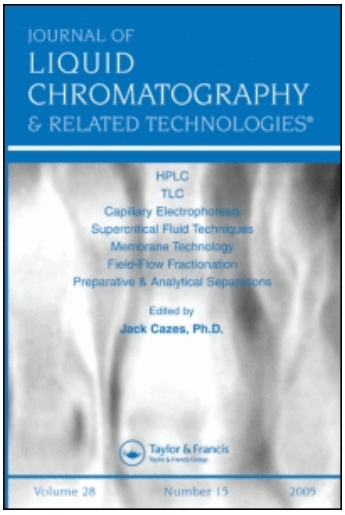

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Higashi, Yasuhiko , Gao, Ranzhi and Fujii, Youichi(2009) 'Determination of Fluoxetine and Norfluoxetine in Human Serum and Urine by HPLC Using a Cholester Column with Fluorescence Detection', Journal of Liquid Chromatography & Related Technologies, 32: 8, 1141 — 1151
To link to this Article: DOI: 10.1080/10826070902841844
URL: http://dx.doi.org/10.1080/10826070902841844

Journal of Liquid Chromatography & Related Technologies®, 32: 1141–1151, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070902841844

Determination of Fluoxetine and Norfluoxetine in Human Serum and Urine by HPLC Using a Cholester Column with Fluorescence Detection

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Abstract: Fluoxetine (FLX), a selective serotonin reuptake inhibitor, is mainly demethylated to norfluoxetine (NFLX). In this study, FLX and NFLX levels in human serum and urine (each 100 μL) were simultaneously analyzed by HPLC fluorescence detection on a Cholester column after pre-column derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). After basic extraction of the samples into pentane, derivatization with NBD-F was conducted in borate buffer (pH 8.5) at 70°C for 2 min. Protriptyline was utilized as an internal standard. The regression equations for FLX hydrochloride and NFLX hydrochloride in human serum showed good linearity in the range of 0.01–0.5 μg/mL with the detection limit of 0.005 μg/mL, and in the range of 0.005–0.5 μg/mL with the detection limit of 0.002 μg/mL, respectively. The corresponding values for human urine were 0.1–0.5 μg/mL with the detection limit of 0.06 μg/mL, and 0.1–0.5 μg/mL with the detection limit of 0.04 μg/mL, respectively. The coefficients of variation were less than 15.7% with good recovery. Our method is useful for a simple and sensitive determination of FLX and NFLX in human serum and urine using a sample volume as small as 100 μL, and should be suitable for therapeutic drug monitoring.

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Keywords: Cholester column, Derivatization, Fluoxetine, Norfluoxetine 4-fluoro-7-nitro-2,1,3-benzoxadiazole

INTRODUCTION

Fluoxetine (FLX), (±)-*N*-methyl-γ-[4-(trifluoromethyl)phenoxy]benzene propanamine, is a highly selective serotonin reuptake inhibitor used as an antidepressant agent.[1,2] After administration, FLX is mainly converted to norfluoxetine (NFLX, the *N*-demethylated metabolite of FLX), which has similar pharmacological activity to FLX.[1,3,4] Both FLX and NFLX have long elimination half lives, ranging from 1 to 6 days and from 5 to 6 days, respectively; about 11% of the dose is excreted as unchanged FLX and about 7% as NFLX.[5,6] Therefore, simultaneous determinations of FLX and NFLX in human serum and urine have been developed and applied for pharmacokinetic studies, therapeutic drug monitoring, and so on.

Many HPLC methods utilizing UV, fluorescence, and MS detection have been established for FLX and NFLX determination.[7–12] In addition, gas chromatographic determinations have been developed using ECD, NPD, and MS.[13–16] However, development of a method with fluorescence detection remains desirable, because of its advantages of sensitivity, simplicity, and low cost.

An HPLC method using dansyl chloride as a fluorescent labeling agent required 45 min for derivatization, as well as a large plasma sample (1 mL).[9] Another method using dansyl chloride required a complicated gradient elution system and a long derivatization time (4 hr).[17] *R*-1-(1-Naphthyl)ethyl isocyanate (derivatization time: 30 min) was used as a labeling agent for chiral separation of FLX and NFLX, and highly sensitive detection was achieved with a gradient system.[5] Guo et al. reported simultaneous detection of FLX and NFLX with 4-(*N*-chloroformylmethyl-*N*-methyl)-amino-7-nitro-2,1,3-benzoxadiazole (NBD-COCl).[18] However, a derivatization time of 2 hr was needed for complete reaction. A simultaneous analysis using 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) also required a long derivatization time (30 min), a gradient system, an expensive internal standard (IS), a large plasma sample volume (1 mL), and two extraction steps.[19]

In this study, we examined the applicability of a Cholester column, and developed an isocratic HPLC assay of FLX and NFLX in human serum and urine (each 100 μL) with fluorescence detection after pre-column derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), which is much more rapidly reactive than NBD-Cl, as the labeling reagent. The reaction scheme is presented in Figure 1. Protriptyline was chosen as an inexpensive IS.

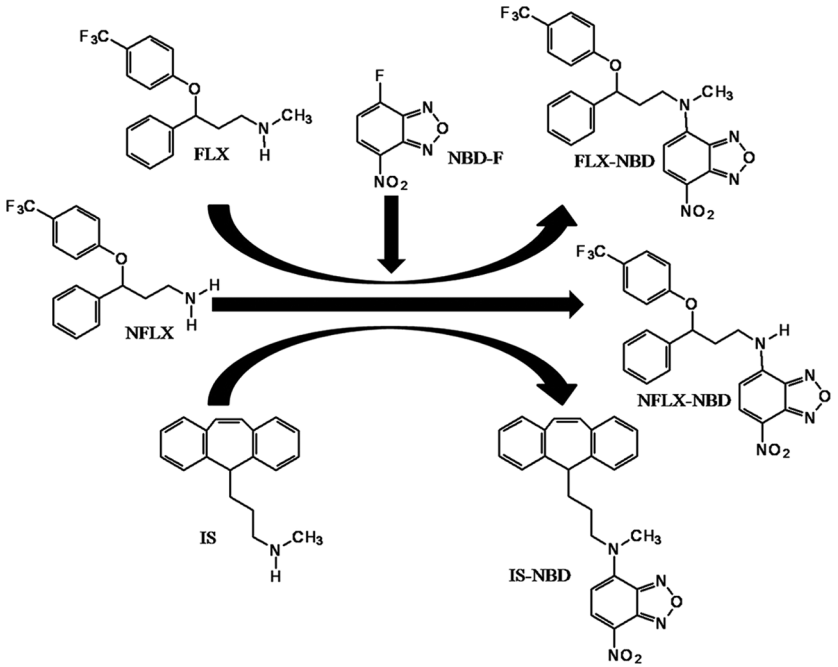

Figure 1. Derivatization scheme of FLX, NFLX, and IS with NBD-F.

EXPERIMENTAL

Reagents

FLX hydrochloride, NFLX hydrochloride, protriptyline hydrochloride, amoxapine, maprotiline hydrochloride, nortriptyline hydrochloride, human serum, and acetonitrile were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Human urine as a freeze dried powder (normal, quantitative urine control) was obtained from Bio-Rad Laboratories (Irvine, CA, U.S.A.). NBD-F, fluvoxamine maleate, paroxetine hydrochloride, and general reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Equipment

The HPLC system comprised a model L-6200 pump (Hitachi, Tokyo, Japan), a Rheodyne injection valve (Cotati, CA, USA) with a 50 μL loop, and a model RF-10A fluorometer (Shimadzu, Kyoto, Japan)

operating at an excitation wavelength of 470 nm and an emission wavelength of 540 nm. The Cholester and C_{18}-MS-II columns (Nacalai tesque, Kyoto) were 150×4.6 mm i.d. with 5 μm particles. Quantification of the peaks was performed using a Chromatopac Model CR-8A integrator (Shimadzu). The mobile phase was prepared by the addition of acetonitrile (450 mL) and ethanol (200 mL) to a solution of trifluoroacetic acid (0.1 v/v%) in 350 mL of water. The samples were eluted from the column at room temperature, at a flow rate of 1.0 mL/min.

Extraction from Human Serum and Urine

An aliquot of 100 μL of sample was rendered alkaline by the addition of NaOH (1 M, 100 μL). Protriptyline hydrochloride solution in water (0.5 μg/mL, 100 μL) was added as an IS to prepare the standard curves for FLX and NFLX. Then, the mixture was vortexed for 1 min and extracted with pentane (3 mL, twice). Each pentane phase was combined and evaporated, and the derivatization was performed as follows.

Derivatization

Borate buffer (0.1 M) was adjusted to pH 8.5 by the addition of NaOH (1 M). Borate buffer (300 μL) was added to the extract. NBD-F solution in acetonitrile (10 mM, 100 μL) was added and vortexed. The mixture was allowed to stand for 2 min at 70°C. Then, it was set on ice for 1 min to stop the derivatization reaction before HCl (0.05 m, 400 μL) was added. The derivatives (50 μL) were injected into the HPLC system.

Calibration Curve

A solution of FLX hydrochloride and NFLX hydrochloride (1 mg/mL) in water was added to human biological fluids. The concentrations of FLX hydrochloride and NFLX hydrochloride were 0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, and 0.5 μg/mL. All samples were extracted and analyzed using the procedures described above. Calibration curves based on the peak area ratios of FLX hydrochloride and NFLX hydrochloride to the IS were constructed. The values of the lower limit of quantification, i.e., the lowest concentration on the standard curve that can be measured with a C.V. value of $<20\%$ (relative standard deviation less than 20%) and a recovery of $100 \pm 20\%$, are listed in Tables 2 and 3.[20,21]

RESULTS AND DISCUSSION

Comparison of Cholester Column with C_{18}-MS-II Column

FLX hydrochloride and NFLX hydrochloride in water (each 0.5 μg/mL) were derivatized with NBD-F and analyzed. As shown in Figure 2, the blank showed a peak close to the NFLX derivative peak eluted from the C_{18}-MS-II column, which is usually the first choice. It was considered that this peak would interfere with the sensitive determination of NFLX. When a Cholester column, usually recommended as a second choice column, was used instead, we found no peak that would interfere with the peaks of interest. Therefore, we judged that the Cholester column is

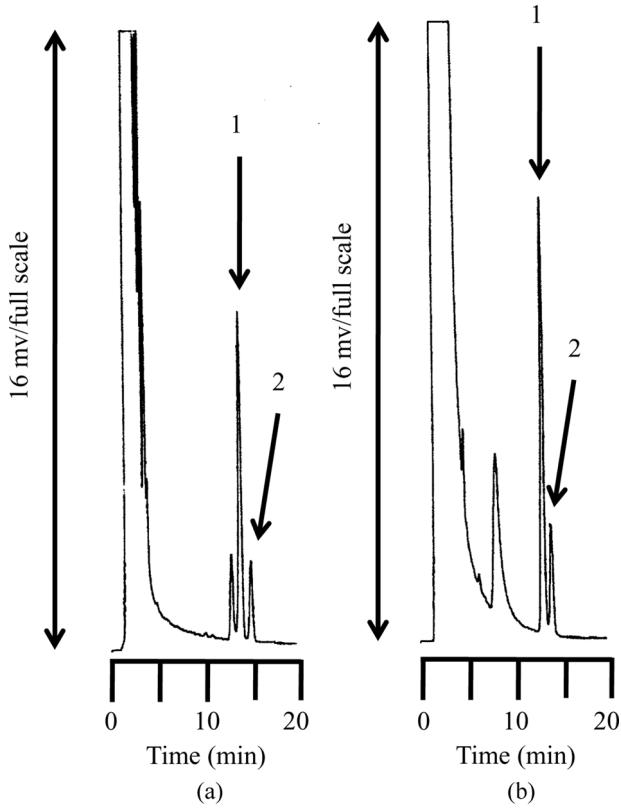

Figure 2. Chromatograms of FLX and NFLX derivatives using C_{18}-MS-II (a) and Cholester column (b). FLX hydrochloride and NFLX hydrochloride (0.5 μg/mL) were derivatized with NBD-F. Peaks: 1 = NFLX derivative, 2 = FLX derivative.

superior to the C_{18}-MS-II column for simultaneous determination of FLX and NFLX.

Chromatograms

Figure 3 shows typical chromatograms obtained from (A-1) blank serum, (A-2) serum spiked with FLX hydrochloride, NFLX hydrochloride, and IS (each 0.5 μg/mL), (B-1) blank urine and (B-2) urine spiked with FLX hydrochloride, NFLX hydrochloride, and IS (0.5 μg/mL). The retention times of the NFLX, FLX, and IS derivatives were 13.7, 14.6, and 17.6 min, respectively. The running time was 20 min.

Standard Curves of FLX and NFLX and Detection Limits

Standard curves of FLX and NFLX were constructed by plotting integrated peak area ratios of FLX hydrochloride and NFLX hydrochloride to IS *vs.* FLX hydrochloride and NFLX hydrochloride concentration

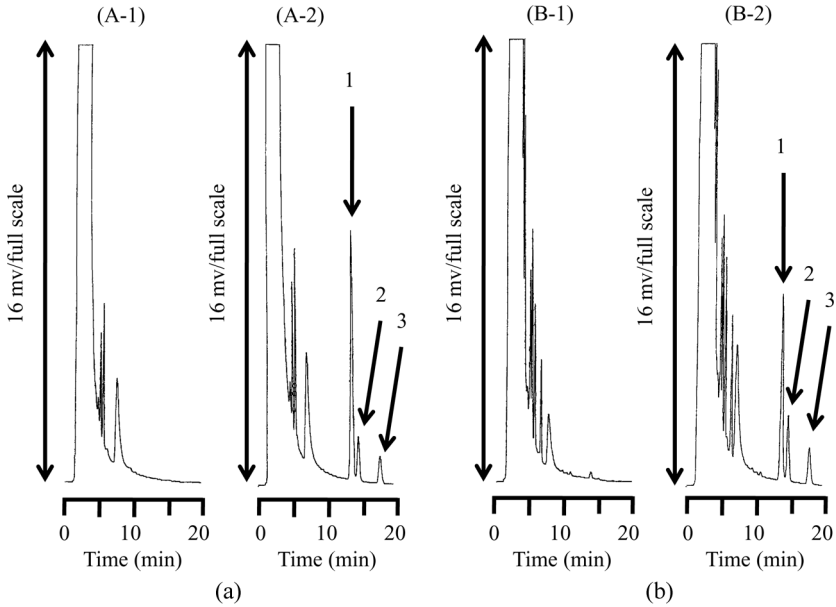

Figure 3. Typical chromatograms of FLX, NFLX and IS derivatives with NBD-F in human serum (a) and urine (b) using Cholester column. (A-1) and (B-1), Blank; (A-2) and (B-2), sample spiked with FLX hydrochloride, NFLX hydrochloride, and IS (each 0.5 μg/mL). Peaks: 1 = NFLX derivative, 2 = FLX derivative, 3 = IS derivative.

Table 1. Linear correlation parameters of FLX hydrochloride and NFLX hydrochloride

	Slope	Intercept	Concentration range	*r*
Serum				
FLX hydrochloride	2.81	+0.010	0.01 to 0.5 μg/mL	0.9961
NFLX hydrochloride	12.8	+0.026	0.005 to 0.5 μg/mL	0.9972
Urine				
FLX hydrochloride	3.05	+0.104	0.1 to 0.5 μg/mL	0.9945
NFLX hydrochloride	7.35	+0.157	0.1 to 0.5 μg/mL	0.9955

(Table 1). In human serum samples, linear relationships were obtained for FLX hydrochloride ($y = 2.81x + 0.010$, concentrations ranging from 0.01 to 0.5 μg/mL, $r = 0.9961$) and for NFLX hydrochloride ($y = 12.8x + 0.026$, concentrations ranging from 0.005 to 0.5 μg/mL, $r = 0.9972$). The lower limits of detection for FLX hydrochloride and NFLX hydrochloride were 0.005 μg/mL (14 nM, 0.0045 μg/mL as FLX) and 0.002 μg/mL (6.0 nM, 0.0018 μg/mL as NFLX), respectively, (signal-to-noise ratio of 3:1). In human urine samples, linear relationships were obtained for FLX hydrochloride ($y = 3.05x + 0.104$, concentrations ranging from 0.1 to 0.5 μg/mL, $r = 0.9945$) and NFLX hydrochloride ($y = 7.35x + 0.157$, concentrations ranging from 0.1 to 0.5 μg/mL, $r = 0.9955$). The lower limits of detection for FLX hydrochloride and NFLX hydrochloride were 0.06 μg/mL (0.17 μM, 0.053 μg/mL as FLX) and 0.04 μg/mL (0.12 μM, 0.036 μg/mL as NFLX), respectively, (signal-to-noise ratio of 3:1).

A previous report using NBD-Cl gave detection limits of 0.5 ng/mL for FLX and 0.02 ng/mL for NFLX in 1 mL of plasma.[19] Although the detection limit using NBD-COCl was not described, the limits of quantification were given as 10 nM in 100 μL of plasma.[18] These methods were applied for pharmacokinetic studies in healthy volunteers (dose of 40 mg of FLX hydrochloride) and in rats (dose of 10 mg/kg of FLX hydrochloride). Our method should be particularly useful for kinetic studies.

Precision and Accuracy

Precision and accuracy for intra-day and inter-day assays of FLX and NFLX derivatives are shown in Tables 2 and 3. In the intra- and inter-day assay using human serum samples, the range of standard deviation of the mean for FLX hydrochloride and NFLX hydrochloride was within 2.9 to 13.8%. The recoveries of FLX hydrochloride and NFLX hydrochloride were within 88.0 to 110.0%. In the intra- and inter-day assays

Table 2. Intra- and inter-day assay reproducibility for determination of FLX hydrochloride and NFLX hydrochloride in human serum

Concentration (μg/mL)	Measured (μg/mL) (Mean ± S.D., $n = 4$)	C.V. (%)	Recovery (%)
Intra-day assay			
FLX hydrochloride			
0.01	0.0107 ± 0.0014	13.1	107.0
0.1	0.109 ± 0.008	7.3	109.0
0.5	0.550 ± 0.070	12.7	110.0
NFLX hydrochloride			
0.005	0.00440 ± 0.00052	11.8	88.0
0.01	0.00957 ± 0.00060	6.3	95.7
0.1	0.103 ± 0.003	2.9	103.0
0.5	0.503 ± 0.019	3.8	100.6
Inter-Day Assay			
FLX hydrochloride			
0.01	0.0107 ± 0.0009	8.4	107.0
0.1	0.0983 ± 0.0089	9.1	98.3
0.5	0.506 ± 0.030	5.9	101.2
NFLX hydrochloride			
0.005	0.00484 ± 0.00067	13.8	96.8
0.01	0.0103 ± 0.0014	13.6	103.0
0.1	0.0959 ± 0.0130	13.6	95.9
0.5	0.505 ± 0.032	6.3	101.0

Table 3. Intra- and inter-day assay reproducibility for determination of FLX hydrochloride and NFLX hydrochloride in human urine

Concentration (μg/mL)	Measured (μg/mL) (Mean ± S.D., $n = 4$)	C.V. (%)	Recovery (%)
Intra-day assay			
FLX hydrochloride			
0.1	0.111 ± 0.005	4.5	111.0
0.5	0.501 ± 0.035	7.0	100.2
NFLX hydrochloride			
0.1	0.102 ± 0.016	15.7	102.0
0.5	0.479 ± 0.029	6.1	95.8
Inter-day Assay			
FLX hydrochloride			
0.1	0.110 ± 0.015	13.6	110.0
0.5	0.473 ± 0.059	12.5	94.6
NFLX hydrochloride			
0.1	0.0932 ± 0.0039	4.2	93.2
0.5	0.501 ± 0.062	12.4	100.2

Table 4. Interference by other antidepressants

Derivatives	Relative retention time
Amoxapine	0.40
Fluvoxamine	0.63
NFLX	0.78
FLX	0.83
Paroxetine	0.99
IS	1.00
Nortriptyline	1.12
Maprotiline	1.24

using human urine samples, the range of standard deviation of the mean for FLX hydrochloride and NFLX hydrochloride was within 4.2 to 15.7%. The recoveries of FLX hydrochloride and NFLX hydrochloride were within 93.2 to 111.0%. The assay exhibited satisfactory precision and accuracy.

Interference

As shown in Table 4, the retention times of other antidepressant derivatives were investigated. The relative retention times of amoxapine, fluvoxamine, paroxetine, nortriptyline, and maprotiline derivatives were 0.40, 0.63, 0.99, 1.12, and 1.24, respectively. The peak of the paroxetine derivative almost fully overlapped with the IS derivative peak.

CONCLUSION

An isocratic HPLC method was developed for quantitation of FLX and NFLX in human serum and urine by means of fluorescence detection after pre-column derivatization with NBD-F. The use of a Cholester column instead of an C_{18}-MS-II column avoided interference of endogenous peaks with the peaks of interest. While the sensitivity of our method is less than has been obtained with other NBD related labeling agents, the derivatization procedure is much quicker in our method.[18,19] Validation data (precision, accuracy, and recovery) were satisfactory for simultaneous FLX and NFLX determination. Among related compounds examined, paroxetine interfered with the IS derivative peak. The established method is expected to be particularly suitable for routine therapeutic drug monitoring of FLX and NFLX during treatment for depression in patients not also receiving paroxetine, as well as for pharmacokinetic studies in experimental animals.

REFERENCES

1. Schmidt, M.J.; Fuller, R.W.; Wong, D.T. Fluoxetine, a highly selective serotonin reuptake inhibitor: a review of preclinical studies. Br. J. Psychiat. Suppl. **1988**, *3*, 40–46.
2. Lemberger, L.; Rowe, H.; Carmichael, R.; Crabtree, R.; Horng, J.S.; Bymaster, F.; Wong, D. Fluoxetine, a selective serotonin uptake inhibitor. Clin. Pharmacol. Ther. **1978**, *23*, 421–429.
3. Wong, D.T.; Bymaster, F.P.; Reid, L.R.; Mayle, D.A.; Krushinski, J.H.; Robertson, D.W. Norfluoxetine enantiomers as inhibitors of serotonin uptake in rat brain. Neuropsychopharmacology. **1993**, *8*, 337–344.
4. Baumann, P. Pharmacokinetic-pharmacodynamic relationship of the selective serotonin reuptake inhibitors. Clin. Pharmacokinet. **1996**, *31*, 444–469.
5. Unceta, N.; Gómez-Caballero, A.; Sánchez, A.; Millán, S.; Sampedro, M.C.; Goicolea, M.A.; Sallés, J.; Barrio, R.J. Simultaneous determination of citalopram, fluoxetine and their main metabolites in human urine samples by solid-phase microextraction coupled with high-performance liquid chromatography. J. Pharm. Biomed. Anal. **2008**, *46*, 763–770.
6. Altamura, A.C.; Moro, A.R.; Percudani, M. Clinical pharmacokinetics of fluoxetine. Clin. Pharmacokinet. **1994**, *26*, 201–214.
7. Cruz-Vera, M.; Lucena, R.; Cárdenas, S.; Valcárcel, M. Combined use of carbon nanotubes and ionic liquid to improve the determination of antidepressants in urine samples by liquid chromatography. Anal. Bioanal. Chem. **2008**, *391*, 1139–1145.
8. Malfará, W.R.; Bertucci, C.; Costa Queiroz, M.E.; Dreossi Carvalho, S.A.; Pires Bianchi Mde, L.; Cesarino, E.J.; Crippa, J.A.; Costa Queiroz, R.H. Reliable HPLC method for therapeutic drug monitoring of frequently prescribed tricyclic and nontricyclic antidepressants. J. Pharm. Biomed. Anal. **2007**, *44*, 955–962.
9. Suckow, R.F.; Zhang, M.F.; Cooper, T.B. Sensitive and selective liquid-chromatographic assay of fluoxetine and norfluoxetine in plasma with fluorescence detection after precolumn derivatization. Clin. Chem. **1992**, *38*, 1756–1761.
10. Kovacevic, I.; Pokrajac, M.; Miljkovic, B.; Jovanovic, D.; Prostran, M. Comparison of liquid chromatography with fluorescence detection to liquid chromatography-mass spectrometry for the determination of fluoxetine and norfluoxetine in human plasma. J. Chromatogr. B. **2006**, *830*, 372–376.
11. Santos-Neto, A.J.; Bergquist, J.; Lanças, F.M.; Sjöberg, P.J. Simultaneous analysis of five. Antidepressant drugs using direct injection of biofluids in a capillary restricted-access media-liquid chromatography-tandem mass spectrometry system. J. Chromatogr. A. **2008**, *1189*, 514–522.
12. Juan, H.; Zhiling, Z.; Huande, L. Simultaneous determination of fluoxetine, citalopram, paroxetine, venlafaxine in plasma by high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-MS/ESI). J. Chromatogr. B. **2005**, *820*, 33–39.

13. Lantz, R.J.; Farid, K.Z.; Koons, J.; Tenbarge, J.B.; Bopp, R.J. Determination of fluoxetine and norfluoxetine in human plasma by capillary gas chromatography with electron-capture detection. J. Chromatogr. **1993**, *614*, 175–179.
14. Martínez, M.A.; Sánchez de la Torre, C.; Almarza, E. A comparative solid-phase extraction study for the simultaneous determination of fluoxetine, amitriptyline, nortriptyline, trimipramine, maprotiline, clomipramine, and trazodone in whole blood by capillary gas-liquid chromatography with nitrogen-phosphorus detection. J. Anal. Toxicol. **2003**, *27*, 353–358.
15. Berzas Nevado, J.J.; Villaseñor Llerena, M.J.; Guiberteau Cabanillas, C.; Rodríguez Robledo, V. Screening of citalopram, fluoxetine and their metabolites in human urine samples by gas chromatography-mass spectrometry. A global robustness/ruggedness study. J. Chromatogr. A. **2006**, *1123*, 130–131.
16. Nerzas Nevado, J.J.; Villaseñor Llerena, M.J.; Guiberteau Cabanillas, C.; Rodríguez Robledo, V.; Buitrago, S. Sensitive capillary GC-MS-SIM determination of selective serotonin reuptake inhibitors: reliability evaluation by validation and robustness study. J. Sep. Sci. **2006**, *29*, 103–113.
17. Clausing, P.; Rushing, L.G.; Newport, G.D.; Bowyer, J.F. Determination of D-fenfluramine, D-norfenfluramine and fluoxetine in plasma, brain tissue and brain microdialysate using high-performance liquid chromatography after precolumn derivatization with dansyl chloride. J. Chromatogr. B. **1997**, *692*, 419–426.
18. Guo, X.; Fukushima, T.; Li, F.; Imai, K. Determination of fluoxetine and norfluoxetine in rat plasma by HPLC with pre-column derivatization and fluorescence detection. Biomed. Chromatogr. **2003**, *17*, 1–5.
19. Ertürk, S.; Cetin, S.M.; Atmaca, S.; Ersoy, L.; Baktr, G. A sensitive HPLC method for the determination of fluoxetine and norfluoxetine in human plasma with fluorescence detection. Ther. Drug Monit. **2005**, *27*, 38–43.
20. Karnes, H.T.; Shiu, G.; Shah, V.P. Validation of bioanalytical methods. Pharm. Res. **1991**, *8*, 421–426.
21. Shah, V.P.; Midha, K.K.; Findlay, J.W.; Hill, H.M.; Hulse, J.D.; McGilveray, I.J.; McKay, G.; Miller, K.J.; Patnaik, R.N.; Powell, M.L.; Tonelli, A.; Viswanathan, C.T.; Yacobi, A. Bioanalytical method validation–a revisit with a decade of progress. Pharm. Res. **2000**, *17*, 1551–1557.

Received October 24, 2008
Accepted December 10, 2008
Manuscript 6436