PHARMACOKINETICS AND ANTIDEPRESSANT ACTIVITY OF FLUOXETINE IN TRANSGENIC MICE WITH ELEVATED SERUM ALPHA-1-ACID GLYCOPROTEIN LEVELS

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ABSTRACT:

Fluoxetine, a novel selective serotonin reuptake inhibitor utilized in the treatment of depression, is avidly bound to serum albumin and alpha-1-acid glycoprotein (AAG). AAG is an acute phase protein, and its serum levels are elevated in a variety of pathophysiological conditions including inflammation, depression, cancer, and acquired autoimmune deficiency syndrome. Further, the pharmacokinetic disposition and pharmacological activity of several highly bound drugs have been reported to be significantly altered as a result of elevated serum AAG. We investigated the effects of elevated serum AAG levels on the pharmacokinetic disposition, antidepressant activity, and steady state profile of fluoxetine and its demethylated metabolite, norfluoxetine. This was approached utilizing a novel strain of transgenic mice that expressed genetically elevated serum AAG levels severalfold over those of control mice. Serum and brain drug concentrations were determined by HPLC after fluoxetine administration. In transgenic mice, the volume of distribution and the terminal elimination half-life of fluoxetine were significantly reduced. Further, significant reductions in brain-to-serum fluoxetine concentration ratios and antidepressant activity were observed in transgenic mice, despite having higher serum drug levels than control mice. This trend in the serum continued at steady state, and brain fluoxetine levels were significantly lower in transgenic mice. The results of this study provide valuable insights regarding the consequences of elevated serum AAG levels, often seen in several disease states, on the pharmacokinetic disposition of fluoxetine.

Fluoxetine, a novel selective serotonin reuptake inhibitor utilized in the treatment of depression, is extensively metabolized by the hepatic cytochrome P450 enzyme system to form its active metabolite, norfluoxetine (Bergstrom et al., 1992). Highly lipophilic in nature, fluoxetine is widely distributed in peripheral tissues with a mean volume of distribution of 36–50 liters/kg in humans (Altamura et al., 1994). Fluoxetine is classified as a high extraction ratio drug and is known to undergo extensive first pass metabolism in humans (Altamura et al., 1994). Further, fluoxetine is known to inhibit the metabolism of other concomitantly administered antidepressants, such as imipramine (Bergstrom et al., 1992; El-Yazigi et al., 1995; Caccia et al., 1990). Fluoxetine is reported to be extensively bound to serum proteins (>94%) including albumin and alpha-1-acid glycoprotein (AAG)1 (Lemberger et al., 1985; Aronoff et al., 1984). Serum AAG levels are not constant and are substantially elevated in several disease states including depression, arthritis, severe burns, and autoimmune deficiency syndrome (Kremer et al., 1984; Luzier and Morse, 1993). Elevated serum AAG levels have been shown to alter the drug disposition and action of several highly bound drugs such as imipramine, prazosin, and lidocaine (Yoo et al., 1996; Chiang and Øie, 1990; DeRick et al., 1987). To date, it is unknown whether the pharmacokinetic disposition of fluoxetine is altered by elevated serum AAG levels.

The goal of this study was to determine what effects elevated serum AAG levels often found in pathophysiological conditions have on the disposition and action of fluoxetine. Historically, the role of AAG in drug disposition and action has been conducted in disease states in which AAG levels were elevated (Yasuhara et al., 1985; Belpaire et al., 1986), after exogenous administration of human AAG in laboratory animals (Chiang and Øie, 1990), or after treatment with AAG inducing agents (DeRick et al., 1987; Abramson and Lutz, 1986). Disadvantages to these approaches include unstable and continuously varying AAG levels (during exogenous AAG administration) and multiple physiological disturbances that may accompany elevated AAG levels. Our approach involves the utilization of a unique transgenic mouse model in which elevated serum AAG 1) is a genetic trait of the animal and invariant among individuals of the same transgenic line, 2) is constant over the lifetime of the individual animal, and 3) involves no other physiological perturbations. Therefore, this model offers obvious advantages over other approaches. The transgenic mice were produced by pronuclear microinjection of the rat AAG gene (Dewey et al., 1990), and all of the AAG transgenic lines thereby produced were found to express supernormal AAG levels. The line used in the present study expressed serum AAG 8.6-fold over normal and has been applied previously to pharmacokinetic studies (Yoo et al., 1996; Holladay et al., 1996).

Materials and Methods

Chemicals. Fluoxetine and norfluoxetine (as hydrochloride salts) were kindly supplied by Eli Lilly Corp. (Indianapolis, IN). Clomipramine hydro-

1 Abbreviation used is: AAG, alpha-1-acid glycoprotein.
chloride, EDTA, triethylamine, and sodium hydroxide were purchased from Sigma. Acetonitrile, methanol, hexane (HPLC grades), and 0.9% saline were obtained from VWR Scientific (Suwanee, GA). Diethyl ether and 85% phosphoric acid were purchased from Mallinckrodt (Paris, KY). Perchloric acid was obtained from J.T. Baker.

**Animals.** Adult (4–5 months old, male and female, 20–30 g) control (C57BL/6) mice (Jackson Laboratories, Bar Harbor, ME) and transgenic mice expressing serum AAG levels severalfold elevated over normal were used in these studies (Dewey et al., 1990). These transgenic mice were produced by standard transgenic technology involving microinjection of a rat AAG genomic clone (containing the entire coding region along with 4.7 kilobases of 5′ flanking sequences) into the pronuclei of (C57BL/6 × DBA/2)F1 embryos. The transgenic founders were identified by Southern analysis and mated to nontransgenic C57BL/6 mice. Positive (transgenic) offspring from this cross were subsequently crossed to each other to produce homozygous mice among the offspring. Mice homozygous for the transgene were identified on the basis of the intensity of the signal in Southern analyses and confirmed by progeny testing. Two of such homozygotes were bred, and the line was maintained by sequential brother-sister mating. Among the resulting transgenic lines, AGP 9.5–5 was observed to express serum AAG levels 15.2-fold over normal (Yoo et al., 1996; Dewey et al., 1990). For this study, (AGP 9.5–5 × C57BL/6) F1 hybrids that expressed serum AAG levels 8.6-fold elevated over normal (2.24 vs. 0.26 mg/ml) and nontransgenic control mice (pure strain C57BL/6) were used. All mice were maintained in a temperature-controlled animal facility with a 12/12-hr light/dark cycle and had free access to food and water.

**Drug Administration.** To characterize the pharmacokinetic disposition of fluoxetine, a bolus dose of fluoxetine (10 mg/kg as free base) dissolved in isotonic saline (2.0 mg/ml) was administered by tail vein injection into control and transgenic mice. Postinjection, whole blood samples were harvested into borosilicate test tubes by cardiac puncture of the anesthetized animal at 0, 15, and 30 min and 1, 3, 6, 12, 24, and 48 hr (N = 4 for each sampling time). Whole blood samples were allowed to stand at room temperature for 1 hr and were subsequently centrifuged at 3000 × g for 5 hr at 4°C until drug analysis.

To examine the steady state disposition in transgenic and control mice, fluoxetine was delivered by sc infusion (1 μl/hr of 12.5 mg/ml fluoxetine in saline) using Alzet osmotic pumps (model 1003D, Alza Corp., Palo Alto, CA). Before the implantation of each osmotic pump, the mice were weighed and anesthetized with diethyl ether. After cleansing the area with isopropyl alcohol, an incision was made left of the spine, and each pump was implanted into a formed pocket in the sc tissue. Each incision was closed with wound clips (11 mm, Propoor Manufacturing Co., Long Island, NY). At 18, 36, 48, 60, and 72 hr after the initiation of the infusion, whole blood was collected by cardiac puncture and allowed to stand for 1 hr. Serum samples were harvested after centrifugation and were kept in borosilicate tubes at −20°C until drug analysis.

**Determination of Antidepressant Activity.** The mouse forced-swimming test was conducted as defined by Porsolt et al. (1978). This test is thought to show a state of despair as the animal, realizing no escape route is plausible, becomes immobile in a “state of despair.” Both groups of mice (N = 10 each group) received either a single ip injection of isotonic saline or fluoxetine (20 mg/kg, 2.0 mg/ml in saline) in a double-blind procedure. Thirty min after injection, each mouse was placed inside a vertical glass cylinder (25 × 20 cm) containing 15 cm of water maintained at 25°C. All animals were forced to swim for a total of 6 min, and the total duration of immobility during the last 4 min was recorded. Animals were tested only once after either saline or fluoxetine treatment.

For the determination of serum and brain drug levels 30 min after ip injection, adult control and transgenic mice (N = 4 each) received fluoxetine (20 mg/kg in saline) by ip injection. Thirty min after injection, whole blood samples were collected and harvested serum stored as previously described. Whole brains were removed from each mouse promptly after cardiac puncture and processed as previously mentioned.

**Serum Protein Binding Determination.** The extent of serum protein binding in transgenic and control serum was determined by equilibrium dialysis. Dialysis was carried out using Plexiglas dialysis cells (Bolab Products, Lake Havasu City, AZ) with a 0.5-ml chamber volume and Spectrapor2 dialysis membrane (molecular weight cutoff, 12,000–14,000) at 37°C. In a preliminary experiment, the optimum equilibration time for fluoxetine binding to serum proteins was determined to be 5 hr. Thereafter, fluoxetine-spiked transgenic and control serum (0.5–100 μg/ml) was dialyzed for 5 hr against phosphate buffer (0.133 M, pH 7.35) containing 0.6% sodium chloride to approximate the osmolality of plasma (Aronoff et al., 1984). At equilibrium, serum and buffer samples were collected into borosilicate test tubes and stored at −20°C until drug analysis. For the determination of the unbound fraction of fluoxetine and norfluoxetine at steady state (C1/2,ss), pooled transgenic and control serum obtained 48, 60, and 72 hr after sc infusion of fluoxetine was dialyzed for 5 hr against stock buffer as previously mentioned. The unbound fraction of drug was calculated as the ratio of the buffer to serum drug concentrations.

**Drug Analysis.** Serum and brain samples were analyzed for fluoxetine and norfluoxetine content by HPLC with UV detection. HPLC drug quantitation was performed on a Shimadzu component system (Shimadzu, Columbia, MD) using a Microsorb MV octadecyl column (Rainin, Woburn, MA). The mobile phase consisted of 55% acetonitrile and 45% distilled water containing 10 mM aqueous triethylamine, with the pH adjusted to 4.8 by dropwise addition of 85% phosphoric acid. The flow rate was set at 1.0 ml/min, and the effluent was monitored for UV absorption at 226 nm. Briefly, 20 μl of internal standard solution (clomipramine, 5.0 μg/ml in methanol), 100 μl of 5.0 M sodium hydroxide, and 2 ml of hexane were added to a borosilicate test tube containing 0.1 ml of serum or 0.2 ml of brain homogenate. The mixture was vortexed for 30 sec and centrifuged at 3000 g for 5 min. The organic layer was transferred to a fresh borosilicate tube and dried under a gentle stream of nitrogen at 20°C using an N-Evac Evaporator (Organamation Association, Inc., Berlin, MA). The resulting drug residue was reconstituted with 50 μl of mobile phase, and an aliquot (20 μl) was injected into the chromatographic system. Drug concentrations were measured as the mean of duplicate samples. The lower limit of quantitation was 25 ng/ml for both fluoxetine and norfluoxetine, with intra- and inter-day coefficients of variation <16%.

**Data Analysis.** Serum fluoxetine concentration vs. time data were analyzed using the least squares nonregression analysis computer program, WinNonlin (Scientific Consulting, Inc., Cary, NC). The CIu and t1/2u were calculated from the following equations: CIu = Dose/AUCu; t1/2u = 0.693/AUCu; Varea = AUC × t1/2u; Cmax = Dose/AUCu × t1/2u; Carea = Cmax × t1/2u; and Ci; Dose/AUCi × R/Ci; Cl; R/Ci. The brain-to-serum drug concentration ratios of fluoxetine and norfluoxetine after ip injection or sc infusion were determined as the quotient of the brain-to-serum drug concentrations. Brain drug concentrations were expressed as nanograms of drug per gram of brain weight. The difference in the mean pharmacokinetic parameters of fluoxetine between transgenic and control mice was performed by a one-way analysis of variance followed by Tukey’s test. An unpaired Student’s t test was used to determine the difference in the swimming immobility between the groups of mice. The significance level was set at p<0.05.

**Results**

Mean serum fluoxetine and norfluoxetine concentration vs. time profiles in transgenic and control mice after iv injection of fluoxetine are shown in figs. 1 and 2, respectively. Pharmacokinetic parameters of fluoxetine obtained after iv injection are shown in table 1. In transgenic mice, there were significant decreases in Varea and t1/2u with no differences in the area under the concentration vs. time curve (AUC) and systemic and unbound systemic clearances. The mean terminal elimination half-life for the demethylated metabolite, norfluoxetine, was also significantly decreased in transgenic mice (12.0 vs. 14.9 hr). The unbound fraction of fluoxetine was not significantly reduced in transgenic mice (2.61 vs. 3.38%) and remained unaltered over the concentration range studied (0.5–100 μg/ml).

To determine the drug effectiveness in both groups of mice, the duration of swimming immobility was measured after saline or fluo-
oxetine treatment. After saline administration, no significant difference was seen in the mean immobility values between transgenic and control mice (155.8 vs. 150.2 sec) (fig. 3). Treatment of fluoxetine resulted in a significant reduction of immobility in control mice (83.0 sec) but did not alter the duration of immobility in transgenic mice (135.0 sec). Mean fluoxetine brain drug levels were significantly lower 30 min after a single ip injection (table 2) in transgenic mice (22,860.9 ng/g vs. 31,289.9 ng/g). However, serum fluoxetine levels were significantly higher in transgenic mice (table 2). Serum and brain norfluoxetine drug levels were similar between transgenic and control mice after ip injection of fluoxetine.

The steady state levels of fluoxetine and norfluoxetine were achieved in the serum and the brain of both groups of mice within 48 hr of initiation of infusion (figs. 4 and 5, respectively). Steady state fluoxetine and norfluoxetine serum and brain concentrations were calculated as the mean of drug concentrations at 48, 60, and 72 hr. Fluoxetine serum concentrations at steady state were significantly higher in transgenic mice as compared with control mice (table 3); however, steady state brain concentrations of fluoxetine were significantly lower in transgenic mice. There was no significant difference in norfluoxetine serum and brain values between the animal groups at steady state.

At steady state, fluoxetine and norfluoxetine were extensively distributed into the brain in both groups of mice (table 3). The brain-to-serum ratios were significantly higher for both norfluoxetine and fluoxetine in control mice. The mean serum unbound fraction ($C_{u,ss}$) of fluoxetine and norfluoxetine was 3.2 and 9.1 for control mice and 2.0 and 7.9 for transgenic mice, respectively. At steady state, the systemic clearance of fluoxetine remained unaltered between transgenic and control mice (33.2 ± 4.2 vs. 42.1 ± 2.3 ml/min/kg, respectively). In both groups of mice, steady state metabolite levels were higher than the parent drug levels, with a mean metabolite to parent drug concentration ratios of 1.9 and 1.7 in the serum and 2.0 and 1.4 in brain in transgenic and control mice, respectively.

Comparison of pharmacokinetic parameters (mean ± SD) between transgenic and control mice obtained after a 10 mg/kg iv fluoxetine injection (N = 4)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Transgenic Mice</th>
<th>Control Mice</th>
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<tbody>
<tr>
<td>AUC (μg * min/ml)</td>
<td>465.8 ± 40.2</td>
<td>409.1 ± 22.7</td>
</tr>
<tr>
<td>$V_{area}$ (ml/min/kg)</td>
<td>16.3 ± 1.8a</td>
<td>27.6 ± 1.9</td>
</tr>
<tr>
<td>$Cl_t$ (ml/min/kg)</td>
<td>21.9 ± 4.0</td>
<td>24.6 ± 2.7</td>
</tr>
<tr>
<td>$Cl_u$ (ml/min/kg)</td>
<td>842.3 ± 77.1</td>
<td>729.7 ± 40.8</td>
</tr>
<tr>
<td>$t_{1/2,1}$ (min)</td>
<td>10.7 ± 2.2a</td>
<td>18.3 ± 1.3</td>
</tr>
<tr>
<td>$t_{1/2,2}$ (hr)</td>
<td>8.6 ± 0.6a</td>
<td>12.9 ± 0.8</td>
</tr>
<tr>
<td>$f_u$ (%)</td>
<td>2.61 ± 0.4</td>
<td>3.38 ± 1.0</td>
</tr>
<tr>
<td>$AUC_{NFL}/AUC_{FLU}$</td>
<td>1.7 ± 0.3</td>
<td>1.5 ± 0.1</td>
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</table>

a Significantly different from control values ($p < 0.05$).
Discussion

Fluoxetine is highly extracted by the liver, and therefore its systemic clearance is likely to be a function of hepatic blood flow rather than changes in serum protein binding (Wilkinson and Shand, 1975). However, the systemic clearance of several highly extracted drugs (i.e., imipramine and disopyramide) has been reported to be significantly reduced during elevated AAG states (Holladay et al., 1996; Huang and Oie, 1985). In our study, the systemic clearance of fluoxetine was not significantly altered in transgenic mice with elevated serum AAG levels. The mean unbound fraction of fluoxetine was not significantly reduced in transgenic mice, despite an 8.6-fold elevation in serum AAG levels. This may be a result of nonspecific binding of fluoxetine to albumin and AAG or perhaps methodological problems with equilibrium dialysis. Alterations in the fluoxetine pharmacokinetic parameters V_{area} and t_{1/2} are consistent with elevated serum AAG levels. Significant alterations in the pharmacokinetic parameters of basic drugs that are highly bound to serum proteins (i.e., imipramine, prazosin, and lidocaine) have been reported during elevated serum AAG states (Yoo et al., 1996; Chiang and Oie, 1990; DeRick et al., 1987). The terminal elimination half-life of fluoxetine in transgenic and control mice (table 1) is considerably different from the range reported in humans (1–4 days) (Benfield et al., 1986). However, the mean unbound fraction of fluoxetine in control mice is similar to that reported in normal human patients (5.5%) (El-Yazigi et al., 1995; Lemberger et al., 1985) and slightly lower than in rats (10%) (Caccia et al., 1990). In rats (Caccia et al., 1990) and humans (Lemberger et al., 1985), norfluoxetine elimination is rate limited. A similar trend was seen in mice (fig. 2), as the terminal elimination half-life of norfluoxetine was higher than that of fluoxetine.

It remains controversial whether the total or unbound serum drug concentration of fluoxetine is a more accurate predictor of response. Kelly, et al. (1989) reported no significant correlation between serum fluoxetine and norfluoxetine concentrations and response to therapy in depressed patients. In control mice, a significant reduction in the duration of swimming immobility was found after fluoxetine administration. This finding is similar to studies performed in rats and mice in which the duration of immobility after a single ip injection of fluoxetine (10–40 mg/kg) was shown to be significantly different from saline treatment in a dose-dependent fashion (Cesana et al., 1993; Fuller and Snoody, 1993; Griebel et al., 1995). Despite having significantly higher total fluoxetine serum levels, the antidepressant activity of fluoxetine was significantly reduced in transgenic mice in our study (fig. 2). Reduced pharmacological activity in transgenic mice was consistent with lower brain drug levels found in these mice. Whether fluoxetine efficacy was modified by mechanisms other than alterations in serum protein binding, i.e., alterations in receptor response (Chiang and Oie, 1990), is unknown.

At steady state, serum fluoxetine concentrations did not correlate with brain drug levels in transgenic mice, as evidenced by its lower brain-to-serum drug concentration ratio as compared with control mice (table 2). The metabolite to parent AUC ratio found in transgenic and control mice (1.7 and 1.5, respectively) is similar to that reported after an iv bolus injection of fluoxetine (10 mg/kg) in rats (Caccia et al., 1990). The consequences of elevations in serum AAG levels on pharmacological activity of fluoxetine and its active metabolite, norfluoxetine, may become even more important at steady state, when brain drug efficacy in humans is noted (Altamura et al., 1994; Lemberger et al., 1985). The contribution of norfluoxetine to clinical response should be considered, as norfluoxetine brain drug levels are more than 2-fold elevated over fluoxetine values at steady state (table 3).

In summary, elevated serum AAG levels resulted in significantly reduced pharmacological activity and pharmacokinetic parameters of fluoxetine (V_{area} and t_{1/2}), and its pharmacological activity did not correspond to serum drug concentrations. Serum elevations of AAG produced increases in serum drug concentrations, while reducing the relative brain drug uptake. This unique animal model provided valuable insights on the influences of endogenously elevated serum AAG levels on the pharmacokinetics and antidepressant activity of fluoxetine.

References


TABLE 2

<table>
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<tr>
<th>Parameter</th>
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<th>Control</th>
<th>Transgenic</th>
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<tr>
<td>C_{30} min, serum (ng/ml)</td>
<td>2568.2 ± 338.8a</td>
<td>1367.6 ± 149.3</td>
<td>218.5 ± 23.5</td>
<td>209.3 ± 14.3</td>
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<tr>
<td>C_{30} min, brain (ng/g)</td>
<td>22860.9 ± 1985.9a</td>
<td>31289.9 ± 4352.3</td>
<td>30345.9 ± 209.9</td>
<td>3974.4 ± 689.1</td>
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<td>Brain-to-serum ratio</td>
<td>8.9 ± 0.3a</td>
<td>22.9 ± 1.7</td>
<td>19.1 ± 6.9</td>
<td>14.1 ± 3.3</td>
</tr>
</tbody>
</table>

*Significantly different from control values (p < 0.05).

TABLE 3

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Control</th>
<th>Transgenic</th>
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<td>C_{ss}, serum (ng/ml)</td>
<td>354.5 ± 61.7</td>
<td>278.9 ± 26.7</td>
<td>681.7 ± 58.7a</td>
<td>466.8 ± 22.3</td>
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<td>C_{ss}, brain (ng/g)</td>
<td>3406.0 ± 348.8a</td>
<td>6302.1 ± 365.2</td>
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<td>Brain-to-serum ratio</td>
<td>11.4 ± 1.4a</td>
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<td>20.6 ± 1.2</td>
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<tr>
<td>Cl_{ss} (ml/min/kg)</td>
<td>33.3 ± 4.2</td>
<td>42.1 ± 2.3</td>
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</table>

*Significantly different from control values (p < 0.05).


