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Souverain, S.; Mottaz, M.; Cherkaoui, Samir; Veuthey, Jean-Luc

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S. Souverain · M. Mottaz · S. Cherkaoui · J.-L. Veuthey

Rapid analysis of fluoxetine and its metabolite in plasma by LC-MS with column-switching approach

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Abstract A rapid and sensitive method was developed for the simultaneous determination of fluoxetine and its primary metabolite, norfluoxetine, in plasma. It was based on a column-switching approach with a precolumn packed with large size particles coupled with a liquid chromatography–electrospray ionisation–mass spectrometry (LC-ESI-MS). After a simple centrifugation, plasma samples were directly injected onto the precolumn. The endogenous material was excluded thanks to a high flow rate while analytes were retained by hydrophobic interactions. Afterwards, the target compounds were eluted in back flush mode to an octadecyl analytical column and detected by ESI-MS. The overall analysis time per sample, from plasma sample preparation to data acquisition, was achieved in less than 4 min. Method performances were evaluated. The method showed good linearity in the range of 25–1000 ng mL⁻¹ with a determination coefficient higher than 0.99. Limits of quantification were estimated at 25 ng mL⁻¹ for fluoxetine and norfluoxetine. Moreover, method precision was better than 6% in the studied concentration range. These results demonstrated that the method could be used to quantify target compounds. Finally, the developed assay proved to be suitable for the simultaneous analysis of fluoxetine and its metabolite in real plasma samples.

Keywords Fluoxetine · Norfluoxetine · Column-switching · On-line sample preparation · Large particles support · LC-MS

Introduction

Fluoxetine is an antidepressant for oral administration and belongs to the selective serotonin reuptake inhibitor (SSRI)

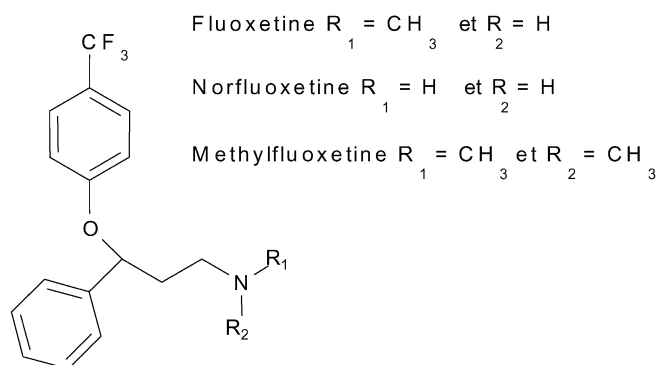


Fig. 1 Chemical structures of fluoxetine, norfluoxetine and methylfluoxetine

family [1, 2]. It is used to treat depression, bulimia and obsessive-compulsive disorders. Through N-demethylation, fluoxetine is extensively metabolised in the liver to norfluoxetine, which is also a potent SSRI and even higher than of the parent drug. After oral administration, fluoxetine is rapidly absorbed and disappears from plasma with a half time of 1–3 days, while norfluoxetine plasma half time is between 7 and 15 days. Therefore, because of its increasing popularity and important role in modern therapy, reliable analytical methods for monitoring fluoxetine and its primary active metabolite in biological fluids are highly desirable. The chemical structures of fluoxetine, norfluoxetine and methylfluoxetine, which is used as internal standard (I.S.), are reported in Fig. 1.

Several methods have been reported for the simultaneous determination of fluoxetine and its metabolite in biological fluids. Gas chromatography coupled with electron capture detection (ECD) [3], nitrogen phosphorus detection (NPD) [4, 5] or mass spectrometry detection (MS) [6, 7, 8] has been described for the analysis of the studied compounds. In recent years, aqueous and non-aqueous capillary electrophoresis methods have been developed for the determination of fluoxetine and other related compounds, including metabolites, by-products and impurities [9, 10]. Nevertheless, the most widely used method is by far liq-

S. Souverain · M. Mottaz · S. Cherkaoui · J.-L. Veuthey (✉)
Laboratory of Analytical Pharmaceutical Chemistry,
School of Pharmacy, University of Geneva,
20 bvd d'Yvoy, 1211 Geneva 4, Switzerland
e-mail: jean-luc.veuthey@pharm.unige.ch

uid chromatography (LC) with ultraviolet [11, 12, 13, 14, 15, 16, 17] or fluorescence detection [12, 18, 19, 20]. In order to achieve high sensitivity, several approaches including a preconcentration step [11, 12, 15, 16, 17, 18], large injection volume [15, 16], derivatisation procedures [21] or the use of more specific and sensitive detectors such as mass spectrometry (MS or MS/MS) [22, 23] were found necessary. More recently, column miniaturisation has evolved as a very suitable alternative for sensitivity improvement [24]. Indeed, reducing the column internal diameter increases sensitivity by decreasing chromatographic dilution [25, 26]. Nevertheless, a sample preparation step is often required to allow the separation of analytes of interest from endogenous compounds such as salts, lipids and proteins. The conventional treatment procedures are liquid-liquid extraction (LLE), solid phase extraction (SPE) or protein precipitation, but these are recognised as labour intensive and time consuming. Therefore, to achieve fast analysis, new extraction methods have been developed allowing the direct injection of biofluids in the analytical system. Among the different strategies applied, the use of supports with large particles size has received wide acceptance [27, 28, 29, 30, 31, 32, 33, 34, 35, 36]. Quinn and Takarewski introduced this concept under the name of turbulent flow chromatography [37]. Indeed, high flow rates can be used without excessive back pressure and, therefore, the extraction time is considerably reduced. This fast and generic extraction procedure is based on the rapid elimination of endogenous components such as proteins while analytes are retained by hydrophobic interactions [38]. Afterwards, the target compounds can be eluted with an organic solvent (acetonitrile or methanol) towards a specific MS detector (single column approach) or an analytical column (column-switching approach). Single column methods have been developed for the direct determination of drugs and their metabolites, with a short overall analysis time, generally less than 2 min [28, 39, 40]. However, due to the low resolution afforded by the large particle supports, the lack of chromatographic separation can become a real problem, especially for complex matrices such as biological fluids. Indeed, despite the high MS detector specificity, it has been demonstrated that, in the bio-analytical field, the separation remains essential to avoid or reduce matrix ionisation suppression [35, 36, 41]. Therefore, an extraction support coupled on-line with an analytical column (i.e. column-switching set-up) is highly recommended.

In the present investigation, a fast, selective and highly sensitive bioanalytical method was developed for the simultaneous quantitation of fluoxetine and its primary metabolite, norfluoxetine, in plasma with a column-switching system. The method was based on the solid phase extraction of a biological sample onto a stationary phase containing large particles coupled on-line with an LC-electrospray ionisation-mass spectrometer detector (ESI-MS). Method performances were evaluated according to ICH recommendations [42] and, the method was applied for the analysis of fluoxetine and its metabolite in real plasma samples.

Experimental

Chemicals

Fluoxetine and methylfluoxetine hydrochlorides were kindly donated by Heumann Pharma (Nürnberg, Germany). Norfluoxetine was purchased from Sigma (Buchs, Switzerland). All reagents and solvents were of analytical grade. Acetonitrile and formic acid were obtained from SDS (Peypin, France) and ultra-pure water was supplied by a Milli-Q Gradient A10 water purification unit from Millipore (Bedford, MA, USA). Blank plasma was obtained from the blood centre of the Geneva hospital (Geneva, Switzerland).

Sample preparation

Stock standard solutions of fluoxetine, norfluoxetine and methylfluoxetine were prepared in acetonitrile at a concentration of 1000 µg mL⁻¹. Stock solutions were stored at 4 °C. Fluoxetine and norfluoxetine spiking solutions of 10, 1 and 0.1 µg mL⁻¹ were prepared by further dilution of each stock solution with water. A solution of 1 µg mL⁻¹ of methylfluoxetine in water was prepared by successive dilution of the stock solution. Calibration samples were obtained by appropriate dilution of the spiking solutions in blank plasma to reach a concentration range of 25–1000 µg mL⁻¹. Calibration samples (25, 50, 100, 250, 500, 750 and 1000 ng mL⁻¹) were prepared extemporaneously for each chromatographic sequence. Calibration curves were based on the peak area ratio of each compound versus the internal standard. Linearity was assessed by unweighted least-squares regression analysis. Using the same procedure, four quality control (QC) plasma samples at 25, 50, 250 and 750 ng mL⁻¹ were prepared and analysed to determine method precision and accuracy.

Spiked plasma was vortex mixed and diluted 1:1 with the solution of 1 µg mL⁻¹ of IS (final concentration of IS 500 ng mL⁻¹). After vortex mixing, samples were centrifuged for 5 min at 6000 g. Finally, 50 µL of supernatant was injected in the analytical system.

Patient plasma

Plasma samples were obtained from patients treated with fluoxetine and were provided by Dr C. Eap of the Biochemistry and Clinical Psychopharmacology Department (Lausanne, Switzerland). Fluoxetine was administered orally with a daily dose range between 20 and 180 mg. Samples were kept in a freezer at –20 °C until analysis. Each plasma sample was prepared according to the procedure described in the previous section.

Instrumentation

The configuration of the column-switching system is schematically shown in Fig. 2. It included an Agilent Series 1100 LC system (Agilent Technologies, Walbronn, Germany) equipped with an autosampler, a binary pump (pump 1), a six-port switching valve and a single wavelength UV detector. An additional Agilent Series 1100 LC isocratic pump (pump 2) was included in the system. The Chemstation software suite (Agilent Technologies) was used for instrument control, data acquisition and data handling. The on-line sample extraction was performed on an Oasis HLB precolumn (50×1 mm I.D., d_p 30 µm) from Waters Corp. (Milford, MA, USA). An in-line filter was placed prior to the precolumn to eliminate particles, which might remain in the sample after the centrifugation step. The analytical column used for analyte separation was a Discovery HS C18 (50×2.1 mm I.D., d_p 3 µm) from Supelco (Bellefonte, PA, USA). The detection was carried out with an Agilent Series 1100 MSD single quadrupole (Agilent Technologies) equipped with an orthogonal electrospray ionisation interface. Nitrogen was used both as nebulising gas at a pressure of 35 psi (1 psi=6894.76 Pa),

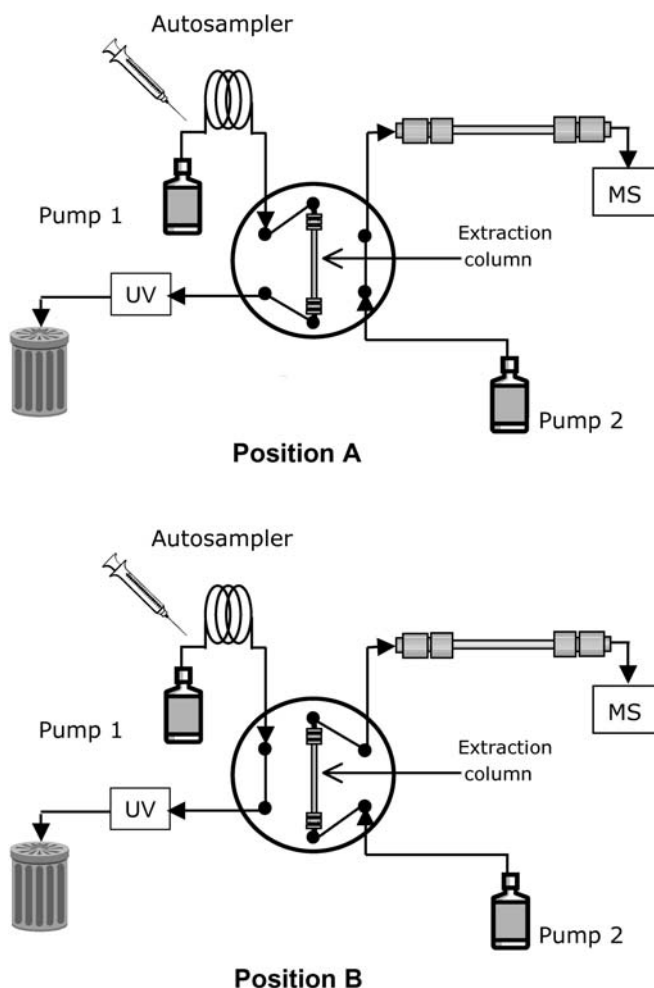


Fig. 2 Column-switching configuration. *A* Extraction, precolumn wash and re-equilibration steps. *B* Transfer step

and as drying gas at a temperature of 350 °C and a flow rate of 10 L min⁻¹. Electrospray voltage was set at 3000 V and skimmer voltage optimised at 40 V. MS detection was carried out in the selected ion-monitoring mode for the protonated molecular ion. The selected masses were acquired with a dwell time of 392 ms on each mass to charge ratio, which were 296 for norfluoxetine, 310 for fluoxetine and 324 for methylfluoxetine.

Analytical conditions

The analytical process involves three main steps: (1) extraction, (2) analytes transfer and (3) separation.

Extraction step

During the extraction, the switching valve was in position A (Fig. 2). The autosampler and pump 1 were used to load 50 µL of treated plasma onto the extraction precolumn. The loading mobile phase consisted of water-acetonitrile (95:5 v/v) containing 0.1% formic acid and the flow rate was set at 4 mL min⁻¹. Elution of the endogenous material was monitored by UV detection at 280 nm. Simultaneously, the octadecyl analytical column was conditioned with water-acetonitrile (65:35 v/v) containing 0.1% formic acid delivered by pump 2 at a flow rate of 400 µL min⁻¹.

Transfer step

After sample extraction ($t=0.4$ min), the valve was switched to position B (Fig. 2). Analytes were rapidly eluted from the extraction support in the back flush mode with the mobile phase and flow rate reported previously, and transferred to the analytical column for 1 min.

Separation, wash and re-equilibration step

After sample transfer ($t=1.4$ min), the valve was switched to its initial position (position A in Fig. 2). Analytes were separated onto the analytical column and detected by ESI-MS. Simultaneously, the precolumn was washed with water-acetonitrile (20:80 v/v) containing 0.1% formic acid delivered by pump 1 at a flow rate of 4 mL min⁻¹ for 2.5 min. At $t=2.9$ min, the mobile phase described for the extraction step at the same flow rate was used to re-equilibrate the extraction precolumn for 2 min and the system was ready for the next injection of plasma sample.

Results and discussion

The aim of the present work was to determine fluoxetine and its primary metabolite in plasma without laborious sample preparation. Zhu et al. developed a LC-MS-MS method for determining paroxetine in human plasma with fluoxetine as internal standard [43]. The analysis was performed in less than 2 min with a sub ng mL⁻¹ limit of quantification (LOQ). However, the sample preparation procedure was based on a conventional liquid-liquid extraction which represented the time-consuming step of the whole analytical process. In this work, the potential of the on-line extraction using a large particle size support coupled to a LC-MS was investigated to reduce the sample preparation step in terms of time and sample handling. For this purpose, the use of large particle supports of hydrophobic polymer (i.e. d_p of 30 to 50 µm) was found to be a suitable

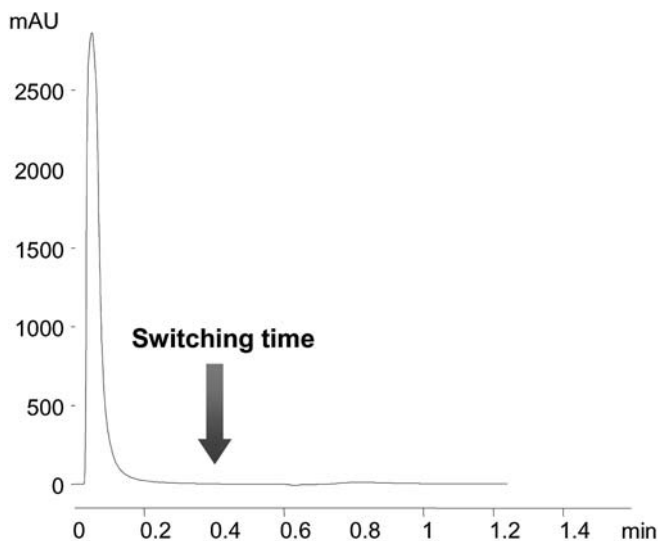


Fig. 3 Endogenous elution profile after injection of 50 µL of plasma diluted 1:1 with water. Detection: UV at 280 nm. Mobile phase: water-acetonitrile (95:5 v/v) with 0.1% formic acid. Flow rate: 4 mL min⁻¹

approach allowing the direct injection of biological fluids in a column-switching configuration. Indeed, this extraction sorbent enabled a fast and efficient separation of analytes from endogenous material. The extraction process was based on the direct injection of biological fluids onto the support with an aqueous mobile phase at a high flow rate without generating excessive backpressure. In order to prevent system clogging, the plasma sample was centrifuged prior to injection. The high flow rate allowed the rapid passage of proteins and other endogenous materials through the support while analytes of interest were retained by means of hydrophobic interactions. The time needed for the washout of endogenous material was monitored by UV detection (280 nm). For 50 μL of plasma diluted 1:1 with water and injected onto the extraction support, endogenous components were eluted in 0.4 min at a flow rate of 4 mL min^{-1} (Fig. 3). In these conditions, the breakthrough volume of fluoxetine, norfluoxetine and methylfluoxetine was evaluated and no analyte elution was observed for 10 min (data not shown). Therefore, under these sample loading conditions, the valve can be switched after 0.4 min. Afterwards, analytes can be eluted from the precolumn to the analytical column and subjected to a chromatographic separation with a hydro-organic solvent.

The mobile phase used for analytes transfer and separation was water–acetonitrile (65:35 v/v) containing 0.1% formic acid. This composition was a good compromise between a satisfactory chromatographic separation and a short transfer time. Oasis HLB was selected as extraction material because it presents hydrophobic and hydrophilic properties. Therefore, the use of a hydrophobic octadecyl stationary phase for analyte separation allowed a fast analytes transfer from the extraction support to the analytical column; which reduced the peak broadening. The transfer was carried out in backflush mode and the time required

for complete elution of the analytes from the precolumn to the analytical column was 1 min. At $t=1.4$ min the valve was switched back to its original position to ensure precolumn washing and re-equilibration. According to our preliminary experiments, a washing step was found necessary to avoid fluoxetine and norfluoxetine carry-over between successive runs. In this context, a precolumn washing step with 80% acetonitrile was found sufficient to suppress any carry-over. This step was performed simultaneously with the chromatographic separation in order to reduce the overall analysis time. Under these conditions, norfluoxetine, fluoxetine and methylfluoxetine were eluted at 1.71, 1.89 and 2.06 min, respectively, as illustrated in Fig. 4. Consequently, the total analysis time was less than 4 min.

Repeatability, linearity, sensitivity and accuracy were assessed to evaluate the developed method. Moreover, to establish the response function, seven standard solutions, over a concentration range from 25 to 1000 ng mL^{-1} , were injected. This range corresponded to the expected concentration found in plasma samples of patients treated with fluoxetine. Linear regression curves were calculated by plotting peak area ratio versus concentration, using the least squares method. The following equations were obtained:

$$\text{fluoxetine: } y = 0.001x - 0.0017; \quad R^2 = 0.9979$$

$$\text{norfluoxetine: } y = 0.0009x - 0.0015; \quad R^2 = 0.9981$$

where y represents the peak area ratio, x the solution concentration (ng mL^{-1}) and R^2 indicates the determination coefficient. The intercepts were not statistically different from zero (Student t test, $\alpha=0.05$) and determination coefficient (R^2) values were higher than 0.99 for both analytes.

Method repeatability was expressed in terms of relative standard deviation and was evaluated by means of quality control samples (QC) at four concentration levels: 25, 50, 250 and 750 ng mL^{-1} . Accuracy was determined by recalculating the four concentration levels using the corresponding response function. Both accuracy and RSD values are reported in Table 1 and results were satisfactory. RSD never exceeded 6% at any of the tested concentrations, indicating good method precision.

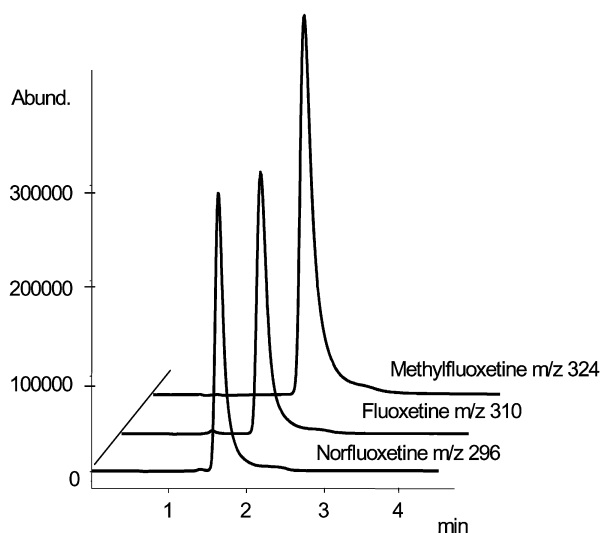


Fig. 4 Chromatograms in SIM mode of spiked plasma at 500 ng mL^{-1} of fluoxetine and norfluoxetine and at 1000 ng mL^{-1} of methylfluoxetine. Analytical conditions as described in the experimental section. *Abund* corresponds to abundance in arbitrary units

Table 1 Accuracy and repeatability values ($k=4$, $n=3$)^a

	Concentration (ng mL^{-1})	Accuracy (%)	Repeatability (CV %)
Fluoxetine	25	105.5	5.1
Norfluoxetine		103.4	1.4
Fluoxetine	50	103.0	2.2
Norfluoxetine		101.3	2.4
Fluoxetine	250	103.3	1.3
Norfluoxetine		104.5	2.6
Fluoxetine	750	104.2	2.0
Norfluoxetine		101.4	2.0

^a k =number of concentrations, n =number of plasma samples from different individuals
CV coefficient of variation

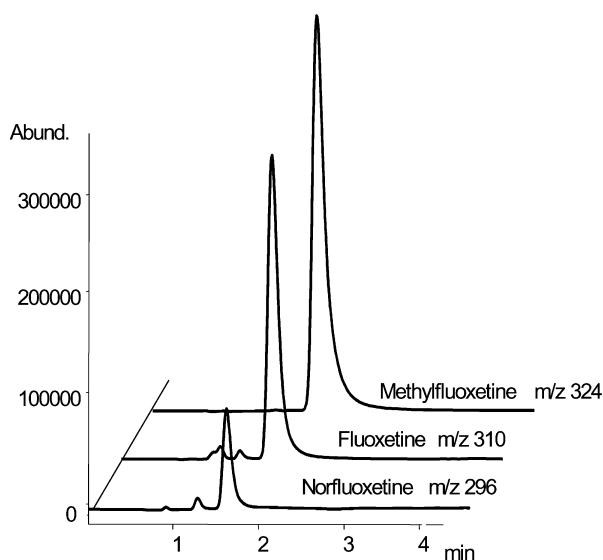


Fig. 5 Chromatograms in SIM mode of a plasma sample from a patient treated with a daily dose of 60 mg of fluoxetine. Analytical conditions as described in the experimental section. *Abund* corresponds to abundance in arbitrary units

The LOQ were determined as being the lowest point on the standard curve with accuracy and repeatability values including $\pm 20\%$. For both compounds, the lower analyte concentration of the calibration range was 25 ng mL^{-1} . At this level, accuracy and precision data were below $100 \pm 20\%$ and 20% CV, respectively. If necessary, a lower LOQ could be easily determined. For both compounds, the LOQ value was estimated at 25 ng mL^{-1} . The method selectivity was assessed by analysing three blank plasma coming from three different sources and no interference was observed on the chromatograms (data not shown). No significant performance degradation of the extraction and analytical columns was observed after the injection of 100 plasma samples (ca. 5 mL of plasma).

The developed method was applied to real samples from patients receiving daily various fluoxetine doses. A typical plasma chromatogram from a patient treated with 60 mg of fluoxetine per day is represented in Fig. 5. As illustrated, fluoxetine and its primary metabolite were clearly detected at 1.7 and 1.9 min. Plasma concentrations, determined by means of the previously obtained calibration, were 526 and 180 ng mL^{-1} for fluoxetine and norfluoxetine, respectively. These preliminary results suggest that the described assay represents a suitable approach for the therapeutic drug monitoring (TDM) of these pharmaceutical compounds in biological fluids.

Conclusion

A rapid and sensitive method with a column-switching approach using a large particle extraction support coupled to an LC-MS system was developed for the direct analysis of fluoxetine and its primary metabolite, norfluoxetine,

in plasma samples. Under the optimised conditions, target analytes were extracted and separated in less than 4 min, which represents a drastic reduction of the overall analysis time in comparison with commonly used methods. Thus, with the described system, up to 360 samples per day can be analysed with minimal manual intervention. The method showed good performances in terms of accuracy and precision. Moreover, the high sensitivity allowed the determination of fluoxetine and its metabolite at low ng mL^{-1} range. In comparison to conventional methods, this procedure represents a significant improvement in terms of labour time saving and allows repeated injection onto the same precolumn. Furthermore, with this automated on-line sample extraction, the manipulation of hazardous biological samples is largely reduced.

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