Chiral High-Performance Liquid Chromatographic Analysis of Fluoxetine and Norfluoxetine in Rabbit Plasma, Urine, and Vitreous Humor Using an Acetylated β-Cyclodextrin Column

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Abstract

Fluoxetine (Prozac) is a potent selective serotonin reuptake inhibitor used for the treatment of major depression. Both fluoxetine (F) and its demethylated metabolite, norfluoxetine (NF), are racemic. S-Fluoxetine (SF) and S-norfluoxetine (SNF) are more potent inhibitors of serotonin reuptake than R-fluoxetine (RF) and R-norfluoxetine (RNF). Quantitation of individual enantiomers may provide a greater understanding of pharmacokinetic properties. The objective of this study was to perform a limited chiral selectivity study using rabbit plasma, urine, and vitreous humor analyzed by a solid-phase extraction protocol and a newly developed chiral analysis with an acetylated β-cyclodextrin (CD) column. Liquid chromatographic parameters for CD were as follows: a mobile phase composition of methanol/0.3% triethylamine buffer, pH 5.6, (30:70), a flow rate of 1 mL/min, detection at 214 nm, and a temperature of 40°C. Elution order was SNF, SF, RNF, and RF with capacity factors of 6, 7, 8, and 9, respectively. The corresponding resolution factors were as follows: R1,2 = 0.8, R2,3 = 1.2, and R3,4 = 0.9. The conditions for solid-phase extraction were optimized for Varian Bond Elut Certify columns. Following sample application, the column was rinsed with water, acetic acid, and then with methanol. Drug enantiomers were eluted with methylene chloride, isopropanol, and ammonium hydroxide (78:20:2). After extract evaporation, the extract residue was reconstituted for high-performance liquid chromatographic analysis. To investigate chiral pharmacology, a biodistribution study was performed by administering 2 mg/kg of F to five rabbits. Blood, urine, and vitreous specimens were collected. Plasma samples collected 45 min postinjection showed nearly equal concentrations of RF and SF. After 24 h, the only metabolite detected in plasma was RNF. Drugs were not detectable in vitreous humor. Urine concentrations of SNF, SF, RNF, and RF were 34, 38, and 8 μg/L, respectively. The CD column along with the described extraction protocol may be used for a chiral selectivity study of fluoxetine.

Introduction

Animal studies have shown enantiomeric differences in pharmacological activities for fluoxetine and norfluoxetine. Figure 1 shows the chemical structures of S- and R-fluoxetine. Synaptosomal preparations of the rat cerebral cortex showed S-fluoxetine to be slightly more potent in inhibiting serotonin reuptake than R-fluoxetine in vitro. Concentrations causing 50% inhibition (IC50, nM) were 34, 25, and 40 for racemic fluoxetine, S-fluoxetine and R-fluoxetine, respectively. Furthermore, S-fluoxetine exhibited a longer duration of action based on the in vivo inhibition of p-chloroamphetamine, a serotonin-depleting drug, in both rats and mice. After a single dose of S-fluoxetine, the duration of serotonin inhibition was 24 h. However, with a single dose of R-fluoxetine, inhibition only persisted up to 8 h. In other words, S-fluoxetine is approximately 1.5 times more potent than R-fluoxetine and displays a longer duration of action.
threefold longer duration of action. As for the metabolite, S-norfloxadine was found to be approximately 20 times more potent in inhibiting serotonin uptake than R-norfloxasine (1–5). Because of differences in the pharmacokinetics of floxasine and norfloxsine enantiomers, individuals may display unequal acts of the four active compounds. Therefore, establishing a relationship between racemic floxasine dosage and its clinical response is difficult. Quantification of individual enantiomers, rather than measuring the sum of enantiomers, may provide a greater understanding of therapeutic and pharmacokinetic activities. Recently, using an analytical method that involved GC–MS and chiral derivatization, a patient mediatized with 20 mg racemic floxasine for nine months showed the following plasma concentrations: S-norfloxsine, 38 ng/mL; R-norfloxsine, 22 ng/mL; R-floroxetine, 11 ng/mL; and S-floroxetine, 31 ng/mL, demonstrating the chiral selectivity in drug metabolism (6).

The objective of this study was to perform a limited chiral selectivity study using rabbit plasma, urine, and vitreous humor with an established solid-phase extraction protocol followed by chiral analysis with an acetylated β-cyclodextrin (CD) column.

Methods

Instrumentation
A Waters (Milford, MA) high-performance liquid chromatography (HPLC) system was used for chiral analysis of floxasine and norfloxsine. It consisted of a model 510 HPLC pump, Waters Intelligent Sampler Processor 710B (WISP), model 486 Tunable Absorbance UV detector, and a model 730 Data Module processing system. A guard column was connected to a chiral analytical column. Separation was achieved with a 25-cm × 4.6-mm chiral analytical column packed with 10 µm acetylated β-cyclodextrin from Astec (Whippany, NJ). A column heater was used for temperature regulation.

Chemical reagents
All chemical reagents were HPLC grade or the highest purity available. Methanol, potassium phosphate, and potassium carbonate were purchased from Fisher Scientific (Pittsburgh, PA). Acetonitrile, ammonium hydroxide, dichloromethane, and isopropanol were obtained from Sigma (St. Louis, MO). Acetic acid and triethylamine were purchased from Aldrich Chemical Company (Milwaukee, WI).

Standards preparation
The preparation of standards and solutions as well as the solid-phase extraction procedure were performed as previously described (7). Fluoxetine, norfluoxetine, and the pure enantiomeric drug standards were kindly provided by Eli Lilly (Indianapolis, IN). Imipramine was obtained from Ciba-Corning (Summit, NJ), and clomipramine was from Altech Associates (State College, PA). Primary drug stock solutions of 1 mg/mL were prepared by dissolving in 10 mg of each drug powder in 10 mL of methanol. A 10-µg/mL working solution was prepared by adding 100 µL of the primary stock to 10 mL of methanol. Imipramine was diluted to a concentration of 2.5 µg/mL solution by adding 1 mL of 10-µg/mL working solution to 3 mL of methanol.

Aqueous triethylamine was prepared by adding 6.6 mL of triethylamine to 2 L of deionized water (3.3 mL/L). The pH was adjusted to the required value using glacial acetic acid. Each liter of mobile phase consisted of 300 mL of methanol and 700 mL of aqueous triethylamine (30:70). In order to maintain a homogeneous mixture, the mobile phase was stirred continuously throughout the assay. For performing the biodistribution study, the ratio was changed to 25:75.

For solid-phase extraction, 0.1M phosphate buffer was prepared by dissolving 13.6 g of KH2PO4 in 1 L of deionized water, and the pH was adjusted to 6.8 with the addition of 10M KOH. The methylene chloride, isopropanol, and ammonium hydroxide (78:20:2) elution solvent was prepared daily.

Fluoxetine drug solution
A 1.86-mg/mL fluoxetine drug solution was prepared by dissolving the contents of 20-mg capsules in water. After alkalizing the solution to a pH between 10 and 11, it was extracted with hexane. The dried residue was suspended with Dulbecco’s phosphate-buffered saline and sterile filtered using a 0.2-µm filter to eliminate bacterial contaminants. The final fluoxetine concentration of 1.86 mg/mL was established by HPLC analysis.

Sample collection
A research protocol was submitted and approved by the Animal Care and Use Committee of the University of Wisconsin-Milwaukee. Six male New Zealand White rabbits were obtained from LSR Industries (Union Grove, WI). Rabbits were weighed in order to calculate a fluoxetine injection volume. The injection volume was calculated by multiplying dosage by weight in kilograms divided by drug concentration. A 2.0-mg/kg racemic mixture of fluoxetine hydrochloride was administered to the animals via a butterfly vein infusion set into the right marginal ear vein. Blood samples were drawn from the left marginal ear vein at time intervals of 0, 0.75, 2, 6, and 24 h postinjection. Approximately 4 mL of blood was drawn and collected into tubes containing 80 µL of 7.5% EDTA. Samples were centrifuged at 3000 × g for 10 min. Plasma and urine samples were transferred to clean polypropylene tubes and stored at −20°C until analysis. Rabbits were euthanized with an overdose of pentobarbital. Urine specimens were collected with a 50-µL syringe, and a 5-µL syringe was used to collect vitreous humor. Approximately 1 mL of vitreous humor was drawn from each eye. The syringe was inserted to a depth of about 1 cm and withdrawn gently to avoid drawing cellular components (8). The vitreous fluid was transferred to polypropylene tubes and centrifuged for 10 min to remove any cellular components or particulate matter.

Biological sample preparation
Standard preparation involved pipetting 2 mL of drug-free plasma into a series of polypropylene screw-cap tubes followed by the addition of 0, 10, 20, 40, and 80 µL of each of the 10-µg/mL working solutions of fluoxetine and norfluoxetine. The
final plasma concentrations were 0, 50, 100, 200, and 400 μg/L for a racemic mixture (0, 25, 50, 100, and 200 μg/L per enantiomer). To each tube, except for the blank, 20 μL of the 2.5-μg/mL imipramine working solution was added as the internal standard for analysis with the acetylated β-cyclodextrin column.

Solid-phase extraction

The conditions for solid-phase extraction were optimized for Varian Bond Elut Certify columns. Columns were conditioned with methanol and deionized water. A 2-mL aliquot of 0.1M phosphate buffer was added to establish a pH of 6.8. Then, 2-mL sample aliquots were mixed with 8 mL of 0.1M phosphate buffer were transferred into separate solid-phase extraction columns and drawn through slowly at a flow rate of about 1 mL/min (5–10 mm Hg). After rinsing the columns with deionized water, 2-mL aliquots of 1.0M acetic acid were used to acidify each column. A 5-mL aliquot of methanol was then introduced to remove lipids and proteins from the columns. After drying the columns by prolonged suctioning, drugs were eluted with 3 mL of methylene chloride, isopropanol, and ammonium hydroxide (78:20:2). The solvent mixtures were evaporated, and the dried residue was reconstituted with 200 μL of mobile phase. A 180-μL aliquot of each extract was injected into the HPLC for analysis.

Chiral analysis

Following extraction, fluoxetine and norfluoxetine concentrations were determined by chiral HPLC analysis. The mobile phase composition was methanol/0.3% triethylamine buffer (pH 5.6) (25:75). While maintaining the column temperature at 30°C, the solvent was pumped into the HPLC column at a flow rate of 1 mL/min. Compounds were detected at 214 nm and 0.005 absorbance units full scale (AUFS). As a protective measure, an achiral C18 column was initially used to determine if drugs were extracted and if the extract was suitable for injection into chiral columns. The mobile phase for the C18 column consisted of 0.05M phosphate buffer (pH 4.7)/acetonitrile (60:40). The flow rate was 2 mL/min at a column temperature of 50°C. The eluent was monitored at 214 nm and 0.005 AUFS. Clomipramine was used as the internal standard with the C18 column because of interference of imipramine with fluoxetine and norfluoxetine drug peaks.

Data analysis

Calibration curves were generated for each enantiomer by plotting the spiked standard drug concentration versus the peak-height ratio (enantiomer peak height/internal standard peak height). Unknown concentrations were determined by extrapolation from standard curves.

Results

The rabbits used in this study weighed 4.35 ± 0.31 kg and the concentration of fluoxetine administered was 1.86 mg/mL. The injection volume was 4.68 ± 0.35 mL to deliver a 2.0 mg/kg dosage. Blood samples were collected at 0, 0.75, 2, 6, and 24 h after injection.
postinjection. A chromatogram of plasma standard containing 100 μg/L of each enantiomer is shown in Figure 2 to demonstrate the separation achieved when both enantiomers of fluoxetine and norfluoxetine are present. In order to maintain adequate peak resolution, mobile phase ratio was changed to 25:75 for methanol/triethylamine buffer. Representative HPLC chromatograms of plasma samples collected from a single rabbit are shown in Figures 3–7. Precision data were presented previously (7).

No interferences were exhibited in the blank chromatograms prior to the injection of fluoxetine as shown by Figure 3. Analysis of plasma samples collected 45 min after injection showed nearly equal concentrations of R- and S-fluoxetine as shown in Figure 4. The concentrations were 32 μg/L and 37 μg/L, respectively. Samples collected at 2 h postinjection showed the presence of R-norfluoxetine at 12 μg/L, and both parent drugs’ levels both decreased to 21 μg/L as shown in Figure 5. At 6 h, R-norfluoxetine continued to increase from 12 to 32 μg/L, whereas R-fluoxetine and S-fluoxetine concentrations decreased to 17 and 13 μg/L, respectively, as shown in Figure 6. Approximately 24 h following injection, R-norfluoxetine was the only enantiomer detected in plasma as shown in Figure 7.

In the 24-h urine, the concentrations of the S-enantiomers were consistently greater than those of the R-enantiomers. The S-norfluoxetine, S-fluoxetine, R-norfluoxetine, and R-fluoxetine concentrations were 51, 76, 34, and 8 μg/L, respectively. Fluoxetine and norfluoxetine were not detected in vitreous humor. Vitreous humor enantiomeric concentrations may have been lower than the detection limit of this assay, which was 8 μg/L. The various plasma and urine concentrations of fluoxetine and norfluoxetine are shown in Table I.

**Discussion**

Application of the acetylated β-cyclodextrin assay in a pharmacology study using rabbits indicates differences in the metabolism and elimination of fluoxetine and norfluoxetine enantiomers. Plasma samples collected 45 min after injection showed nearly equal concentrations of R- and S-fluoxetine. After 2 h, R-norfluoxetine was detected and continued to peak at 6 h. R- and S-fluoxetine diminished at 6 h and were not detected at 24 h. S-Norfluoxetine was not detected in rabbit plasma. The only metabolite that was detected after 24 h was R-norfluoxetine. It is possible that stereospecific metabolism and pharmacokinetics may be observed for fluoxetine and norfluoxetine enantiomers.

Some examples of compounds that may undergo stereospecific metabolism are methamphetamine, amphetamine, and phenytoin. The d-enantiomer of methamphetamine is preferentially metabolized over l-methamphetamine during demethylation to d-amphetamine. In addition, d-amphetamine is usually metabolized at a faster rate than l-amphetamine (9). As for phenytoin, one of the metabolites is chiral and it appears that

| Table I. Plasma and Urine Levels of Fluoxetine and Norfluoxetine in Rabbits |
|-----------------------------|-----------------------------|-----------------------------|
|                            | Plasma (n = 5)               | Urine (n = 4)               |
|                            | 0 h | .75 h | 2 h | 6 h | 24 h | 24 h |
| RF (μg/L ± SD)             | ND* | 32 ± 10 | 21 ± 9 | 17 ± 4 | ND | 8 ± 1 |
| SF (μg/L ± SD)             | ND  | 37 ± 10 | 21 ± 8 | 13 ± 4 | ND | 76 ± 27 |
| RNF (μg/L ± SD)            | ND  | ND    | 12 ± 4 | 32 ± 13 | 29 ± 5 | 34 ± 6 |
| SNF (μg/L ± SD)            | ND  | ND    | ND    | ND    | ND | 51 ± 24 |

* ND, not detected
the metabolism of the S-enantiomer is favored over the R-enantiomer by a ratio of 9:1 (10). Although S-norfluoxetine was not detected in plasma, its presence in urine after 24 h suggests that differences in metabolism and/or excretion may exist. A previous study by Potts and Parli (11) showed that fluoxetine and norfluoxetine concentrations were greater in the dog brain than in plasma. In addition, S-norfluoxetine concentrations were found to be greater than R-norfluoxetine concentrations in the rat brain, whereas the opposite was true in rat plasma. In a preliminary study of depressed patients by Perel et al. (12), concentrations of S-enantiomers of fluoxetine and norfluoxetine were found to be greater than the R-enantiomers in patient plasma samples. A recent single patient study also confirmed this chiral selectivity (6). Differences in the rate of metabolism, excretion and/or affinity for serotonin receptors or transporters may be observed for fluoxetine and norfluoxetine enantiomers (11,12). Thus, measurement of fluoxetine and norfluoxetine concentrations in biological fluids may prove to be useful in understanding the metabolism and pharmacokinetics of each enantiomer (12,13).

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References