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Pharmacokinetics and pharmacodynamics of norfluoxetine in rats: Increasing extracellular serotonin level in the frontal cortex

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ABSTRACT

Norfluoxetine is the most important active metabolite of the widely used antidepressant fluoxetine. Although the pharmacokinetics/pharmacodynamics (PK/PD) relationship and neurochemical profile of fluoxetine is well characterized in human and in animals, little is known about the effect of its metabolite. The aim of this study was to characterize extracellular level of serotonin (5-hydroxytryptamine, 5-HT)-time profile of norfluoxetine after acute administration over 18 h post dose and to establish the relationship between this pharmacodynamic (PD) profile and its pharmacokinetic (PK) properties. Following subcutaneous administration of fluoxetine in rats, plasma and brain PK of fluoxetine and norfluoxetine were monitored respectively by liquid chromatography/tandem mass spectrometry (LC/MS/MS). The extracellular level of 5-HT in the frontal cortex was measured by microdialysis as a PD endpoint. Norfluoxetine when directly administrated to rats caused a significant increase in extracellular level of 5-HT in the frontal cortex and maintained for 18 h. This result is correlated well with higher plasma and brain concentration and longer plasma and brain retention time of norfluoxetine. Our results showed that norfluoxetine contributes to 5-HT transporter inhibition and extends fluoxetine efficacy.

1. Introduction

Fluoxetine (Prozac) is widely used for the treatment of depression (Fuller, 1995; Masand and Gupta, 1999). Its biochemical and pharmacological profiles have been studied extensively in animals and human (Fuller, 1995; Qu et al., 2006, 2003; Stahl, 1998; Wong et al., 1995). However the mechanism of drug action related to its clinical efficacy has not been fully understood. It is generally believed that a significant part of the therapeutic activity of fluoxetine is attributable to its most important active metabolite norfluoxetine (Fuller et al., 1992), since clinical pharmacokinetic (PK) study has shown that plasma norfluoxetine level were 100–130% those of fluoxetine (Tulloch and Johnson, 1992). Fluoxetine is a selective serotonin (5-hydroxytryptamine, 5-HT) transporter inhibitor, which exerts its behavioral and clinical therapeutic effect by blocking the transport of 5-HT at the 5-HT transporter, thereby increasing extracellular level of 5-HT in serotonergic synaptic cleft of many brain regions, which mediate a variety of behaviors. *In vivo* microdialysis has been extensively used to document the changes of extracellular level of 5-HT in rat brain after administration of fluoxetine (Beyer et al., 2002; Boothman et al., 2006; Bymaster et al., 2002; Fuller, 1994; Kobayashi et al., 2008; Koch et al., 2002;). It has been reported that norfluoxetine is as potent as the parent drug itself as an inhibitor of 5-HT uptake *in vitro* (Fuller, 1995; Wong et al., 1995) and *in vivo* (Fuller et al., 1992). However, the effect of norfluoxetine on the extracellular level of 5-HT in rat brain has never been reported.

In clinical study, fluoxetine has a mean half-life of 1–3 days and is subject to hepatic metabolism by cytochrome P450 enzymes (Mandrioli et al., 2006). Norfluoxetine is its N-demethylated metabolite with a longer half-life of 4–16 days (Mandrioli et al., 2006). We hypothesize that the time course of extracellular 5-HT level in following the acute norfluoxetine treatment may also relate to longer plasma half-life of norfluoxetine.

Therefore we designed the following experiments to evaluate both PK and pharmacodynamic (PD) features of norfluoxetine. 1). The effect of acute systemic administration of norfluoxetine (3 and 10 mg/kg s.c.) on extracellular level of 5-HT in the frontal cortex of freely moving rats was examined by performing a 21-hour *in vivo* microdialysis experiment. 2). A comparison of plasma and brain PK of fluoxetine and norfluoextine in rat after acute administration (3 and 10 mg/kg, s.c.) of fluoxetine. Based on the findings of these experiments, we established the PK/PD relationship of norfluoxetine, which is indicated by extracellular level of 5-HT and the plasma and brain concentration of the norfluoxetine.

2. Methods

2.1. Drugs

Fluoxetine hydrochloride, norfluoxetine hydrochloride and 5-hydroxytryptamine were purchased from Research Biochemicals

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International of Sigma (Natick, MA, USA). Other chemicals and reagents were of HPLC or analytical grade of purity. HPLC water was purchased from J. T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA) and purified by passing through a C18 solid phase extraction column (Sep-Pak C18, Waters, USA).

2.2. Microdialysis experiments

Male Sprague–Dawley rats (Charles River Laboratories, MA, USA) weighing 300–350 g were used. Each rat was given a 0.05 ml s.c. injection of Buprenex 0.06 mg/kg (buprenorphine hydrochloride) 5 min prior to anesthesia. Animals were anesthetized with an isoflurane/air mixture and stereotaxically implanted with a guide cannula (Eicom, Japan) in the prefrontal cortex (incisor bar −3.5 mm, +3.2 mm anterior, 0.8 mm lateral and 1 mm ventral to Bregma) (Paxinos and Watson, 1997). The guide cannula was secured in place with skull screws and dental cement. Animals were allowed at least 3 days to recover from surgery prior to experimentation.

Dialysis experiments were conducted between 8:00 am and 4:00 am the following day, in a controlled environment. The animals remained in their home cage throughout experimentation. Dialysis probes (Eicom, molecular weight cut-off 5,5000 Da, 0.22 mm out diameter, 4 mm active membrane length) were perfused with aCSF (147 mM NaCl, 4 mM KCl, 0.85 mM $MgCl_2$, 2.3 mM $CaCl_2$, pH 7.4) at a flow rate of 1 µl/min and implanted the afternoon prior to sample collection. The probe was connected via FEP tubing to a liquid swivel (QM, Instech, USA) mounted on a counter-balance arm. The following morning 3 h of baseline samples were collected into a 96-well plate (Sarstedt, 96 well multiply PCR, USA) via a four-channel fraction collector (Eicom). Norfluoxetine (3 or 10 mg/kg s.c.) was subcutaneously administrated to the animal. Samples were collected every 60 min for 18 h into the 96-well plate maintained at 4 °C containing 15 µl of the antioxidant (1 mM oxalic acid and 3 mM l-cysteine in 0.1 M acetic acid). The 60 µl microdialysis sample plus 15 µl antioxidant was aliquot to 30 µl for 5-HT analysis. Vehicle animals received 5% N-methyl-2-pyrrolidone solution (v/v, 1 ml/kg, s.c.) injection. New probes were used every time without determining *in vitro* recovery.

2.3. Analysis of extracellular serotonin levels

Quantification of extracellular levels of 5-HT in microdialysis samples of the frontal cortex was achieved by high-performance liquid chromatography (ESA Model 582) coupled to electrochemical detector (CoulArray Coulometric, ESA) with dual channel coulometric microdialysis cell (ESA 5014B) (Barbier et al., 2007). Separation was performed on a C18 column (Hypersil, 150×3.2 mm I.D.) at room temperature. The mobile phase consisted of 75 mM NaH_2PO_4, 0.5 mM Disodium-EDTA, 350 mg/L 1-octanesulfonic acid, pH 3.1, 1.0% THF, and 9.0% ACN. Flow rate was 0.4 ml/min. 22 µl microdialysate was injected by an autosampler (ESA Model 540). The first electrode of the detector was set at −90 mV (reduction) and the second at +280 mV (oxidation). All values for microdialysis studies were calculated as percentage change at each time point compared with the average of three baseline values. The overall effect of norfluoxetine treatments on extracellular levels of 5-HT was determined by a two way ANOVA analysis with treatment as the independent variable and time as the repeated measurement. If significant, the ANOVA was followed by post-hoc Duncan's multiple range test (SigmaStat, SPSS Inc., www.spss.com). Student paired *t*-test was used to compare the extracellular 5-HT level in light and dark phases.

2.4. Plasma sample preparation

Following administration of 3 or 10 mg/kg fluoxetine (s.c.), 250 µl blood samples via venipuncture of the lateral tail vein were collected into heparinized tubes over a time course as follow: 0.5, 1, 2, 4, 6, 8, 9, 10, 12 and 24 h. Then centrifuged at 14,000 rpm for 5 min. The plasma was retained and kept frozen in a −20° C freezer waiting for liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis.

The thawed plasma samples (50 µl) were mixed with 50 µl dimethyl sulfoxide (DMSO) and 150 µl acetonitrile, at room temperature for 10 min and centrifuged for 10 min at 14,000 rpm. 150 µl of supernatant was diluted with 450 µl of 10 mM ammonium acetate (pH = 7) for LC/MS/MS injection.

2.5. Brain sample preparation

Following administration of 3 or 10 mg/kg (s.c.) fluoxetine, brains were taken out at 1, 6, 24, or 48 h immediately after scarified by CO_2 asphyxiation and homogenized in 6 ml of phosphate buffered saline (Dulbecco HyQ DPBS/MODIFIED, HyClone, Utah, USA) and stored at −20° C. For LC/MS/MS analysis, the thawed homogenized brain samples (50 µl) were mixed with 50 µl DMSO, and 150 µl methanol. After incubation at room temperature for 10 min, 250 µl acetonitrile was added and waiting at room temperature for another 10 min, the samples were centrifuged for 10 min at 14,000 rpm. The supernatants were diluted 1:1 with 10 mM ammonium acetate (pH = 7) for LC/MS/MS injection.

2.6. Drug analysis by using LC/MS/MS

LC/MS/MS system consisted of Agilent 1100 series LC (Agilent Technologies, Wilmington, DE, USA) including a vacuum degasser, a binary pump, and an autosampler coupled to an Applied Biosystems/MDS SCIEX API-4000 triple–quadrupole mass spectrometer equipped with a TurboIonSpray source (Foster City, CA, USA). LC separations were achieved using 5-µm C18 column (50×2.1 mm i.d.; MAC-MOD http://www.mac-mod.com) at 40 °C with gradient elution. Mobile phase A was prepared by mixing 5% acetonitrile with 95% 10 mM ammonium acetate buffer (pH = 7) (v/v) and mobile phase B was prepared by mixing 95% acetonitrile with 5% 10 mM ammonium acetate buffer (pH 7) (v/v). A gradient elution over 7 min at a flow rate of 0.4 ml/min was used. Fluoxetine and norfluoxetine were monitored using the single-reaction monitoring (SRM) mode. The SRM transition m/z 310.1 → 44.1 and 296.1 → 134.0 were sequentially monitored for the detection of fluoxetine and norfluoxetine, respectively.

2.7. PK data analysis

WinNonlin software was used to analyze the data. A non-compartmental pharmacokinetic model was used to determine the following pharmacokinetic parameters: C_{max} (µM), peak plasma or brain concentration; T_{max} (h), time to maximum plasma or brain concentration; $AUC_{0-\infty}$ (µM h), area under the plasma or brain-time curve extrapolated to infinity; MRT (h), mean retention time; $t_{1/2}$ (h), half life.

3. Results

3.1. Effects of norfluoxetine on extracellular level of 5-HT

The effects of vehicle or norfluoxetine (3, 10 mg/kg s.c.) administration on time course of extracellular level of 5-HT in the frontal cortex of the freely moving rat are shown in Fig. 1. The acute administration of norfluoxetine (3.0 mg/kg s.c., $n = 5$) evoked a significant increase of 5-HT from basal levels in the frontal cortex of rats, to a maximum of 346 ± 50% at 10 h post dose and the average over the 18-hour treatment period was sustained at 277 ± 15% of basal values. Acute administration of a higher dose of norfluoxetine (10 mg/kg s.c., $n = 6$) evoked a larger increase of extracellular 5-HT level, to a maximum of 432 ± 56% at 9 h and the average over the 18-hour treatment period was