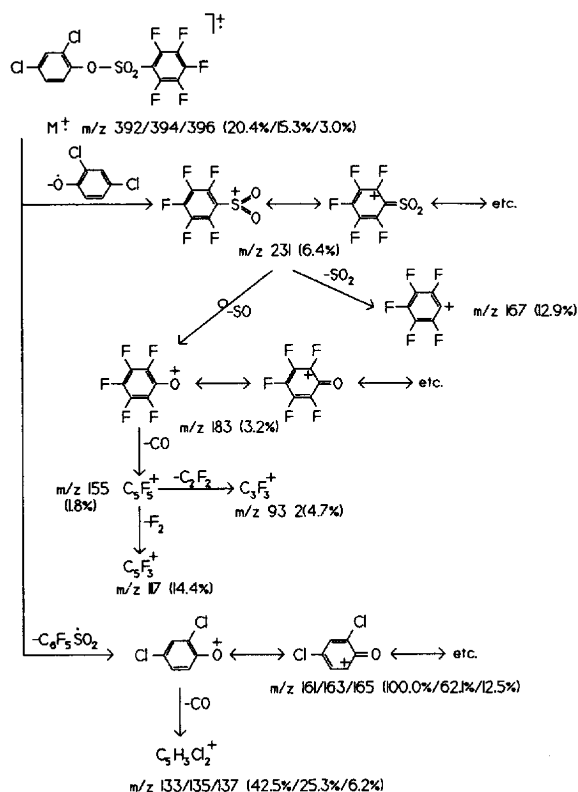

Fig. 5. Proposed electron impact mass spectrometric fragmentation pattern for the pentafluorobenzenesulfonyl derivative of the internal standard, 2,4-dichlorophenol. The % relative abundance values are shown in parentheses.

would involve O-dealkylation, a reaction which could involve a CYP isozyme, as is the case with drugs such as dextromethorphan [18].

The procedure described here is a useful method for the analysis of TFMP in biological samples and has been applied to the separation, detection and quantitation of TFMP in rat brain and liver tissues, human and rat urine and human plasma samples.

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Table 1
Concentrations of free TFMP in rat brain, liver and urine and in human urine and plasma (values represent means±S.E.M.)

Tissue or body fluid	Concentration	Sample size (*n*)	FLU Dosage
Rat urine	10.5±3.6 μg/24 h	8	10 mg/kg[1]
Rat brain	17.0±4.1 ng/g	6	10 mg/kg[2]
Rat liver	236±85 ng/g	8	10 mg/kg[2]
Human plasma	8.4±0.95 ng/ml	4	10–20 mg/day[3]
Human urine	229±41 μg/24 h	4	10–20 mg/day[3]

Rat urines were collected for 24 h[1] after a single i.p. injection of FLU. Following 10 drug-free days, the rats were reinjected with the drugs and sacrificed 5 h[2] post injection. The human plasma and urine samples were collected from patients who had been taking FLU for a period of 3 weeks[3].

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