Fluoxetine Bioequivalence Study: Quantification of Fluoxetine by Liquid Chromatography Coupled To Mass Spectrometry

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***Abstract*-A liquid chromatography method was developed and validated for the determination of fluoxetine in human plasma using paroxetine as internal standard. The drugs were extracted from plasma by liquid-liquid extraction and separated isocratically on a C18 analytical column, with water:acetonitrile as mobile phase, run at a flow rate of 0.20 mL/min. The method was linear in the range of 0.2-50 ng/mL and demonstrated acceptable results for the precision, accuracy and stability studies. The method was successfully applied for the bioequivalence study of two tablet formulations of fluoxetine 20 mg after single oral dose administration to healthy human volunteers.**

Keywords: human plasma; liquid chromatography; fluoxetine.

I. Introduction

Fluoxetine is a potent and highly selective inhibitor of 5-HT (5-hydroxytryptamine) uptake (selective serotonine reuptake inhibitor [SSRI]).1 The drug is commercially available in tablets containing 20 mg of fluoxetine hydrochloride.The absorption of fluoxetine is not affected by the dosage form, and any presystemic metabolism is thought to be clinically insignificant.2 Fluoxetine is relatively highly bound to plasma proteins (80%-90%) and has a large volume of distribution (20-42 l kg–1). This results in a slow rate of elimination, with a halflife of 47 to 72 hours. N-demethylation is the main pathway for metabolism, and norfluoxetine, the most important metabolite, is as potent as fluoxetine in inhibiting serotonin uptake and has a much longer half-life (7-14 days). The N-demethylation pathway has an intersubject variability probably linked to genetic factors. Fluoxetine and norfluoxetine are potent inhibitors of cytochrome P4502D6 (CYP2D6) and hence may cause important interactions with drugs metabolized by this isoenzyme.3,4Adverse reactions include anxiety, insomnia, dizziness, tremor and headache.5 Nausea/vomiting is the most common (~20%) adverse reaction of fluoxetine. Diarrhoea, anorexia, xerostomia and dyspepsia are also fairly common (~10%) and may require medical attention.6

Fluoxetine is unrelated to tricyclic, tetracyclic or other available antidepressant agents and propylamine designated (±)-N-methyl-3-phenyl-3-[a,a,a-trifluoro-p-tolyl) hydrochloride. It has the empirical formula of C17H18F3NO.HCl. Its molecular weight is 345.79. 7The aim of the present article was to develop and validate a simple, specific and sensitive LC method, using a single step liquid-liquid extraction procedure. This method was applied to a pharmacokinetic analysis of fluoxetine in human plasma supporting a bioequivalence study of two pharmaceutical formulations.

II. Experimental

1) *Chemicals and reagents*

The test and reference formulations containing 20 mg of fluoxetine were manufactured by the Medley S.A. Indústria Farmacêutica (Campinas, SP, Brazil), and Eli Lilly Ltda. (São Paulo, SP, Brazil), respectively, within their shelf life period. Fluoxetine reference substance (Lot FLUOCAP20TE02) and paroxetine (Lot AO98628) as internal standard (IS) were purchased from Libbs (Brazil). HPLC grade acetonitrile, hexane and ethyl acetate. Water was purifield using a MilliQ®.

2) *Chromatographic conditions*

An aliquot (15 □L) of each plasma extract was injected into a Polaris C18 (5 □m, 50 x 20 mm) analytical column (150 mm x 4.6 mm i.d.) coupled with SECURITYGUARDTM precolunm (4,0 x 3,0 mm) operated at room temperature (□20ºC). The compounds were eluted by pumping the mobile phase (acetonitrile and water 60/40, v/v, containing 0.1% formic acid) at a flow rate of 0.20 mL/min (total run time 2.8 min).

3) *Mass-spectrometric conditions*

It was used the mass spectrometer model Quattro Micro™ (Micromass dados do fabricante) and ionization source by electrospray operating in positive mode (ESI+), and set up in Multiple Reaction Monitoring (MRM), monitoring the transitions 310.0 > 43.9 and 330.4 > 70.0 for fluoxetine and paroxetine, respectively. The source and desolvatation temperature were 100°C and 350°C, respectively. The gas flow of cone and desolvatation were 40 and 250 L/h, respectively. The values of capillary voltage, cone voltage and collision energy were 4.0 kV, 10.0 V and 10.0 eV for fluoxetine and 4.0 kV, 20.0 V and 35.0 eV for paroxetine. The gas pressure (argon) was 2.5 x 10^{-3} Torr.The mass spectrometer was set as follows: m/z 310.0 for fluoxetine and 330.4 for paroxetine as the precursor ions and m/z 43.9 and 70.0 as the respective daughter ions in the MRM mode. No peak was observed in the mass chromatogram of blank human plasma under the LC-MS-MS conditions described. Also, the mass chromatograms of a sample (LOQ) are shown in Figure 1, where it can be observed that the retention times of fluoxetine and paroxetine were 1.15 and 1.05 min, respectively.

4) Standard solutions and calibration curves

The stock solutions of fluoxetine and IS were prepared by weighing 10 mg of reference material into a 50 mL individual volume¬tric flask and dissolving to volume with methanol:water, obtaining a concentration of 1 µg/mL. The prepared stock solutions were stored at 2-8 ºC protected from light. Analytical curves of fluoxetine were prepared by spiking blank plasma at concentrations from 0.2 to 50.0 ng/mL. The quality control (QC) samples were prepared in blank plasma at concentrations of 0.60 (low), 20.0 (medium), and 40.0 ng/mL (high), and then divided in aliquots that were stored at –20 ºC until analysis. The spiked plasma samples (standards and quality controls) were extracted on each analytical batch along with the unknown samples.

5) Plasma extraction

Fluoxetine and its internal standard paroxetine were extracted from human plasma by liquid-liquid extraction with hexane/ethyl acetate 1:1 (v/v). Plasma fluoxetine concentrations were quantified by combined reversed phase liquid chromatography tandem mass spectrometry with positive ion electrospray ionization using selected daughter ion monitoring (MRM). Briefly, 500 □L of plasma was added to Eppendorff tube with 50 □L of 0.1 mol/L NaOH, 25 □L of internal standard (500.0 ng/mL paroxetine) and 1000 □L of hexane/ethyl acetate 1:1 (v/v). The tube was homogenized in orbital shaker (Finemixer, Fine PCR, speed 9) by 5 min. After centrifugation (5 min at 14000 rpm, 4ºC) the upper layer (700 □L of organic phase) was removed to other tube and evaporated under compressed air flow. The dry residue was reconstituted with 150 □L of mobile phase and homogenized in orbital shaker (speed 9). The solutions were then transferred to the auto-injector microvials.

6) Validation of the bioanalytical method

The method was validated by the determination of the following parameters: specificity, linearity and range, recovery, accuracy, precision, lower limit of quantitation (LLOQ), and stability studies, following the bioanalytical method validation guidelines.8,9Specificity was assessed using six blank human plasma samples, randomly selected, from different sources (including haemolysed and lipemic plasma), that were subjected to the extraction procedure and chromatographed to determine the extent to which endogenous plasma components could interfere in the analysis of fluoxetine or the IS. The results were compared to a solution containing fluoxetine. The analytical curves were constructed from a blank sample (plas¬ma sample processed without IS), a zero sample (plasma processed with IS), and concentrations of fluoxetine, including the LLOQ, ranging from 0.2 to 50.0 ng/mL. The peak area ratio of the drug to the IS against the respective standard concentrations was used for plotting the graph and the linearity evaluated by least square regres¬sion analysis. The acceptance criteria for each calculated standard concentration was not more than 15% deviation from the nominal value, except for the LLOQ, which was set at 20%.

The recovery was evaluated by the mean of the response of three concentrations of fluoxetine (0.60, 20.0, and 40.0 ng/mL), each one with addition of 500 ng/mL of the IS, dividing the mean of the extracted sample by the mean of the unextracted sample (spiked with the extracted blank plasma residues) at the same concentration level. To eliminate the matrix effects, a comparison of the extracted to the unextracted sample was performed, giving the true recovery.To evaluate the inter-day precision and accuracy, QC samples were analyzed together with one independent analytical standard curve for 3 days, while intra-day precision and accuracy were eva¬luated through analysis of the QC samples in six replicates in the same day. Inter- and intra-day precision were expressed as relative standard deviation (RSD). The evaluation of precision and accuracy was based on the criteria that the RSD of each concentration level should be within 15% of the nominal concentration. 9 The lowest standard concentration on the analytical curve should be accepted as the limit of quantitation if the following conditions are met: the analyte response at the LLOQ should be at least five times the response compared to blank response and analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120%. The limit of detection (LOD) was defined by the concentration with a signal-to-noise ratio of 3.The stability of fluoxetine in human plasma was evaluated after each storage period and related to the initial concentration as zero cycle (samples that were freshly prepared and processed immediately). The samples were considered stable if the deviation (expressed as percentage bias) from the zero cycle was within ±15%. The freeze-thaw stability of fluoxetine was determined at low, medium, and high QC samples (n = 3) over three freeze thaw cycles within 3 days. In each cycle, the frozen plasma samples were thawed at room temperature for 2 h and refrozen for 24 h. After completion of each cycle the samples were analyzed and the results compared with that of the zero cycle. The short term stability was evaluated using three aliquots each of the low, medium, and high unprocessed QC samples kept at room temperature (25 ± 5 ºC) for 6 h, and then analyzed. For the processed sample stability, three aliquots each one of the low, medium, and high QC samples were processed and placed into the autosampler at 5 ºC and analyzed after 24 h. For the long term stability analysis of fluoxetine, three aliquots of each QC samples were frozen at –20 ºC for 410 days and then analyzed.

7) Bioequivalence study

Twenty eight healthy volunteers of both sexes, aged between 20 and 40 years old and within the 15% of the ideal body weight were included. The male group was composed of 14 volunteers (25 □ 4.4 yeras, (mean + S.D).; range 20 – 34 years), height between 160 and 182 cm (171.8 □ 0.07 cm), weighing between 55.8 and 88.8 kg (65.2 □ 8.7 kg). The female group was composed by 14 volunteers (30.4 □ 6.9 years; range: 21 – 40 years), height between 152 and 169 cm (161.0 □ 0.04 cm), weighing between 46.0 and 79.5 kg (62.5 □ 8.6 kg). The volunteers were instructed to abstain

from taking any drug including over-the-counter (OTC) medications for 2 weeks prior to and during the study period.This study was performed according to the revised Declaration of Helsinki (1964-2000) for biomedical research involving human beings and Resolution 196/96 from Anvisa - Brazil. The study protocol was approved by M.M. Assert Serviços Médicos S/A Ltda (Escola Assertiva) ethics committee ACCORDING TO BRAZILIAN LAW OF ETHICS COMMITTEE (196/96). All subjects gave written informed consent prior to their inclusion. All volunteers were healthy as assessed by physical examination, electrocardiogram (ECG), and the following laboratory tests: blood glucose, urea, creatinine, uric acid, alanine and aspartate aminotransferases (ALT and AST), gamma-gluthamil transferase (□-GT), alkaline phosphatase, total billirubin, albumin and total protein, trygliceride, total cholesterol, hemoglobin, hematocrit, total and differential white cell counts, red blood cell counts, platelet counts, routine urinalysis and parasitological exam of feces. All subjects were negative for human immunodeficiency virus, and B (except for serological scar) and C hepatitis virus. All female volunteers were negative for □-HCG (pregnancy test). It was an open-label, randomized, two-period, two-sequence crossover study with a two-month washout period. During each period, the volunteers were hospitalized at 7:00 p.m. They had the usual evening meal until 9:00 p.m., and an overnight fast (minimum of 10 hours). At 7:00 a.m., they received a single oral dose of 20 mg (one capsule) of either fluoxetine formulation. Water (200 mL) was given immediately after oral drug administration. All volunteers were then fasted for 4 h following the drug administration; afterwards a standard lunch was consumed. Standard snack, dinner and supper meals were provided respectively 7-8h, 10-12h and 13-14h post dosing. No other food was permitted during the confinement period. Liquid consumption was allowed ad libitum after 2 h following the drug administration, however xanthine-containing drinks including tea, coffee, and cola were avoided.Systolic and diastolic arterial blood pressure (measured non-invasively with a sphygmomanometer), heart rate and temperature were recorded just before and 1, 2, 4, 6, 8, 10, 12, 14, 24, 48, 72, 96, 120, 168, 192, 240, 288 and 360 hours after drug administration.Blood samples (8 mL) from a suitable forearm vein were collected into heparin containing tubes before and at 1, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11, 12, 14, 24, 48, 72, 96, 120, 168, 192, 240, 288, 360 h after the administration of each fluoxetine formulation. Blood samples were centrifuged at 3 000 rpm for 10 min at 4ºC. Plasma was decanted and stored at -20°C until assayed for their fluoxetine contents.Systolic and diastolic arterial blood pressure (measured non-invasively with a sphygmomanometer), heart rate and temperature were recorded just before and 1, 2, 4, 6, 8, 10, 12, 14, 24, 48, 72, 96, 120, 168, 192, 240, 288 and 360 hours after drug administration.It was an open-label,

8) Pharmacokinetic and statistical analysis

Maximum plasma concentration (Cmax) and the time taken to reach this concentration (Tmax) were obtained directly from the curves of fluoxetine plasma concentrations vs. time for each volunteer. The area under the curve to the last measurable concentration (AUC0-t) was calculated by the linear trapezoidal method. Extrapolation to infinity (AUC0-□) was calculated by adding the Ct/ke to AUC0-t (where Ct = the last detectable concentration). The terminal elimination rate constant (ke) was estimated by linear regression from the points describing the elimination phase on a log-linear plot. Half-life (t1/2) was derived as t1/2 = ln(2)/ke.Bioequivalence between the formulations was assessed by the classic 90%- confidence interval (90%-CI) for the ratio of geometric means of Cmax, AUC0-t and AUC0-□. 90%-CI for Tmax difference was calculated as well but was not used in evaluating bioequivalence. Nonparametric method was used to build Tmax confidence interval for the individual differences. Mixed models were fitted using SAS® release 9.1.3 to analyze the log-transformed data. Mean square errors were used to build the parametric confidence intervals.Software used was SAS® version 9.1.3, Microsoft Office® Excel 2003 version 7 and GraphPad Prism version 3.00.

III. Results and Discussion

a) Optimization of the method

In order to obtain a simple and effective analytical method, many pretreatment procedures were assayed. To obtain the best chromatographic separation, the mobile phase was optimized to provide sufficient specificity and sensitivity in a short separation time. Various combinations of organic solvents (me¬thanol and acetonitrile) and water were evaluated as mobile phase components. The mobile phase selected resulted in higher specificity, better sensitivity, short analysis time, improving the peak symmetry. The Polaris C18 (5 □m, 50 x 20 mm) analytical column was selected as it provides the best chromatographic performance and acceptable peak characteristics, including tailing factor, number of theoretical plates and capacity factor. The optimized conditions of the liquid chromatography method were validated for the analysis of fluoxetine in human plasma, due to the capability and application for the bioequivalence study. The proposed method is based on a simple, rapid and efficient sample pre-treatment which allows the determination of fluoxetine in biological matrix, thus fulfilling the criteria required for pharmacokinetic and bioequivalence studies.

b) Validation of the method

Linearity was evaluated in range of 0.2-50.0 ng/mL. The values of the determination coefficient (r2 > 0.990) indicated signi¬ficant linearity of the analytical curves for the method. The LLOQ was evaluated in an experimental assay and was found to be 0.20 ng/mL with precision and accuracy lower than 20%. Fluoxetine in human plasma was directly extracted with hexane/ethyl acetate by liquid-liquid extraction. The mean extraction recoveries for the three concentration levels of the QC samples were 53.40% for fluoxetine and 74.45% for the IS showing the method suitability (Table 1).The intra-day accuracy of the method

was within 97.67 and 104.18% with a precision of 1.58-7.59%. The inter-day accuracy was within 101.19 and 95.11% with RSD of 4.09-7.01% (Table 2). The data show that the method possesses adequate repeatability and reproducibility.Fluoxetine was stable in neat plasma for up to 6 h at room temperature (short-term) and also after three freeze thaw cycles, de¬monstrating that human plasma samples could be thawed and refrozen without compromising the integrity of the samples. Plasma samples were stable for at least 410 days at –20 ºC (long-term). The results demonstrated that extracted samples could be analyzed after being kept in the autosampler for at least 24 h with acceptable precision and accuracy. The results of stability of fluoxetine in human plasma are shown in Table 3.

c) Pharmacokinetic and statistical analysis

The mean pharmacokinetic parameters after a single 20 mg oral dose administration of test and reference products to 27 healthy volunteers are presented in Table 4.The curve of the mean fluoxetine plasma concentration versus time obtained after a single oral dose of each fluoxetine formulation is shown in Figure 2.

The observed fluoxetine pharmacokinetics parameters were similar to those reported in the literature.10 Times to achieve maximum concentration were equivalents for both formulations as shown by the 90% CI of individual Tmax differences (test – reference) that included the zero value (Table 5). No period effect was observed (data not shown), except for Cmax (p = 0.0118). The observed period effect can be explained by very low variance (SD = 0.04631 for both periods; coefficients of variation of 1.84% and 1.78% for period 1 and 2, respectively). Since the difference (log-transformed means of 2.5190 and 2.6058 for period 1 and 2, respectively) was very low, this period effect was considered irrelevant.At any of the evaluation times, the mean values and the concen¬trations of fluoxetine seemed non significant differences between the individual subjects studied after the administration of each of the 2 formulations. The mean Cmax , obtained at 4.50 and 5.00 h, were 13.30 and 13.34 ng/mL for test and reference formulations, respectively. Further statistical analysis of pharmacokinetic variables that described the early and total exposure to fluoxetine showed point estimates of the geometric means ratios of Cmax and AUC(0-t) (fluoxetine test vs. Prozac® reference) to be 101.15% (90% CI: 95.77-106.82) and 94.40% (90% CI: 86.63-102.87), respectively (Table 5). Bioequivalence between the formulations was assessed by the classic 90%- confidence interval (90%-CI) for the ratio of geometric means of Cmax, AUC0-t and AUC0-∞. 90%-CI for Tmax difference was calculated as well but was not used in evaluating bioequivalence. Nonparametric method was used to build Tmax confidence interval for the individual differences. Mixed models were fitted using SAS® release 9.1.3 to analyze the log-transformed data. Mean square errors were used to build the parametric confidence intervals.Software used was SAS® version 9.1.3, Microsoft Office® Excel 2003 version 7 and GraphPad Prism version 3.00.

IV. Conclusion

The present study showed that 90% CI of mean Cmax (after log-transformation of individual ratios) was included into the bioequivalence range (80-125%), consequently, the two formulations of fluoxetine are equivalent for the rate of absorption.The AUC0-t and AUC0-∞ are both recognized as an uncontaminated measurement of the extent of absorption. The present study showed that 90% CI of mean AUC0-t and AUC0-∞ (after log-transformation of individual ratios) were included into the bioequivalence range (80-125%), consequently, the two formulations of fluoxetine are equivalent for the extend of absorption.The statistical comparison of Cmax, AUC0-t and AUC0-∞ clearly indicated no significant difference in the two formulations of fluoxetine 20 mg capsules. 90% confidence intervals for the mean ratio (T/R) of Cmax, AUC0-t and AUC0-∞ were entirely within the US Food and Drug Administration acceptance range. Based on the pharmacokinetic and statistical results of this study, we can conclude that fluoxetine 20 mg capsules (Medley S.A, Brazil) is bioequivalent to Prozac® 20 mg capsules (Eli Lilly, Brazil), and that then the test product can be considered interchangeable in medical practice.

V. References

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Figure 1. MRM chromatograms of spiked human plasma at a final concentration of: (a) 0.2 ng/mL fluoxetine and (b) 500.0 ng/mL paroxetine. The peaks illustrate the retention times

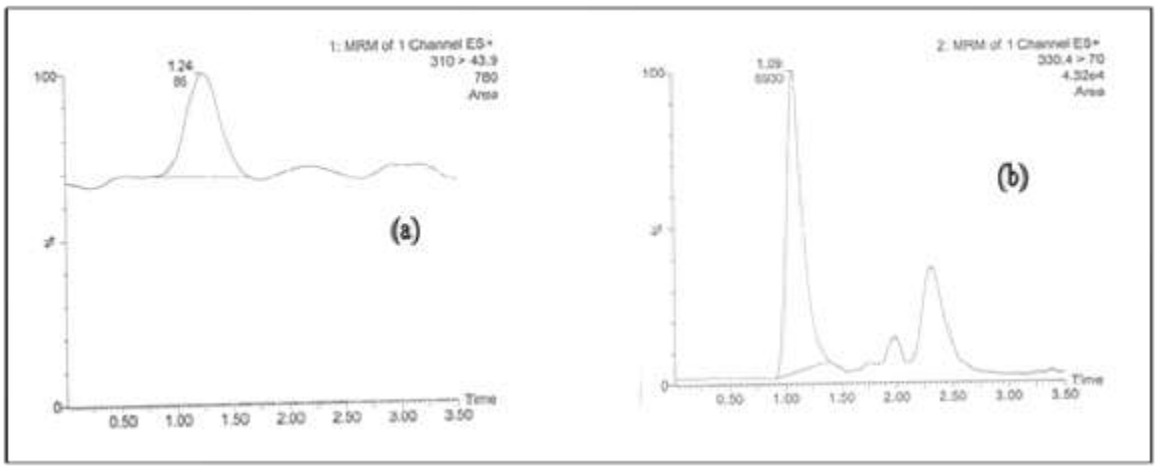

Figure 2. Mean plasma concentration – time profile of fluoxetine after a single 20 mg oral dose administration to 27 healthy volunteers.

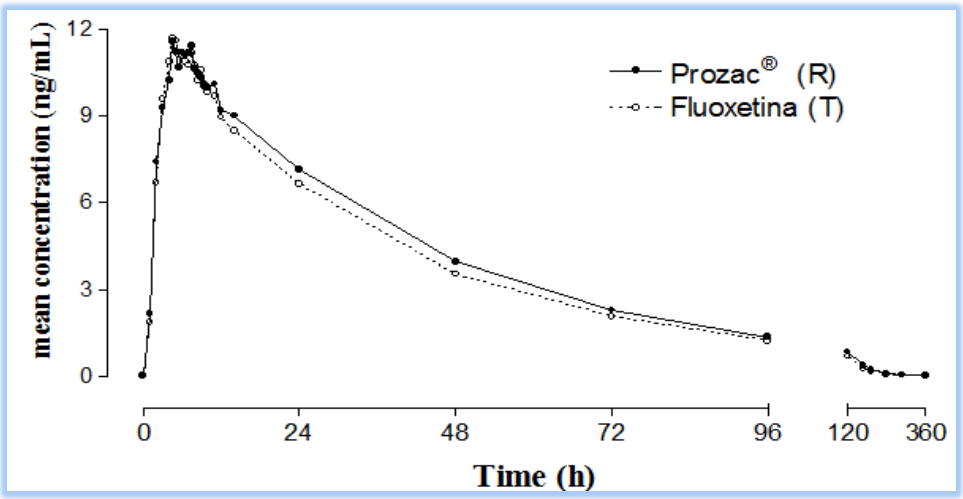

Table 1. Recovery of fluoxetine and paroxetine from human plasma after the extraction procedure

Nominal concentration (ng/mL)	% Recovery (mean ± RSDa %)	
	Fluoxetine	Paroxetine
0.60	58.45 ± 8.66	-
20.0	48.58 ± 1.32	-
40.0	53.17 ± 3.67	-
500.0	-	74.45 ± 5.08

ARSD = Relative standard deviation. BMean of six replicates

Table 2. Inter-day and intra-day precision and accuracy for the determination of fluoxetine in human plasma

Nominal concentration (ng/mL)	RSDa (%)		Accuracy (%)	
	Intra-dayb	Inter-dayc	Intra-dayb	Inter-dayc
0.60	7.59	7.01	97.67	95.11
20.0	1.58	5.68	104.18	101.19
40.0	3.47	4.09	98.11	95.71

ARSD = Relative standard deviation. BMean of six replicates. CMean of three days

Table 3. Stability of human plasma samples of fluoxetine

Stability condition	0.60 ng/mL (meana ± RSDb)	20.0 ng/mL (meana ± RSDb)	40.0 mg/mL (mean ± RSDb)
Fresh samples (zero cycle, %)	93.67 ± 2.64	106.02 ± 2.85	102.36 ± 2.38
Freeze-thaw stability (three cycles, -20 °C, %)	96.22 ± 7.42	97.86 ± 2.34	95.98 ± 3.74
Short-term stability (6 h, room temperature, %)	90.33 ± 0.74	93.43 ± 2.78	93.79 ± 2.03
Long-term stability (410 days, -20 °C, %)	100.00 ± 4.16	103.76 ± 2.63	100.95 ± 4.90

Amen of three replicates. BRSD = Relative standard deviation

Table 4. Mean pharmacokinetic parameters obtained from 27 healthy volunteers after a single 20 mg oral dose administration of fluoxetine

	Prozac®		Fluoxetine	
	Mean	SD[a]	Mean	SD[a]
AUC_{0-t} (ng*h/mL)	527.74	300.42	485.63	244.47
$AUC_{0-\infty}$ (ng*h/mL)	545.62	307.21	502.74	252.30
Ke	0.02	0.01	0.02	0.01
C_{max} (ng/mL)	13.34	3.48	13.30	2.81
$T_{1/2}$(h)	26.95	48.74	27.18	73.35
T_{max}(h) - Median (range)	5.00	7.53	4.50	8.00

[a]SD = Standard deviation.

Table 5. Bioequivalence analysis of two fluoxetine capsule formulations.

Fluoxetine/Prozac® *percent ratios*	Geometric Mean	90% CI
$AUC_{0-\infty}$ % ratio	94.50	86.68 - 103.02
AUC_{0-t} % ratio	94.40	86.63 - 103.87
C_{max} % ratio	101.15	95.77 - 106.82
T_{max} % difference	-0.50[a]	-1.00 - 0.00[b]

[a] Median for individual differences

[b] 90% CI for individual differences