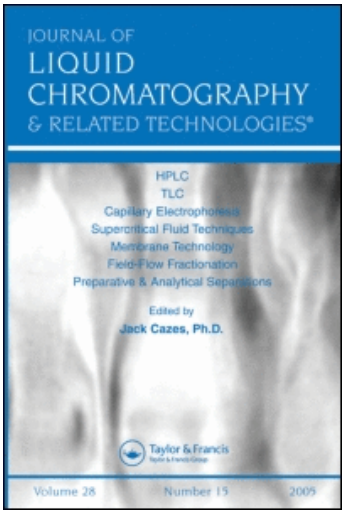

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A SENSITIVE AND HIGH-THROUGHPUT LC/MS/MS METHOD USING A SILICA COLUMN AND AN AQUEOUS-ORGANIC MOBILE PHASE FOR THE ANALYSIS OF FLUOXETINE AND NORFLUOXETINE IN HUMAN PLASMA

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ABSTRACT

A sensitive and high-throughput LC/MS/MS method was developed and validated for the determination of fluoxetine and its metabolite norfluoxetine in human plasma. Analytes of interest were extracted from alkalized human plasma using liquid/liquid extraction. The extract was injected onto a silica column with an aqueous-organic mobile phase consisting of acetonitrile, water, trifluoroacetic acid (TFA), and ammonium acetate. The chromatographic run time was 2.0 min per injection, with retention time of 1.1 min for both fluoxetine and norfluoxetine, and 1.2 min for the internal standard (IS), fentanyl-d_5. Fluoxetine was monitored at m/z 310 $\rightarrow$ 44, norfluoxetine at 296 $\rightarrow$ 134, and IS at m/z 342 $\rightarrow$ 188, respectively.

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The standard curve range was 0.5–250 ng/mL and low limit of quantitation (LLOQ) was 0.5 ng/mL for both fluoxetine and norfluoxetine. The inter-day precision and accuracy of the quality control (QC) samples were <5.1% relative standard deviation (RSD) and <7.3% relative error (RE).

INTRODUCTION

Fluoxetine is a widely used antidepressant drug.[1] The chemical structures of fluoxetine, its metabolite norfluoxetine, and internal standard fentanyl-d_5 are shown in Figure 1. A sensitive (0.5 ng/mL) and high-throughput (>200 samples

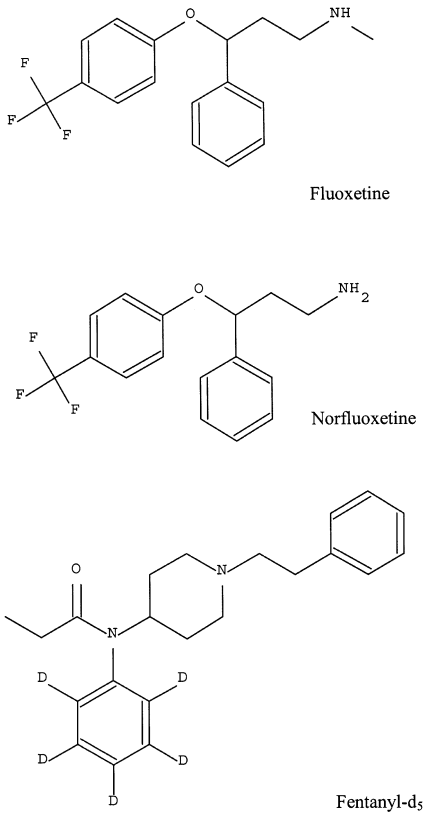

Figure 1. Chemical structures of fluoxetine, norfluoxetine and internal standard (fentanyl-d_5).

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per day) bioanalytical method was needed to analyze samples generated from clinical trials.

Numerous analytical methods for assaying fluoxetine and norfluoxetine in biological fluids, which included HPLC,[2–14] GC,[15–18] GC/MS[19,20] and LC/MS/MS,[21,22] have appeared in literature. HPLC, GC, and GC/MS methods did not appear to have adequate sensitivity for measuring as low as 0.5 ng/mL of fluoxetine and norfluoxetine. Improved detection sensitivity was achieved in the published LC/MS/MS methods. However, tedious extraction procedures coupled with long run time used in these published LC/MS/MS methods, were not conducive for high-throughput analysis of large numbers of clinical samples.

In this paper, a sensitive and high-throughput LC/MS/MS method for assaying fluoxetine and norfluoxetine in human plasma is presented. Particular effort was made to improve the method sensitivity and throughput by our novel approach of using silica column and aqueous-organic mobile phase.

EXPERIMENTAL

Chemicals and Reagents

Fluoxetine hydrochloride (purity 100%) was from USP (Rockville, MD, USA); norfluoxetine hydrochloride (purity 100%) and internal standard, fentanyl-d_5 (purity 99%), was supplied by Sigma (St. Louis, MO, USA). Methyl-tert butyl ether (MTBE) and ammonium hydroxide of ACS reagent grade were from Fisher Scientific (St. Louis, MO, USA). Acetonitrile, methanol, TFA, and water of HPLC grade were also from Fisher Scientific. Control human plasma, sodium heparin anticoagulant, was from Biochemed (Winchester, VA, USA) and was stored in a freezer at -20°C.

Calibration Standards, Quality Control (QC) Samples and Sample Extraction

Standards and QC samples were made from separate stock solutions (0.1 mg/mL) of fluoxetine and norfluoxetine in a mixture of methanol and water (1 : 1, v/v). Calibration standards of 0.500, 1.00, 2.50, 10.0, 25.0, 100, 225, and 250 ng/mL were prepared by fortifying the control plasma with fluoxetine and norfluoxetine. QC samples at levels of 0.500, 1.50, 15.0, 185, and 400 ng/mL were prepared by adding appropriate amounts of fluoxetine and norfluoxetine QC stock solutions to blank human plasma. All QCs were aliquotted and stored frozen at -20°C.

Aliquots of 100 µL of the standard, QC and blank plasma samples were transferred into 16 × 100-mm glass tubes and 20 µL of internal standard solution (100 ng/mL in 1 : 1 methanol–water) was added to all tubes except blank plasma samples. One hundred (100) µL of 2% ammonium hydroxide solution was added to each tube and samples were vortex-mixed for 30 seconds. Two-(2) mL of MTBE was added to each tube and samples were vortex-mixed for 2 minutes. The tubes were then centrifuged at 3000 rpm at room temperature for 5 minutes using a Beckmann Centrifuge Model J6-MC (Fullerton, CA, USA). The lower aqueous layer was frozen in a dry ice/acetone bath and the upper organic layer was decanted into a 12 × 75-mm glass tube. The organic solvent was evaporated to dryness under nitrogen in a Zymark TurboVap LV Evaporator (Hopkinton, MA, USA) set at 30°C and approximately 10-psi nitrogen pressure. The samples were reconstituted with 200 µL of acetonitrile—40-mM ammonium acetate—TFA (95 : 5 : 0.05, v/v/v) by vortex mixing for 1 minute. The samples were transferred to limited-volume polypropylene vials.

LC/MS/MS

The LC/MS/MS system consisted of a Shimadzu HPLC system (Kyoto, Japan) and a PE Sciex API 3000 tandem mass spectrometer (Concord, Ontario, Canada) with (+) ESI. The analytical column, Betasil silica of 5 µm, 50 × 3.0 mm I.D., was from Keystone Scientific (Bellefonte, PA, USA). The mobile phase was acetonitrile—40-mM ammonium acetate—TFA (94 : 6 : 0.05, v/v/v). The injection volume was 10 µL. The flow rate was 0.5 mL/min. Autosampler carryover was determined by injecting the highest calibration standard, then an extracted blank sample. No carryover was observed. Without any column-regeneration, one column could be used for at least 500 injections of the extracted samples.

Sensitivity of the multiple reaction mode (MRM) was optimized by testing with an infusion of 0.1 µg/mL fluoxetine, norfluoxetine, or fentanyl-d_5 in a mixture of acetonitrile, water, and TFA (50 : 50 : 0.05, v/v/v). The Ionspray needle was maintained at 5 kV. The turbo gas temperature was 400°C and the auxiliary gas flow was 8.0 L/min. Nebulizing gas, curtain gas, and collision gas flows were at instrument settings of 8, 8, and 4, respectively. The mass spectrometer was operated under MRM mode with a collision energy (CE) of 35 eV for fluoxetine and IS, and 11 eV for norfluoxetine. The transitions (precursor to product) monitored were m/z 310 → 44 for fluoxetine, 296 → 134 for norfluoxetine and 342 → 188 for IS. The dwell time was 200 msec. Both quadrupoles were maintained at unit resolution. A weighted 1/concentration^2 linear regression was used to generate calibration curves from standards and calculate the concentrations of quality control samples.

RESULTS AND DISCUSSION

Development of the LC/MS/MS Method

In this paper, a LC/MS/MS method using silica column and aqueous-organic mobile phase to analyze fluoxetine and norfluoxetine in human plasma was presented. The selection of silica column and aqueous-organic mobile phase was based on our previous experiences with other polar compounds.[23,24] Bare silica columns operated with aqueous-organic mobile phases are viable means of analyzing polar compounds in biological fluid. In comparison with reversed-phase LC/MS/MS, the sensitivity of our method was significantly improved due to the increased organic content (94% acetonitrile) in the mobile phase. Unlike classic normal-phase LC where the trace amount (in the ppm range) of water in the mobile phase has to be strictly controlled, our mobile phase can be very easily prepared, and the LC/MS/MS condition is completely compatible with the contemporary biological sample extraction techniques. The acidic mobile phase nature ensures the protonation and sensitivity of the basic analyte such as fluoxetine and norfluoxetine in the mobile phase. Water is the stronger eluting solvent than acetonitrile. The reconstitution contained 5% of water while the mobile phase contained 6% of water. The advantage of using a reconstitution solution with elution strength weaker than the mobile phase has been discussed.[25] The peak shape was improved by using an injection solvent with the eluting strength weaker than the mobile phase.

Figures 2 and 3 show the chromatograms of 0.500 ng/mL (LLOQ) in plasma and plasma blank, respectively. Excellent signal to noise ratio was obtained with the LLOQ samples. Known amounts of analytes at 1.50 ng/mL and 185 ng/mL were spiked into each of the six lots of biological fluids. These samples, together with blank samples, were run together with one set of calibration standards extracted from one lot of the biological fluid. All six lots of human plasma were shown to be free of interference for the analytes of interest and the IS. All of the spiked samples have calculated concentration within 20% of the nominal value. This indicated that there is no significant matrix effect difference among the tested lots. The silica column demonstrated superior stability under the chromatographic conditions. At least 500 samples can be analyzed on a single silica column.

IS should mirror the analytes during extraction, chromatography, and MS detection. IS should have similar extraction recovery as the analytes. To compensate for any potential inconsistent response due to matrix effects, IS should elute close to the analytes on the column. Whenever possible, stable isotope internal standard should be used. However, stable isotopes are not always easily available due to the high cost or due to the technical difficulty during synthesis. As demonstrated by this paper, a rugged LC/MS/MS method could still be developed

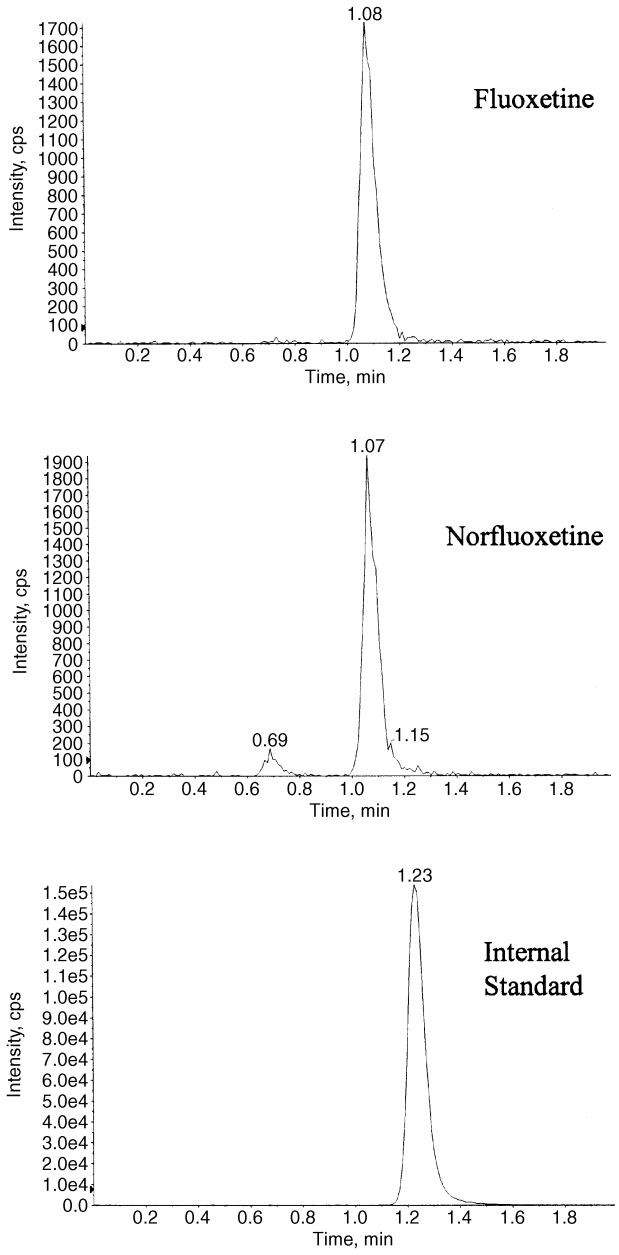

Figure 2. Chromatogram of a blank plasma sample spiked with fluoxetine and norfluoxetine at LLOQ (0.5 ng/mL) and IS.

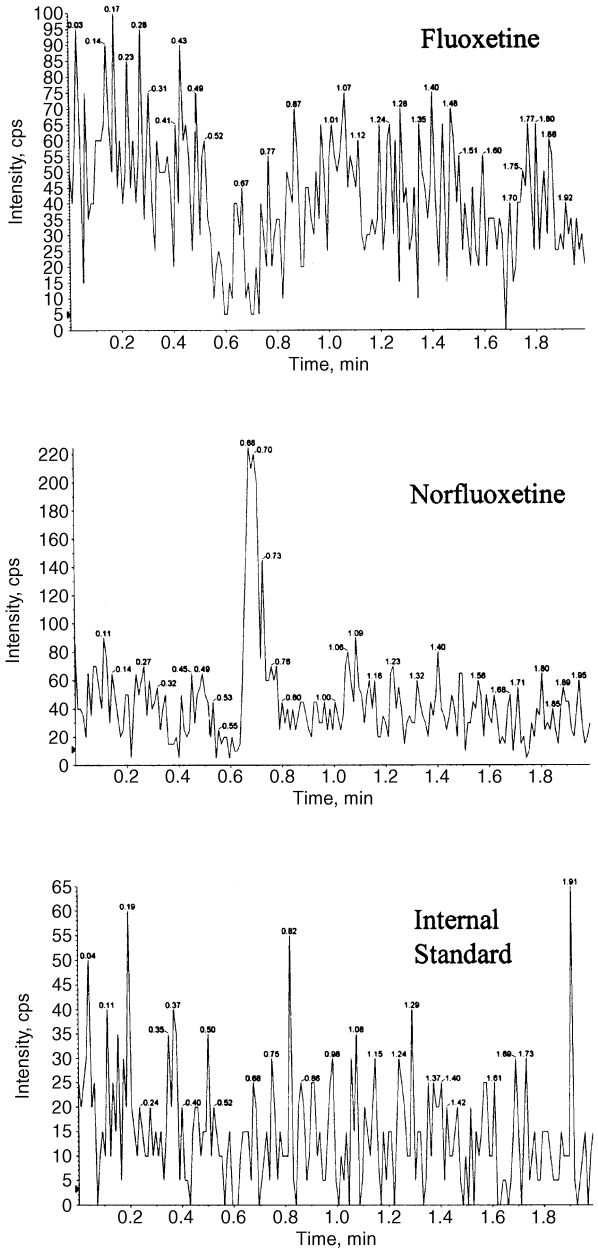

Figure 3. Chromatogram of a blank plasma sample.

using non-stable isotope. Fentanyl-d_5 was chosen as the IS because of its similar extraction recovery and retention time as fluoxetine and norfluoxetine.

Since fluoxetine and norfluoxetine have basic groups, they could be extracted from alkalized human plasma using liquid/liquid extraction or using mixed-mode solid phase extraction. Initially, utilization of a mixed-mode sorbent (Certify® by Varian Sample Preparation Products) to extract analytes was investigated. This sorbent consists of both reversed-phase interaction sites (octyl and benzene) and strong cation exchange sites (sulfonic acid) and is, therefore, capable of retaining basic compounds via both mechanisms. However, consistent recovery was not achieved for fluoxetine and norfluoxetine. A liquid/liquid extraction method was therefore used. Since the sample size was only 0.1 mL, liquid/liquid extraction in 96-well format on the Tomtec Quadra® 96–320 workstation was tried. Successful 96-well liquid/liquid extraction to extract other polar compounds, such as fluconazole from human plasma, was achieved in our laboratory. However, for fluoxetine and norfluoxetine, this attempt failed because of inconsistent recovery. Finally, a simple one-step liquid/liquid extraction was used. Unlike a previously published dual-step extraction procedure used for a LC/MS/MS method, which was tedious and prone to errors,[22] this manual extraction procedure was easy to use and was suitable for high-throughput sample analysis. Extraction recoveries were determined by comparing the peak areas of extracted QC samples with peak areas of post-extraction fortified plasma blanks at corresponding concentrations. Acceptable and consistent recoveries (>80%) were obtained for fluoxetine, norfluoxetine, and IS.

Validation Results

The accuracy and precision data were generated using 3 validation batches. Calibration curve parameters and data are listed in Table 1. The correlation coefficients of the three validation curves were all >0.997. The standards show a linear range of 0.5–250 ng/mL, using weighted ($1/\text{concentration}^2$) least-square linear regression. The precision and accuracy data for QC samples are summarized in Table 2. The data show that this method is consistent and reliable with low RSD and RE values. For the LLOQ QC, the RSD (n = 6) of the measured concentration was 4.0% for fluoxetine and 3.9% for norfluoxetine, respectively. For the same QC samples, the RE of the mean of the measured concentrations was −2.8% for fluoxetine and −5.2% for norfluoxetine.

Stability of sample processing (freeze–thaw and benchtop), and chromatography (extracts) were tested and established. Three freeze/thaw cycles and ambient temperature storage of the QC samples for up to 24 hours prior to analysis appeared to have little effect on the quantitation. QC samples stored in a freezer at −20°C remained stable through the course of the validation. Extracted

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Table 1. Precision and Accuracy of Calibration Standards

	0.500	1.00	2.50	10.0	25.0	100	225	250	Slope	r^2
Fluoxetine (ng/mL)										
Batch 1	0.496	1.03	2.39	9.76	25.3	100	220	262	1.79E–02	0.9994
Batch 2	0.515	0.967	2.32	9.64	27.3	99.7	228	252	2.08E–02	0.9984
Batch 3	0.509	0.976	2.43	9.56	27.6	101	220	247	1.87E–02	0.9986
Mean	0.507	0.991	2.38	9.65	26.7	100	223	254	1.91E–02	0.9988
RSD (%)	1.9	3.4	2.3	1.0	4.7	0.7	2.1	3.0	7.9	
RE (%)	+1.4	−0.9	−4.8	−3.5	+6.8	0.0	−0.9	+1.6		
Norfluoxetine (ng/mL)										
Batch 1	0.487	1.05	2.50	9.97	25.2	102	210	255	1.79E–02	0.9992
Batch 2	0.509	0.988	2.36	9.31	27.4	101	232	245	2.13E–02	0.9983
Batch 3	0.501	1.01	2.42	9.55	28.4	99.4	225	234	1.95E–02	0.9977
Mean	0.499	1.02	2.43	9.61	27.0	101	222	245	1.96E–02	0.9984
RSD (%)	2.2	3.1	2.9	3.5	6.1	1.3	5.0	4.3	8.7	
RE (%)	−0.2	+2.0	−2.8	−3.9	+8.0	+1.0	−1.3	−2.0		

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Table 2. Precision and Accuracy of Quality Control Samples

	Intraday (n = 6)						Interday (n = 18)		
	0.500	1.50	15.0	185	185*	400*	1.50	15.0	185
Fluoxetine (ng/mL)									
Mean	0.486	1.53	15.7	190	195	429	1.54	15.9	193
RSD (%)	4.0	5.2	3.2	3.4	3.3	3.4	5.1	4.0	4.0
RE (%)	−2.8	+2.0	+4.7	+2.7	+5.4	+7.3	+2.7	+6.0	+4.3
Norfluoxetine (ng/mL)									
Mean	0.474	1.53	15.9	190	193	420	1.55	16.1	192
RSD (%)	3.9	6.0	2.3	4.9	4.0	3.8	5.1	3.3	4.0
RE (%)	−5.2	+2.0	+6.0	+2.7	+4.3	+5.0	+3.3	+7.3	+3.8

*Samples were diluted five fold with blank plasma prior to analysis.

calibration standards and QC samples were allowed to stand at 4°C for 48 hours prior to injection. No effect on quantitation of the calibration standards or QC samples was observed. The stock solutions were stable for at least 3 weeks.

The method was successfully cross validated to human plasma, potassium EDTA anticoagulant and has been successfully used to analyze samples from a clinical trial.

CONCLUSION

An easy, sensitive, and reliable LC/MS/MS method for the measurement of fluoxetine and norfluoxetine in human plasma has been successfully developed and validated. A silica column and an aqueous-organic mobile phase were used to improve the sensitivity. The LLOQ is 0.5 ng/mL using only 0.1-mL plasma sample and the analytical run time is only 2.0 minutes per sample.

REFERENCES

1. *Physicians' Desk Reference*®, 54th Ed.; Medical Economics: Montvale, NJ, USA, 2000; 962–966.
2. Raggi, M.A.; Mandrioli, R.; Casamenti, G.; Volterra, V.; Desiderio, C.; Fanali, S. Improved HPLC Determination of Fluoxetine and Norfluoxetine in Human Plasma. Chromatographia **1999**, *50* (7–8), 423–427.
3. Gupta, R.N.; Steiner, M. Determination of Fluoxetine and Norfluoxetine in Serum by Liquid Chromatography with Fluorescence Detection. J. Liq. Chromatogr. **1990**, *13* (19), 3785–3797.
4. Wong, S.H.Y.; Dellafera, S.S.; Fernandes, R.; Kranzler, H. Determination of Fluoxetine and Norfluoxetine by High-Performance Liquid Chromatography. J. Chromatogr. **1990**, 499, 601–608.
5. Gennaro, M.C.; Abrigo, C.; Angelino, S.; Albert, U.; Bogetto, F.; Maina, G.; Prolo, P.; Ravizza, L. Determination of Fluoxetine and Norfluoxetine in Human Plasma by Ion-Interaction RP-HPLC. J. Liq. Chromatogr. Rel. Technol. **1997**, *20* (18), 3017–3028.
6. Maya, M.T.; Domingos, C.R.; Guerreiro, M.T.; Morais, J.A. Determination of the Antidepressant Fluoxetine in Human Plasma by LC with UV Detection. J. Pharm. Biomed. Anal. **2000**, *23*, 989–996.
7. Kristoffersen, L.; Bugge, A.; Lundanes, E.; Slordal, L. Simultaneous Determination of Citalopram, Fluoxetine, Paroxetine and their Metabolites in Plasma and Whole Blood by High-Performance Liquid Chromatography with Ultraviolet and Fluorescence Detection. J. Chromatogr. B **1999**, *734* (2), 229–246.

8. Alvarez, J.C.; Bothua, D.; Collignon, I.; Advenier, C.; Spreux-Varoquaux, O. Determination of Fluoxetine and its Metabolite Norfluoxetine in Serum and Brain Areas Using High-Performance Liquid Chromatography with Ultraviolet Detection. J. Chromatogr. B **1998**, *707* (1–2), 175–180.
9. Holladay, J.W.; Dewey, M.J.; Yoo, S.D. Quantification of Fluoxetine and Norfluoxetine Serum Levels by Reversed-Phase High-Performance Liquid Chromatography with Ultraviolet Detection. J. Chromatogr. B **1997**, *704* (1–2), 259–263.
10. Misztal, G.; Hopkala, H. Determination of Fluoxetine in Human Plasma Using Reserved Phase HPLC. Pharmazie **1997**, *52* (11), 854–856.
11. 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* (2), 419–426.
12. Nichols, J.H.; Charlson, J.R.; Lawson, G.M. Automated HPLC Assay of Fluoxetine and Norfluoxetine in Serum. Clin. Chem. **1994**, *40* (7), 1312–1316.
13. Thomare, P.; Wang, K.; Van Der Meersch-Mougeot, V.; Diquet, B. Sensitive Micromethod for Column Liquid Chromatographic Determination of Fluoxetine and Norfluoxetine in Human Plasma. J. Chromatogr. **1992**, *583* (2), 217–221.
14. 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* (9), 1756–1761.
15. Dixit, V.; Nguyen, H.; Dixit, V.M. Solid-Phase Extraction of Fluoxetine and Norfluoxetine from Serum with Gas Chromatography–Electron-Capture Detection. J. Chromatogr. B **1991**, *101* (2), 379–384.
16. Urichuk, L.J.; Aspeslet, L.J.; Holt, A.; Silverstone, P.H.; Coutts, R.T.; Baker, G.B. Determination of p-Trifluoromethylphenol, a Metabolite of Fluoxetine, in Tissues and Body Fluids Using an Electron-Capture Gas Chromatographic Procedure. J. Chromatogr. B **1997**, *698* (1–2), 103–109.
17. Fontanille, P.; Jourdil, N.; Villier, C.; Bessard, G. Direct Analysis of Fluoxetine and Norfluoxetine in Plasma by Gas Chromatography with Nitrogen-Phosphorus Detection. J. Chromatogr. B **1997**, *692* (2), 337–343.
18. 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* (1), 175–179.
19. Addison, R.S.; Franklin, M.E.; Hooper, W.D. Sensitive, High-Throughput Gas Chromatographic–Mass Spectrometric Assay for Fluoxetine and

Norfluoxetine in Human Plasma and its Application to Pharmacokinetic Studies. J. Chromatogr. B **1998**, *716* (1–2), 153–160.

20. Crifasi, J.A.; Le, Nha X.; Long, C. Simultaneous Identification and Quantitation of Fluoxetine and its Metabolite, Norfluoxetine, in Biological Samples by GC–MS. J. Anal. Toxicol. **1997**, *21* (6), 415–419.
21. De Nucci, G.; Bezerra, F.A.F.; Corso, G.; Lerner, F.E.; Moraes, M.E.A.; Moraes, M.O. Fluoxetine Bioequivalence Study: Quantification of Fluoxetine and Norfluoxetine by Liquid Chromatography Coupled to Mass Spectrometry. J. Clin. Pharmacol. **1999**, *39* (10), 1053–1061.
22. Sutherland, F.C.; Badenhorst, D.; de Jager, A.D.; Scanes, T.; Hundt, H.K.; Swart, K.J.; Hundt, A.F. Sensitive Liquid Chromatographic–Tandem Mass Spectrometric Method for the Determination of Fluoxetine and its Primary Active Metabolite Norfluoxetine in Human Plasma. J. Chromatogr. A **2001**, *914* (1–2), 45–51.
23. Naidong, W.; Shou, W.; Chen, Y.-L.; Jiang, X. Novel Liquid Chromatographic-Tandem Mass Spectrometric Methods Using Silica Columns and Aqueous-Organic Mobile Phases for Quantitative Analysis of Polar Ionic Analytes in Biological Fluids. J. Chromatogr. B **2001**, *754*, 387–399.
24. Shou, Z.; Jiang, X.; Beato, B.B.; Naidong, W. A Highly Automated 96-Well Solid Phase Extraction and Liquid Chromatography/Tandem Mass Spectrometry Method for the Determination of Fentanyl in Human Plasma. Rapid Commun. Mass Spectrom. **2001**, *15*, 466–476.
25. Naidong, W.; Chen, Y.-L.; Shou, W.; Jiang, X. Importance of Injection Solution Composition for LC–MS–MS Methods. J. Pharm. Biomed. Anal. **2001**, *26* (5/6), 753–767.

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