Analytical characterization and pharmacological evaluation of the new psychoactive substance 4-fluoromethylphenidate (4F-MPH) and differentiation between the (±)-*threo*- and (±)-*erythro*- diastereomers.

Gavin McLaughlin, [a,b*] Noreen Morris, [a] Pierce V. Kavanagh, [b] John D. Power, [b,c] Geraldine Dowling, [b,d] Brendan Twamley, [e] John O'Brien, [e] Gary Hessman, [e] Brian Murphy, [d] Donna Walther, [f] John S. Partilla, [f] Michael H. Baumann, [f] and Simon D. Brandt [g]

[a] *Department of Life and Physical Sciences, School of Science, Athlone Institute of Technology, Dublin Road, Westmeath, Ireland*

[b] *Department of Pharmacology and Therapeutics, School of Medicine, Trinity Centre for Health Sciences, St. James's Hospital, Dublin 8, Ireland*

[c] *Forensic Science Ireland, Garda HQ, Phoenix Park, Dublin 8, Ireland*

[d] *School of Chemical and Pharmaceutical Sciences, Dublin Institute of Technology, Dublin 2, Ireland*

[e] *School of Chemistry, Trinity College Dublin, Dublin 2, Ireland*

[f] *Designer Drug Research Unit of the Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD 21224, USA*

[g] *School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, UK*

*Correspondence to: Gavin McLaughlin, Department of Pharmacology & Therapeutics, School of Medicine, Trinity Centre for Health Sciences, St. James's Hospital, Dublin 8, Ireland. E-Mail: gmclaug@tcd.ie or gavinmclaughlin@research.ait.ie

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Abstract

Misuse of (±)-*threo*-methylphenidate (methyl-2-phenyl-2-(piperidin-2-yl)acetate; Ritalin®, MPH) has long been acknowledged, but the appearance of MPH analogs in the form of 'research chemicals' has only emerged in more recent years. 4-Fluoromethylphenidate (4F-MPH) is one of these recent examples and this study presents the identification and analytical characterization of two powdered 4F-MPH products that were obtained from an online vendor in 2015. Interestingly, the products appeared to have originated from two distinct batches given that one product consisted of (±)-*threo*-4F-MPH isomers whereas the second sample consisted of a mixture of (±)-*threo* and (±)-*erythro* 4F-MPH. Monoamine transporter studies using rat brain synaptosomes revealed that the biological activity of the 4F-MPH mixture resided with the (±)-*threo*- and not the (±)-*erythro* isomers based on higher potencies determined for blockage of dopamine uptake (IC_{50} 4F-$MPH_{mixture}$ = 66 nM vs. IC_{50} (±)-*threo* = 61 nM vs. IC_{50} (±)-*erythro* = 8,528 nM) and norepinephrine uptake (IC_{50} 4F-$MPH_{mixture}$ = 45 nM vs. (±)-*threo* = 31 nM vs. IC_{50} (±)-*erythro* = 3,779 nM). In comparison, MPH was three times less potent than (±)-*threo*-4F-MPH at the dopamine transporter (IC_{50} = 131 nM) and around 2.5-times less potent at the norepinephrine transporter (IC_{50} = 83 nM). Both substances were catecholamine selective with IC_{50} values of 8,805 nM and >10,000 nM for (±)-*threo*-4F-MPH and MPH at the serotonin transporter. These findings suggest that the psychostimulant properties of (±)-*threo*-4F-MPH might be more potent in humans than MPH.

Introduction

Methylphenidate (methyl-2-phenyl-2-(piperidin-2-yl)acetate; MPH; Ritalin®) is a substituted phenethylamine that was first synthesized in 1944 and subsequently recognized as a psychostimulant (Figure 1).[1,2] MPH acts primarily by inhibiting the re-uptake of dopamine and norepinephrine resulting in elevated concentrations of these monoamine neurotransmitters within the synaptic cleft.[3,4] MPH is widely used in the treatment of attention deficit hyperactivity disorder (ADHD), as the symptoms of this condition are believed to be associated with suppressed levels of these neurotransmitters.[5,6] Originally, it was marketed as a mixture of two racemates, consisting of 80% (±)-*erythro* and 20% (±)-*threo* isomers, but successive studies demonstrated that the pharmacologically active diastereomers are associated with the (±)-*threo* form.[7-10] Subsequent studies focused on the separation of the two diastereomers and interconversion of (±)-*erythro* racemate to its (±)-*threo* counterpart. The (±)-*threo* racemate exists as two enantiomers, (+)-*threo*-MPH (*2R,2'R*) and *(-)-threo*-MPH (*2S,2'S*), and the absolute stereochemistry

of the most pharmacologically active enantiomer of MPH ((+)-*threo*) has been characterized[11-13] and developed as a medication to treat ADHD in its own right.[14] There have also been efforts to prepare enantiomerically pure *(2R,2'R)-(+)-threo*-MPH.[7] *In vivo*, (±)-*threo*-MPH undergoes enantioselective metabolism, which results in substantial differences in the plasma concentrations of its isomers, depending on route of administration and formulation.[15] Methylphenidate is listed as a Schedule II substance in the 1971 United Nations Convention on Psychotropic Substances.[16] In the United Kingdom (UK), it is controlled as a Class B substance under the Misuse of Drugs Act 1971.[17] In Ireland, it is controlled as a Schedule 2 substance under the Misuse of Drugs Regulations, 1988.[18]

Methylphenidate and an array of its analogs form a class of compounds that are well documented in the patent and scientific literature.[19-23] Some studies have focused on the evaluation of structure-activity relationships whereas others have studied these analogs for other therapeutic indications, including cocaine addiction, depression, fatigue-related disorders, cerebral palsy and fibromyalgia.[19-23] However, misuse of methylphenidate has been observed among recreational drug users, presumably due to its psychostimulant properties. For example, ingestion/insufflation of methylphenidate is popular among university students to increase concentration, improve alertness, or solely for recreational purposes.[24,25] In the last decade, several unregulated substances that are very closely related to methylphenidate have been launched on the new psychoactive substances (NPS) market. As with the majority of NPS launched by vendors, many of these methylphenidate analogs have already been described in the pharmaceutical research literature. For example, ethylphenidate, 3,4-dichloromethylphenidate (3,4-CTMP), methylnaphthidate (HDMP-28), isopropylphenidate (IPP/IPPD) and propylphenidate are methylphenidate analogs that have been detected on the 'research chemicals' market.[6,26] In April 2015, the UK government imposed a temporary drug control order (TCDO) on all these analogs.[27] Furthermore, the sale and supply of substances with psychoactive properties are now controlled in the UK under the newly introduced New Psychoactive Substances Act 2016.[28] Comprehensive analytical characterizations have recently been described for 4-methylmethylphenidate, 3,4-CTMP, ethylphenidate, 3,4-dichloroethylphenidate, *N*-benzyl-ethylphenidate and ethylnaphthidate (HDEP-28), respectively.[29] Recent studies from the UK associated ethylphenidate use to adverse reactions including deaths.[30-32] In addition, it was reported that injection was a common route of administration, thus adding the risk of harms associated with this method of administration. Interestingly, co-ingestion of ethanol and methylphenidate has been reported to form ethylphenidate *in vivo* by transesterfication.[26,33] In Ireland, the sale and supply of analogs of methylphenidate would be controlled under the Criminal Justice (Psychoactive Substances) Act 2010.[34]

4-Fluoromethylphenidate (4F-MPH) (Figure 1) is a methylphenidate analog that was developed within the pharmaceutical setting. However, in November 2015, it was first notified by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) Early Warning System following its detection on the recreational market.[35] Previous *in vitro* studies indicated that the addition of a fluorine atom to the *para*-position of the phenyl ring in the methylphenidate parent structure led to slight increases in potency related to displacement of [^{3}H]WIN-35,428 and [^{3}H]dopamine uptake inhibition.[4]

This study describes the analytical characterization of two powdered samples and a set of tablets of 4-fluoromethylphenidate that were obtained from the same vendor based in the United Kingdom in 2015. The study was triggered following the receipt of two powdered 4F-MPH samples from an online retailer. Various chromatographic, spectroscopic and mass spectrometric analysis methods were employed, followed by x-ray crystal structure analysis. One of the vendor samples was found to contain (±)-*threo*-4F-MPH, which was consistent with the expected racemate based on the current knowledge of the biological activity of (±)-*threo*-MPH. Unexpectedly, the analysis of the second sample revealed that it consisted of a mixture of (±)-*threo* and (±)-*erythro*-4F-MPH. This suggested that the two powdered vendor products might have originated from different batches. The two racemates were isolated from the mixture, purified, and converted to HCl salts prior to full analytical characterization. In addition, 4F-MPH tablets were also obtained from the same vendor and analysed for the presence of the (±)-*threo* and/or (±)-*erythro* form.

It was hypothesized that the distinct forms of 4F-MPH encountered in these products would result in different pharmacological properties similar to what has been reported for MPH, thus, potentially resulting in different effects in consumers. For this reason, the ability of the test drugs to inhibit uptake of [^{3}H]dopamine, [^{3}H]norepinephrine and [^{3}H]serotonin was investigated using synaptosomal preparations from rat brain. The isolated diastereomeric racemates (±)-*threo* and (±)-*erythro*-4F-MPH, the (±)-*threo*/(±)-*erythro* mixture, and MPH were included to study the effects at dopamine transporters (DAT), norepinephrine transporters (NET) and serotonin transporters (SERT), respectively.

Experimental

Chemicals

All reagents and dry solvents used in the syntheses were obtained from Sigma Aldrich Ltd (Arklow, Co. Wicklow, Ireland). LC-MS grade solvents were

obtained from Fisher Scientific (Dublin, Ireland). Preparative silica gel thin layer chromatography plates (UV_{254}, GF 20 x 20 cm, 2000 microns) were obtained from Analtech (Newark, NJ, USA). Two powdered samples of 4-fluoromethylphenidate (4F-MPH) and tablets were obtained from an online vendor based in the UK in 2015.

Isolation of 4-fluoromethylphenidate (4F-MPH) diastereomers

A solution of 4-fluoromethylphenidate (568 mg, vendor sample containing both diastereomers) in water (15 mL) was made basic with sodium hydroxide (pH 10-11). This was extracted with dichloromethane (2 x 10 mL). Drying (anhydrous magnesium sulphate) and removal of the solvent afforded a colorless oil (355 mg). Preparative TLC (silica gel, 2 mm; mobile phase: ethyl acetate; extraction solvent: ethanol) afforded two fractions, both colorless viscous oils (23 and 84 mg). The HCl salts were formed (2M hydrogen chloride in diethyl ether) and crystallized from ethanol for x-ray crystallography.

(±)-threo-4F-MPH freebase (bottom band of preparative TLC)

^{1}H NMR ($CDCl_3$) δ 7.36-7.22 (m; 2H; H-2'/6'), 7.14-6.98 (m; 2H; H-3'/5'), 3.70 (s; 3H; CH_3), 3.61 (d; *J* = 10.2 Hz; 1H; H-2), 3.25-3.13 (m; 2H; H-2'' and one H from H-6''), 2.75 (td; *J* = 12.0, 2.9 Hz; 1H; one from H-6''), 1.76-1.69 (m; 1H; one H from H-4''), 1.66-1.58 (m; 1H; one H from H-5''), 1.46 (dddd; 1H; *J* = 16.1, 12.4, 8.4, 3.8 Hz; one H from H-5''), 1.33 -1.19 (m; 2H; one H each from H-4'' and H-3'') and 1.08-0.97 (m; 1H; one H from H-3'') ppm; ^{13}C NMR ($CDCl_3$) δ 173.53 (C=O), 162.24 (d; J_{CF} = 246.5 Hz; C-4'), 131.98 (C-1'), 130.08 (d; J_{CF} = 7.8 Hz; C-2'/6'), 115.63 (d; J_{CF} = 21.5 Hz; C-3'/5'), 59.01 (C-2''), 57.07 (C-2), 52.32 (CH_3), 46.69 (C-6''), 29.29 (C-3''), 25.43 (C-5'') and 24.05 (C-4'') ppm; ^{19}F NMR ($CDCl_3$) δ -114.63 ppm.

(±)-threo-4F-MPH hydrochloride

Melting point: 202-204 ^{o}C. ^{1}H NMR (DMSO) δ 7.38-7.32 (m; 2H; H-2'/6'), 7.28-7.21 (m; 2H; H-3'/5'), 4.12 (d; *J* = 9.6 Hz; 1H; CH; H-2), 3.83-3.75 (m; 1H; CH; H-2'') 3.32 (s; 3H; CH_3), 3.28 (apparent d; *J* = 13.1 Hz; 1H; one H from H-6''), 2.96 (apparent t; *J* = 11.6 Hz; 1H; one H from H-6''), 1.74-1.64 (m; 2H; one H each from H-4'' and 5''), 1.63-1.57 (m; 1H; one H from H-5''), 1.47-1.38 (m; 1H; one H from H-4'') and 1.46-1.27 (m; 2H; H-3'') ppm; ^{13}C NMR (DMSO) δ 171.48 (C=O), 162.21 (d; J_{CF} = 244.9 Hz; C-4'), 131.03 (d; J_{CF} = 8.4 Hz; C-2'/6'), 130.52 (C-1'), 116.25 (d; J_{CF} = 21.5 Hz; C-3'/5'), 56.93 (C-2''), 53.05 (CH_3), 52.68 (C-2), 44.84 (C-6''), 26.03 (C-3''), 21.94 (C-5'') and 21.55 (C-4'') ppm; ^{19}F NMR (DMSO) δ -113.87 ppm. HR-APCI-MS observed *m/z* 252.139854 (theory $[M + H]^+$: $C_{14}H_{19}FNO_2$ *m/z* 252.139433, Δ = 1.7 ppm).

(±)-erythro-4F-MPH freebase (top band of preparative TLC)

^{1}H NMR ($CDCl_3$) δ 7.47-7.34 (m; 2H; H-2'/6'), 7.12-6.99 (m; 2H; H-3'/5'), 3.68 (s; 3H; CH_3), 3.56 (d; *J* = 10.3 Hz; 1H; H-2), 3.11 (td; *J* = 10.1, 2.2 Hz; 1H; H-2''), 3.01 (apparent d; *J* = 11.6 Hz; 1H; one H from H-6''), 2.55 (td; *J* = 11.6, 2.9 Hz; 1H; one H from H-6''), 1.87-1.68 (m; 2H; one H from H-3'' and H-4''), 1.66-1.58 (m; 1H; one H from H-5'') and 1.55-1.25 (m; 3H; one H each from H-3'', 4'' and 5'') ppm; ^{13}C NMR ($CDCl_3$) δ 172.84 (C=O), 162.50 (d; J_{CF} = 246.5 Hz; C-4'), 131.46 (C-1'), 130.36 (d; J_{CF} = 7.9 Hz; C-2'/6'), 115.95 (d; J_{CF} = 21.0 Hz; C-3'/5'), 59.09 (C-2''), 57.04 (C-2), 52.06 (CH_3), 46.94 (C-6''), 30.58 (C-3''), 25.39 (C-5'') and 24.22 (C-4'') ppm; ^{19}F NMR ($CDCl_3$) δ -114.28 ppm.

(±)-erythro-4F-MPH hydrochloride

Melting point: 198-200 °C.^{1}H NMR (DMSO) δ ppm 7.51-7.44 (m; 2H; H-2'/6'), 7.32-7.24 (m; 2H; H-3'/5'), 4.11 (d, *J* = 9.4 Hz; 1H; CH; H-2), 3.70-3.66 (m; 1H; CH; H-2''), 3.65 (s; 3H; CH_3), 3.14 (apparent d, *J* = 13.0 Hz; 1H; one H from H-6''), 2.83-2.80 (m; 1H; one H from H-6''), 1.91-1.85 (m; 1H; one H from H-3''), 1.82-1.69 (m; 2H; H-4''/H-5''), 1.68-1.59 (m; 2H; one H each from H-4''/H-5'' and H-3'') and 1.55-1.46 (m; 1H; one H from H-4''/H-5'') ppm; ^{13}C NMR (DMSO) δ 171.36 (C=O), 162.81 (d; J_{CF} = 244.5 Hz; C-4'), 131.55 (d; J_{CF} = 8.5 Hz; C-2'/6'), 129.85 (C-1'), 116.65 (d; J_{CF} = 21.6 Hz; C-3'/5'), 57.55 (C-2), 53.55 (C-2''), 53.00 (CH_3), 45.26 (C-6''), 27.39 (C-3''), 22.10 (C-5'') and 21.93 (C-4'') ppm; ^{19}F NMR (DMSO) δ -113.80 ppm. HR-APCI-MS observed *m/z* 252.139659 (theory $[M + H]^+$: $C_{14}H_{19}FNO_2$ *m/z* 252.139433, Δ = 0.9 ppm).

Instrumentation

Gas chromatography-mass spectrometry (GC-MS)

Samples were prepared to give a 1 mg/mL solution in methanol and analyzed on an Agilent 6890N GC coupled to 5975 Mass Selective Detector (Agilent, Little Island, Cork, Ireland). A HP-ULTRA 1 column (12 m × 0.2 mm × 0.33 µm) was used with helium carrier gas at a constant flow of 1 mL/min and a split ratio of 1:1. The injector was set at 250 °C and the transfer line at 280 °C. The initial oven temperature was 50 °C, held for 2 min, then ramped at 10 °C/min to 100 °C with no hold time. The oven temperature was further ramped at 5 °C/min to 200 °C and then finally ramped at 20 °C/min to 295 °C with a hold time of 1 min. The mass spectra were collected after a 4.0 min solvent delay time. The ionization energy was set at 70 eV and the mass range was *m/z* 40-550. The total run time was 32.75 min.

Liquid chromatography-mass spectrometry (LC-MS)

LC-MS analyses were performed on an Agilent 1100 HPLC system equipped with a G13795 degasser, G1312A BinPump, a G1313A ALS and G1316A column oven (COLCOM) (Agilent, Little Island, Cork). Separation was obtained on an Allure PFP Propyl column (5 µm, 50 x 2.1 mm) Restek (Bellefonte, PA, USA). Mobile phase A consisted of 0.1% formic acid in water, whereas, mobile phase B consisted of 0.1% formic acid in acetonitrile. The Agilent LC-MSD settings were as follows: positive electrospray mode, capillary voltage 3500 V, drying gas (N_2) 12 L/min at 350 ^oC, nebulizer gas (N_2) pressure 50 psi, SIM *m/z* 252 and scan mode *m/z* 70-500, fragmentor voltage 50 and 110 V. Samples for LC-MS analysis were dissolved in acetonitrile/water (1:1, containing 0.1% formic acid) at a concentration of 10 µg/mL. The injection volume was 5.0 and 10.0 µL, flow rate was 0.8 mL/min and the column temperature was 30 ^oC. Total run time was 25 min. The following gradient elution program was used: 0-2 min 2% B, followed by an increase to 60% B within 15 min, followed by another increase to 80% B within 18 min before returning to 2% B within 25 min.

High resolution mass spectrometry (HR-MS)

APCI experiments were carried out on a Bruker micrOTOF-Q III spectrometer interfaced to a Dionex UltiMate 3000 LC. The Agilent tuning mix APCI-TOF was used for mass calibration. Masses were recorded over a range of *m/z* 100-1600. Operating conditions were as follows: capillary voltage 4000 V, corona 4000 nA, nebulizer gas 2.0 bar, drying gas flow rate 3.0 L/min, drying gas temperature 100-200 ^oC, vaporizer temperature 100-400 ^oC. MicroTof control and HyStar software were used for data analysis.

Nuclear magnetic resonance spectroscopy (NMR)

The free base samples were prepared in deuterated chloroform ($CDCl_3$) at a concentration of 20 mg/mL. The hydrochloride salt samples were prepared in deuterated dimethyl sulfoxide (DMSO-d_6) at a concentration of 20 mg/mL. ^1H (600 MHz) and ^{13}C (150 MHz) spectra were recorded on a Bruker AV600 NMR spectrometer using a 5 mm TCI cryoprobe. ^1H NMR spectra were referenced to an external TMS reference at δ = 0 ppm. ^{19}F (376 MHz) spectra were recorded on a Bruker DPX400 NMR spectrometer and the external reference was trifluorotoluene set at δ = - 64 ppm.

X-ray crystallography

Data for samples (±)-*erythro* and (±)-*threo*-4F-MPH were collected on a Bruker APEX DUO using Cu Kα radiation (λ = 1.54178 Å) at 100(2) K (Oxford

Cobra Cryosystem) with a Mitegen holder. Bruker APEX[36] software was used to collect and reduce data, determine the space group. XT[37] was used to solve and XL[38] in OLEX 2[39] refine the structure. Absorption corrections were applied using SADABS 2014.[40] All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were assigned to calculated positions using a riding model with appropriately fixed isotropic thermal parameters. N-H hydrogens were located and refined with restraints and with the U_{iso} = 1.2U_{eq} of the carrier atom in both structures. In the *threo*-4F-MPH structure, the fluorophenyl ring was disordered in two positions with occupancies of 85/15%. Restraints were used in the model (DFIX, RIGU, SADI).

Crystal Data for (±)-*erythro*-4F-MPH, $C_{14}H_{19}NO_2FCl$ (*M* =287.75 g/mol): monoclinic, space group $P2_1/n$ (no. 14), *a* = 10.4556(5) Å, *b* = 8.5173(4) Å, *c* = 15.7304(7) Å, *β* = 90.6392(15)°, *V* = 1400.76(11) Å^3, *Z* = 4, *T* = 100.0 K, μ(CuKα) = 2.510 mm^{-1}, *Dcalc* = 1.364 g/cm^3, 18265 reflections measured (10.106° ≤ 2Θ ≤ 137.044°), 2577 unique (R_{int} = 0.0376, R_{sigma} = 0.0232) which were used in all calculations. The final *R_1 was 0.0355 (I > 2σ(I)) and *w*R_2 was 0.1108 (all data). CCDC No. 1503223.

Crystal Data for (±)-*threo*-4F-MPH, $C_{14}H_{19}ClFNO_2$ (*M* =287.75 g/mol): monoclinic, space group $P2_1/n$ (no. 14), *a* = 11.289(3) Å, *b* = 7.2232(16) Å, *c* = 18.494(5) Å, *β* = 106.169(7)°, *V* = 1448.4(6) Å^3, *Z* = 4, *T* = 100(2) K, μ(CuKα) = 2.428 mm^{-1}, *Dcalc* = 1.320 g/cm^3, 20393 reflections measured (8.286° ≤ 2Θ ≤ 136.614°), 2656 unique (R_{int} = 0.0813, R_{sigma} = 0.0513) which were used in all calculations. The final *R_1 was 0.0525 (I > 2σ(I)) and *w*R_2 was 0.1357 (all data). CCDC No. 1503224. * $R_1 = \Sigma||F_o| - |F_c||/\Sigma|F_o|$, w$R_2 = [\Sigma w(F_o^2 - F_c^2)^2/\Sigma w(F_o^2)^2]^{1/2}$.

Infrared Spectroscopy

IR spectra were recorded on a Perkin Elmer Spectrum 100 FT-IR with Universal ATR sampling accessory (Perkin Elmer, Waltham, MA, USA). The wavelength resolution was set to 2 cm^{-1}. IR spectra were collected in a range of 650 - 4000 cm^{-1} with 16 scans per spectrum. The IR data were processed using Spectrum Perkin Elmer Version 6.3.4 Software (Perkin Elmer, Waltham, MA, USA).

Monoamine transporter assays

Male Sprague-Dawley rats (250-300 g, Charles River Laboratories, Wilmington, MA, USA) were housed 2 per cage and maintained on a 12 h light-dark cycle. Food and water were provided ad libitum. Animal use procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Care and Use Committee of

the Intramural Research Program of the National Institute on Drug Abuse (Baltimore, MD, USA). Rats were euthanized by CO_2 narcosis and brains were processed to yield synaptosomes as previously described.[41,42] For uptake assays, synaptosomes were incubated with different concentrations of the test drugs in the presence of 5 nM [^{3}H]dopamine, 10 nM [^{3}H]norepinephrine, or 5 nM [^{3}H]serotonin. The uptake assays were terminated by vacuum filtration and retained radioactivity was quantified by scintillation counting.

Results and Discussion

Manufacturers and entrepreneurs dedicated to the commercial exploration of 'research chemicals' destined for recreational drug markets, are utilizing the scientific and patent literature to generate ideas for launching new compounds. Since methylphenidate and a series of its analogs have been well documented in the literature, e.g.[19-23], it was not surprising to see that several methylphenidate-based NPS have also been encountered on the 'research chemicals' market. One of the more recent methylphenidate-based NPS was 4-fluoromethylphenidate (4F-MPH). 4F-MPH is one of the methylphenidate analogs described in the literature in studies evaluating ring substituted methylphenidate analogs as potential therapeutics for cocaine addiction and fibromyalgia.[19-21] 4F-MPH did not appear to have a history of human usage prior to its distribution online through vendors of 'research chemicals'.

In this study, two powdered samples and a set of tablets were obtained from the same vendor based in the United Kingdom in 2015. Initially, it was noticed that the two powdered products showed different solubility in solvents such as methanol and water. As the analyses progressed, it was noticed that one product contained the *threo*-racemate, whereas the other included a second compound suspected to represent a diastereomeric form based on similar mass spectra. Analysis by gas chromatography-mass spectrometry (GC-MS) suggested that this was the case and it was hypothesized that this product was most likely the racemic *erythro*- form of 4F-MPH. In order to investigate further, the 4F-MPH sample containing both diastereomers was dissolved in water and made alkaline with sodium hydroxide. This was extracted with dichloromethane, dried with anhydrous magnesium sulphate, followed by solvent removal to afford a colorless oil. Preparative thin-layer chromatography (TLC) afforded two fractions as colorless viscous oils. The hydrochloride salts were formed (2M hydrogen chloride in diethyl ether), crystallized from ethanol and subjected to extensive analytical characterization.

From examination of the methylphenidate synthesis literature, it seemed plausible that the presence of the *erythro*-racemate might have reflected a lack of purification at the end of the synthesis procedure. For example, one synthesis procedure employed for a range of methylphenidate analogs[4] involved the reaction of 2-phenylacetonitrile and 2-bromopyridine with potassium *tert.*-butoxide in tetrahydrofuran (THF). This led to the formation of a nitrile intermediate and a ketone by-product, which could be easily removed in the subsequent steps. The nitrile intermediate was hydrolysed under acidic conditions leading to the formation of an acetamide species. Hydrogenation of the pyridine ring to piperidine followed, leading to the formation of two racemic *erythro*- and *threo* acetamide intermediates. At this stage, the *erythro* species was present at a higher ratio compared to the *threo*-species. This mixture was then hydrolysed to form *erythro*- and *threo*-acetic acid entities. An epimerization process with potassium hydroxide flipped these ratios leading to a larger percentage of the *threo*-acetic acid intermediate. Esterification with methanol leads to the formation of the final *threo*/*erythro* acetate species, which was then purified by recrystallization to isolate the (±)-*threo*-racemate.[4] From this perspective, and provided a similar synthetic route was used by the manufacturers, failure to isolate the (±)-*threo*-racemate by appropriate recrystallization might have resulted in the presence of both racemates in the final product. An alternative explanation might be that the manufacturers decided to skip the recrystallization step on purpose.

Analytical Features

Gas chromatography mass spectrometry

The gas chromatographic method used was able to achieve satisfactory separation between the (±)-*threo*- (18.13 min) and (±)-*erythro*- racemates (18.04 min) (Figure 2). The EI mass spectra obtained for both racemates were identical. The base peak was observed at *m/z* 84 and it is suggested that this was due to the formation of a tetrahydropyridinium species ($C_5H_{10}N^+$), following the loss of a methyl 4-fluorophenylacetate moiety from the molecular ion. This fragment was consistent with the EI mass spectral data for methylphenidate, ethylphenidate and 4F-MPH available in the literature.[26,43-46] The fragment observed at *m/z* 190 was consistent with the formation of a 4-fluorophenyl(piperidin-2-ylidene)methylium species ($C_{12}H_{13}FN^+$). The detection of *m/z* 168 may be rationalized by the loss of the tetrahydropyridine moiety from the parent structure, which gave rise to a radical cation and formation of a methyl fluorophenylacetate species ($C_9H_8FO_2^{\bullet+}$). This fragment was also consistent with the EI-MS data for methylphenidate and ethylphenidate.[26,43-44] However, in the case of methylphenidate and ethylphenidate, this ion would be encountered at *m/z* 150 (methyl

phenylacetate moiety) and *m/z* 164 (ethyl phenylacetate moiety), respectively. The fragment at *m/z* 109 can be described by the formation of a tropylium ion, consistent with the formation of tropylium ion reported in the EI-MS of methylphenidate and ethylphenidate at *m/z* 91. The fragment at *m/z* 56 could represent the formation of a dihydroazetium ion. During GC analysis, a second product at 7.26 min was also observed in the chromatogram (Supporting Information) and it was believed that this product might have been a degradant resulting from the thermal decomposition of 4F-MPH within the GC system. This GC-induced reaction was thought to involve the loss of the tetrahydropyridine moiety and the formation of an enol tautomer of methyl 4-fluorophenylacetate, which subsequently tautomerized to methyl 4-fluorophenyl acetate. Interestingly, the formation of the GC-induced product, methyl 4-fluorophenyl acetate, was found to be slightly higher (53.6 %) in the chromatogram of the (±)-*threo*- 4FMPH racemate, compared to that of the (±)-*erythro*- 4FMPH racemate (43.7%). This type of degradation was consistent with literature reports on methylphenidate and its analogs[29,47-48], however, the suggested identity remains tentative as a reference standard was not available.

Liquid chromatography-mass spectrometry

Analysis of both (±)-*threo*- and (±)-*erythro*- racemates of 4F-MPH using high performance liquid chromatography (HPLC) confirmed satisfactory separation for identification purposes. A retention time of 8.90 min was obtained for the (±)-*erythro* form, whereas a retention time of 9.81 min was obtained for the (±)-*threo* racemate (Figure 3). The electrospray ionization (ESI) single quadrupole mass spectra obtained from in-source collision-induced dissociation (CID) (fragmentor 110 V) of the 4F-MPH racemates shared two key ions. The suggested pathways are shown in Figure 3. In the mass spectra of both racemates, the fragment observed at *m/z* 252 represented the protonated molecule with 100% relative abundance. The product ion observed at *m/z* 84 might have represented a loss of the methyl 4-fluorophenylacetate moiety from the protonated molecule resulting in the formation of a tetrahydropyridinium species ($C_5H_{10}N^+$) and this was also present at approximately 95% abundance.

Nuclear magnetic resonance spectroscopy (NMR)

The chemical structures of the (±)-*threo*- and (±)-*erythro*- 4F-MPH racemates and powdered vendor samples were elucidated using both one-dimensional and two-dimensional NMR experiments (Figure 4, Supporting Information). The NMR spectra associated with the 4F-MPH HCl salt forms of the (±)-*erythro*- and (±)-*threo*- racemates shared similar characteristics but significant differences could also be observed between some of the proton and carbon

chemical shifts that facilitated differentiation between the racemates. For example, in the ^{1}H spectrum of the (±)-*erythro*- racemate, the signals associated with the protons on the aromatic ring were observed as multiplets between 7.51-7.44 and 7.32-7.24 ppm. However, in the spectrum of the (±)-*threo*- counterpart these proton resonances were shifted upfield to 7.38-7.32 and 7.28-7.21 ppm, respectively. Furthermore, the proton signal for the methyl group of the (±)-*threo*- racemate was shifted upfield (3.32 ppm) compared to the same signal associated with the (±)-*erythro* racemate (3.65 ppm). The proton environment around the piperidine ring provided further distinguishing features. For example, in the ^{1}H spectrum of the (±)-*erythro*- racemate, the proton signals associated with H-2" were observed as a multiplet between 3.70-3.66 ppm. The same proton resonances in the (±)-*threo*- counterpart were also observed as a multiplet but were shifted downfield to 3.83-3.75 ppm. Moreover, one proton associated with H-6" was observed as a doublet at 3.14 ppm in the proton spectrum of (±)-*erythro*- 4F-MPH. In comparison, the same proton signal in the (±)-*threo*- counterpart was shifted downfield and observed at 3.28 ppm (Figure 4). The second proton associated with H-6" also provided a distinguishing feature as it appeared as a triplet at 2.96 ppm in the (±)-*threo*- spectrum. However, the same proton signal in the (±)-*erythro*- counterpart was observed as a multiplet between 2.83-2.80 ppm.

Some distinguishing features were also observed in the ^{13}C NMR spectra (Supporting Information). For example, the ^{13}C spectrum of the (±)-*erythro*- racemate revealed the carbonyl chemical shift at 171.36 ppm, whereas the same signal was observed at 171.48 ppm in the spectrum of the (±)-*threo*- counterpart. The aromatic region also provided distinct differences. In the spectrum of the (±)-*erythro*- racemate, the aromatic carbon signals (C-2'/6', C-3'/5') were observed at 131.55 and 116.65 ppm. In the spectrum of the (±)-*threo*- racemate, the same carbon signals were shifted upfield to 131.03 and 116.21 ppm, respectively. However, another aromatic carbon signal (C-1') was observed at 129.85 ppm in the (±)-*erythro*- spectrum, whereas the same carbon signal was shifted downfield and observed at 130.52 ppm in the (±)-*threo*- spectrum. Moreover, the carbon of the methine group (C-2) was observed at 57.55 ppm in the spectrum of the (±)-*erythro*- racemate, which shifted upfield in the (±)-*threo*- counterpart and observed at 52.68 ppm. The piperidine carbon (C-2") was observed at 53.55 ppm in the spectrum of the (±)-*erythro*- racemate, which shifted downfield in the (±)-*threo*- counterpart and observed at 56.93 ppm. In general, all carbon signals associated with the piperidine ring (44.84, 26.03, 21.94 and 21.55 ppm) were shifted upfield in the (±)-*threo*- spectrum compared to the same signals in the (±)-*erythro*- spectrum (45.26, 27.39, 22.10 and 21.93 ppm). The mixture contained the characteristics of both racemates.

X-Ray crystallography

Both salts were crystallized from a water/ethanol mixture to give colorless needle shaped crystals. The crystal structures of both diastereomers are shown in Figures 5A and 5B. Both crystallized in the monoclinic space group P2(1)/n as the chloride salt. The (±)-*erythro* salt is shown as the (*R*,*S*)-enantiomer (C2, C3) and the (±)-*threo* as the (*S*,*S*) enantiomer. As these molecules crystallized in a centrosymmetric space group, the inverse enantiomer was also equally present. The piperidine ring in both compounds was in the chair conformation. In both diastereomers, the piperidine was substituted in an equatorial position. The plane of piperidine ring to the fluorophenyl ring was 69.9° in the *erythro* and almost orthogonal at 84.5° in the *threo* salt. Each NH_2 group was involved in hydrogen bonding. In the *erythro* salt the Cl^- anion was strongly hydrogen bound to two NH_2 groups (N1…Cl1, 3.0704(12)Å, 170.1(15)°; N1…Cl1$^{\#4}$, 3.2414(12)Å, 146.8(16)°, symmetry transformation #4 = 1-x, 1-y, 1-z) with the carbonyl oxygen more loosely held by weak C-H interactions (C3…O1$^{\#1}$, 3.3239(17)Å, 150°, symmetry transformation #1 = -x, 1-y, 1-z). This formed a symmetric dimer (Supporting Information). The *threo* salt also displayed NH…Cl interactions (N1…Cl1 3.134(2)Å, 167(3)°; N1…Cl1$^{\#1}$ 3.215(2)Å, 164(2)°, symmetry transformation #1 = 3/2-x, 1/2+y, -1/2-z). However, N1 formed a bifurcated hydrogen bond to the carbonyl oxygen (N1…O1$^{\#1}$ 3.011(3)Å, 113(2)°). Unlike the *erythro* salt, the *threo* salt formed an extended ribbon parallel to the b – axis (Supporting Information) These structural motifs are also seen in similar congeners which also form hydrogen bonded dimers or ribbons extending parallel to the b-axis.[49]

Infrared spectroscopy

The (±)-*threo*- and (±)-*erythro*- racemates of 4F-MPH were also subjected to analysis by infrared (IR) spectroscopy. The IR spectra associated with the 4F-MPH HCl salt forms of the (±)-*erythro*- and (±)-*threo*- racemates shared similar characteristics although one difference was observed that facilitated differentiation (Supporting Information). The absorbance for the carbonyl group was observed at 1743 cm^{-1} for the (±)-*threo*- racemate compared to 1726 cm^{-1} for the (±)-*erythro*- racemate. The vendor sample consisting of both racemates contained two carbonyl absorbance values consistent with those of the isolated (±)-*erythro*- and (±)-*threo*- 4FMPH racemates. The second powdered vendor sample and the tablet shared the same characteristics as those of the (±)-*threo*- 4FMPH racemate. It was encouraging to observe that the infrared spectroscopic analysis was able to provide an important distinguishing feature between the 4F-MPH racemates, which would be beneficial when working within a forensic science laboratory setting.

Monoamine transporter activity

When the analytical investigation revealed that two distinct 4F-MPH products were present, further questions arose about the potential for distinct pharmacological properties associated with the individual (±)-*threo*- and (±)-*erythro*-form of 4F-MPH. Figure 6 shows the effects of the (±)-*threo*/*erythro* mixture, (±)-*threo*-4F-MPH, (±)-*erythro*-4F-MPH and methylphenidate (MPH) on the uptake of [^{3}H]dopamine (DA), [^{3}H]norepinephrine (NE) and [^{3}H]serotonin (5-HT) by their respective transporters DAT, NET and SERT (Figure 6A-6D). The corresponding IC_{50} values for inhibition of uptake are provided in Table 1 and MPH was included for comparison. As revealed from the dose response curves, all test drugs were fully effective uptake inhibitors at DAT and NET, with little activity at SERT, thus, displaying catecholamine selectivity.

The 4F-MPH mixture was about twice as potent as MPH at DAT and NET, although addition of the fluorine atom to the 4-position of the phenyl ring did not increase potency at SERT. Importantly, the data shown in Table 1 indicate that the biological activity of the 4F-MPH mixture predominantly resided with the (±)-*threo*- and not the (±)-*erythro* isomers given that higher potencies were determined for dopamine uptake (IC_{50} *threo* = 61 nM vs. IC_{50} *erythro* = 8,528 nM) and norepinephrine uptake (IC_{50} *threo* = 31 nM vs. IC_{50} *erythro* = 3,779 nM) at DAT and NET, respectively. The reduced activity of (±)-*erythro* isomers at DAT has been reported previously on other ring-substituted analogs.[4] Previous investigations also demonstrated that (±)-*threo*-4F-MPH showed nanomolar binding affinity toward DAT (IC_{50} = 35 nM, [^{3}H]WIN-35,428) and that it inhibited [^{3}H]dopamine uptake via DAT in rat brain synaptosomal preparations (IC_{50} = 142 nM)[4] consistent with the findings reported in this study. Consistent with the data shown in Table 1, (±)-*threo*-4F-MPH did not show any appreciable binding affinity at SERT (IC_{50} > 10 µM, [^{3}H]citalopram). Furthermore, (±)-*threo*-4F-MPH was shown to be about three times more potent than MPH in a drug discrimination assay.[50] Overall, it appears that the acetate group and the piperidine ring must be oriented in the opposite direction for the drugs to interact optimally with transporter proteins.

Conclusion

The analytical characterization of two 4F-MPH powders obtained from the same vendor of 'research chemicals' revealed the presence of (±)-*threo* isomers in one and a mixture of (±)-*threo* / (±)-*erythro*-4F-MPH racemates in the other, which suggested a failure to isolate the (±)-*threo*-racemate that may have formed during the synthesis procedure of the main (±)-*threo* product. Various chromatographic, spectroscopic and mass spectrometric platforms were employed followed by x-ray crystal structure analysis. (±)-*threo*-4F-MPH

was shown to be more potent than methylphenidate in its ability to inhibit uptake of [^{3}H]dopamine and [^{3}H]norepinephrine into rat brain synaptosomes, with significantly less activity associated with inhibition of the serotonin transporter. The significantly reduced potency of (±)-*erythro*-4F-MPH was consistent with (±)-*erythro*-MPH and other (±)-*erythro*-MPH analogs reported in the literature. These findings suggest that the psychostimulant properties of (±)-*threo*-4F-MPH might be more potent in humans than MPH. Since the biological activity resides in the (±)-*threo* form, it is anticipated that other MPH-derived 'research chemicals' on the market might also display this configuration.

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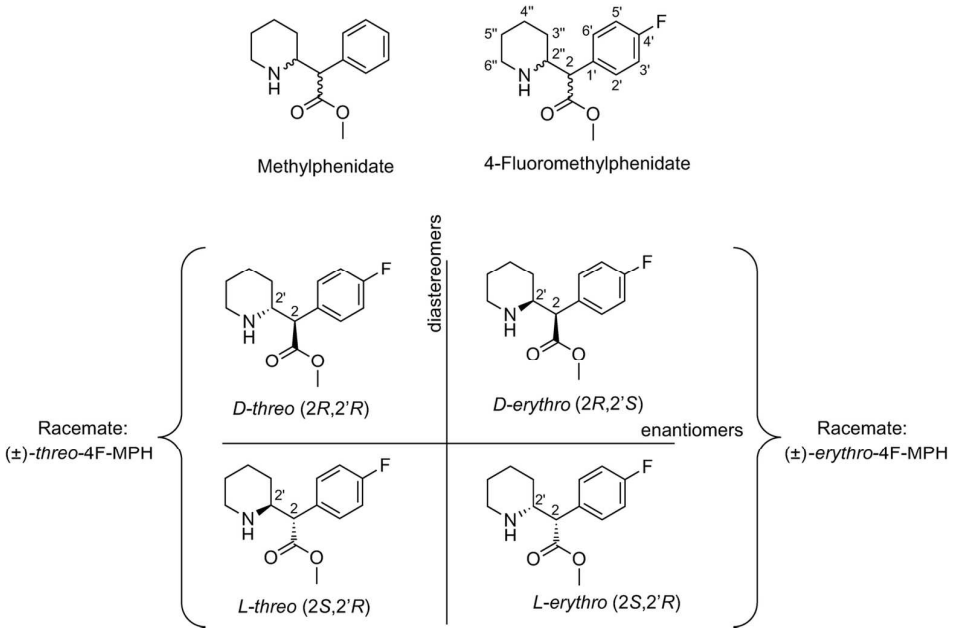

Figure 1. Chemical structures of methylphenidate, (±)-*threo*-racemate and (±)-*erythro*-racemate of 4-fluoromethylphenidate

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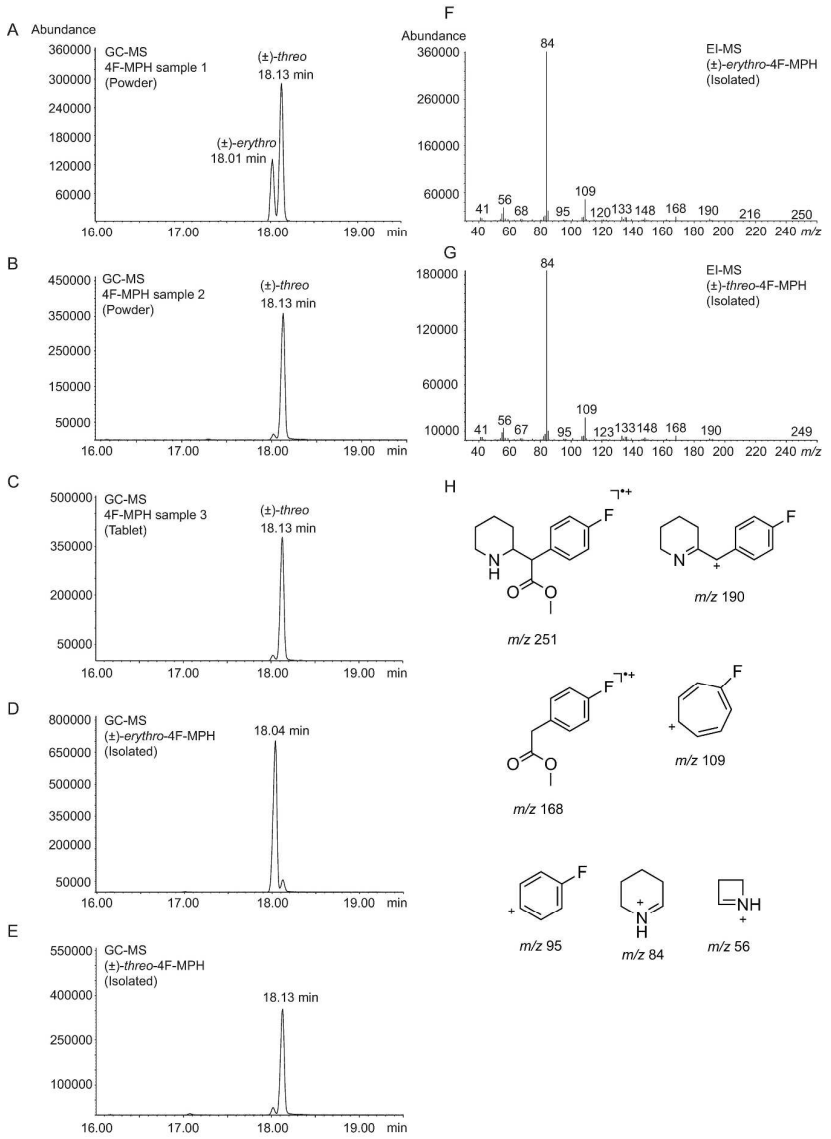

Figure 2. A-E: Gas chromatographic (GC) separation obtained for the isolated (±)-*threo*-racemate, isolated (±)-*erythro*-racemate of 4F-MPH and vendor samples. F-G: Electron ionization mass spectra obtained for the isolated (±)-*threo*- and (±)-*erythro*- racemates of 4F-MPH. H: Proposed fragmentation pattern for both racemates.

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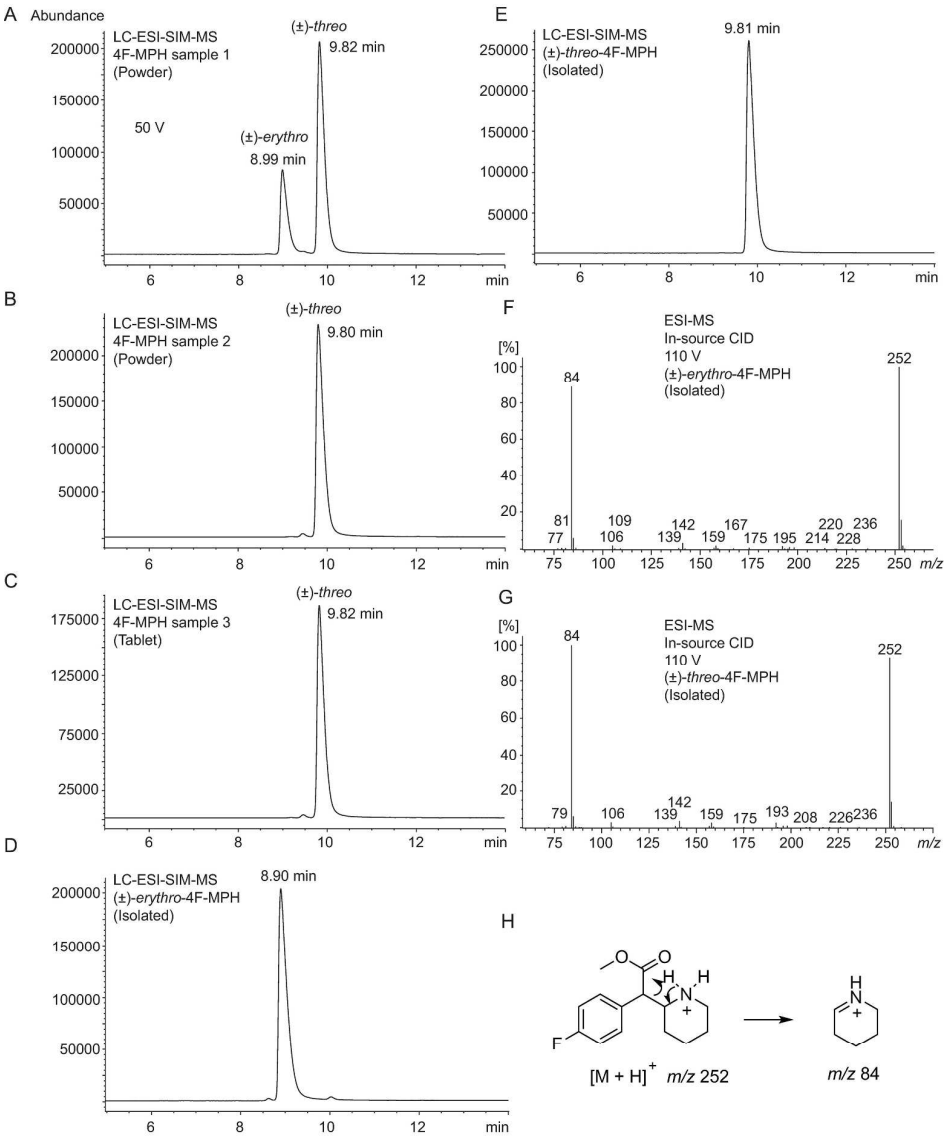

Figure 3. A-E: HPLC separation achieved for the isolated (±)-*threo*-racemate, isolated (±)-*erythro*-racemate and vendor samples of 4F-MPH. F-G: Product ion spectra obtained for isolated (±)-*threo*- and (±)-*erythro*-racemates of 4F-MPH obtained from in-source collision induced dissociation at increased fragmentor voltage (110 V). H: Proposed fragments for both racemates.

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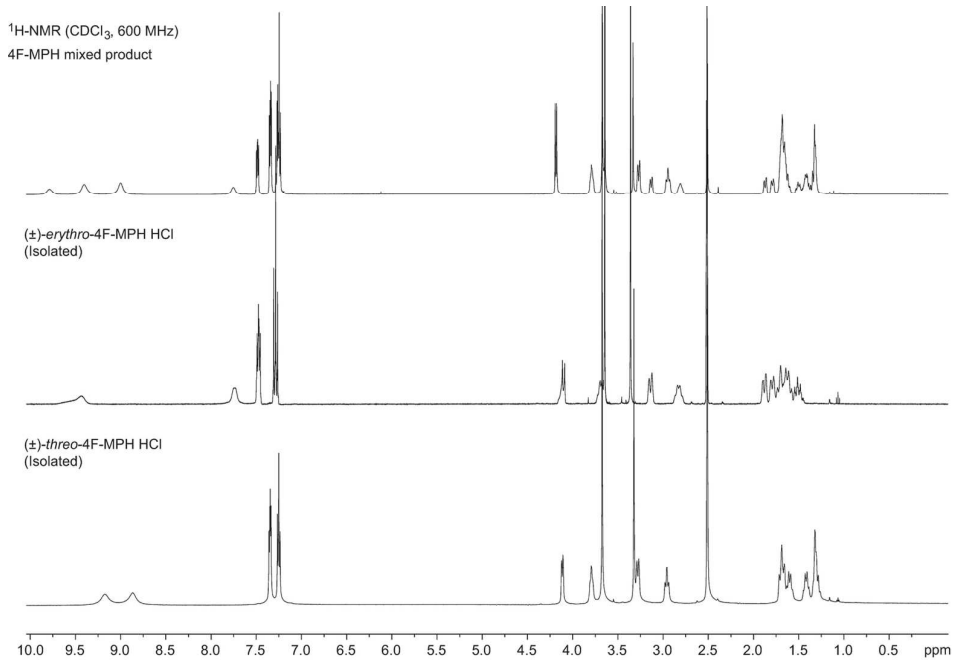

Figure 4. ^{1}H NMR spectra obtained for the mixed 4F-MPH product, the isolated (±)-*threo*-racemate and isolated (±)-*erythro*-racemates of 4F-MPH.

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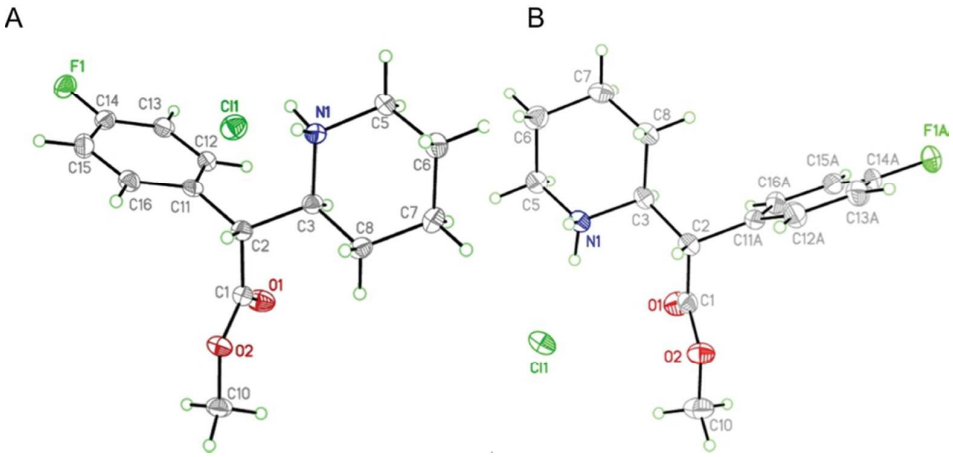

Figure 5. A: Structure of (±)-*erythro* and B: (±)-*threo* salts of 4F-MPH. Atomic displacement parameters shown at 50% probability and hydrogen atoms drawn as spheres of arbitrary radius. In B, only the major disordered fluorophenyl moiety is shown.

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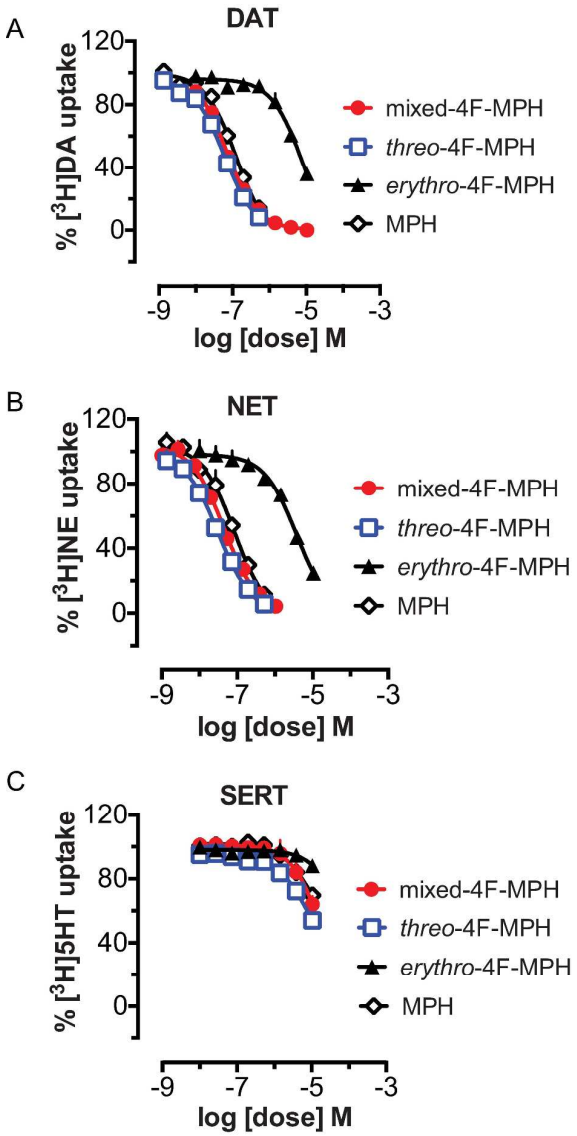

Figure 6. Effects of 4-fluoromethylphenidate isomers (4F-MPH) and comparison with methylphenidate (MPH) on inhibition of uptake at DAT, NET, and SERT in rat brain synaptosomes. Synaptosomes were incubated with different concentrations of test drugs in the presence of 5 nM [^{3}H]dopamine (A, for DAT), 10 nM [^{3}H]norepinephrine (B, for NET), or 5 nM [^{3}H]serotonin (C, for SERT). Data are percentage of [^{3}H]transmitter uptake expressed as mean ± s.e.m. for n = 3 experiments.

242x486mm (300 x 300 DPI)

Table 1. Effects of 4-fluoromethylphenidate (4F-MPH) and methylphenidate (MPH) on transporter-mediated uptake in rat brain synaptosomes [a]

Test Drug	[^{3}H]DA uptake via DAT IC_{50} (nM)	[^{3}H]NE uptake via NET IC_{50} (nM)	[^{3}H]5-HT uptake via SERT IC_{50} (nM)
Diastereomeric mixture of 4F-MPH	66.35 ± 3.27	44.6 ± 4.17	>10,000
(±)-*threo*-4F-MPH	60.96 ± 4.6	30.68 ± 2.64	8,805 ± 2475
(±)-*erythro*-4F-MPH	8,528 ± 1753	3,779 ± 570.5	>10,000
MPH	131.0 ± 14.2	82.85 ± 11.145	>10,000

[a] Data are expressed as nM concentrations (mean ± SD) for n = 3 separate experiments performed in triplicate. For dose-response curve, see Figure 6.