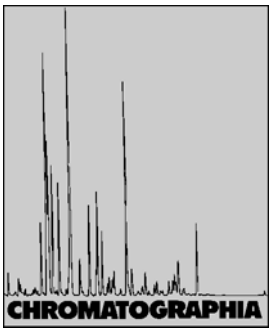
An On-Line Solid Phase Extraction – Liquid Chromatography – Tandem Mass Spectrometry Method for the Analysis of Citalopram, Fluvoxamine, and Paroxetine in Human Plasma



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Key Words

Column liquid chromatography
MS-MS detection
On-line sample clean-up
Citalopram, fluvoxamine and paroxetine
Human plasma

Summary

A specific and sensitive direct-injection high performance liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (HPLC-APCI-MS-MS) method has been developed for the rapid identification and quantitative determination of citalopram, fluvoxamine, and paroxetine in human plasma. After dilution with 0.1% formic acid, plasma samples were injected into the LC-MS-MS system. Proteins and other large biomolecules were removed during an on-line sample cleanup step. The inter- and intra-assay coefficients of variation for all compounds were < 11%. The total analysis time was 6 min per sample. The proposed method permits direct analysis of plasma samples without time-consuming sample preparation.

Introduction

Citalopram, fluvoxamine, and paroxetine are psychoactive drugs of the class of selective serotonin reuptake inhibitors (SSRIs). SSRIs are indicated for the treatment of depression, anxiety, obsessive-compulsive and panic disorders [1, 2]. Their clinical efficiency is comparable to that of the tricyclic antidepressants, with the SSRIs generally having a better side effect profile [1, 3]. Measurement of SSRI plasma concentrations may be useful in cases where patients do not respond at clinically relevant doses, for patients with organic diseases, to assure compliance, as well as for pharmacokinetic studies.

Several analytical methods have been developed for the determination of citalopram, fluvoxamine, and paroxetine in human plasma using either gas chromatography (GC) coupled with mass spectrometric (MS) [4–6] or nitrogen phosphorus detection (NPD) [7, 8], or liquid chromatography (LC) with either ultraviolet (UV) [9–17] or fluorescence detection [7, 14, 18–23]. All of these methods described either require rather laborious manual extraction techniques, or long chromatographic run times, or have limited specificity. The coupling of HPLC to tandem mass spectrometry with atmospheric pressure ionization (API) leads not only to a very specific and sensitive analytical technique, but also significantly reduces the chromatographic run times compared to HPLC methods using traditional detectors such as UV.

A promising approach to HPLC with integrated fully automated sample extraction is high speed on-line SPE [24–26]. This technique enables direct injection of plasma samples without prior extraction by using large particle size stationary phases (30–50 μm) with an extremely high linear flow velocity of the mobile phase (6–10 cm s^{-1}). Owing to the combination of a small diameter LC column packed with large particles and a high flow rate of the mobile phase, large biomolecules rapidly pass through the column while the small analyte molecules are retained. This on-line sample extraction, for which the universality is comparable to that of the manual protein precipitation technique, eliminates the need for time-consuming sample pretreatment.

The purpose of this work was to overcome the limitations of alternative GC and HPLC methods by combining the high selectivity and sensitivity of a benchtop tandem MS system with the feature of HPLC integrated sample preparation for the unambiguous identification and quantitative determination of citalopram, fluvoxamine, and paroxetine in human plasma.

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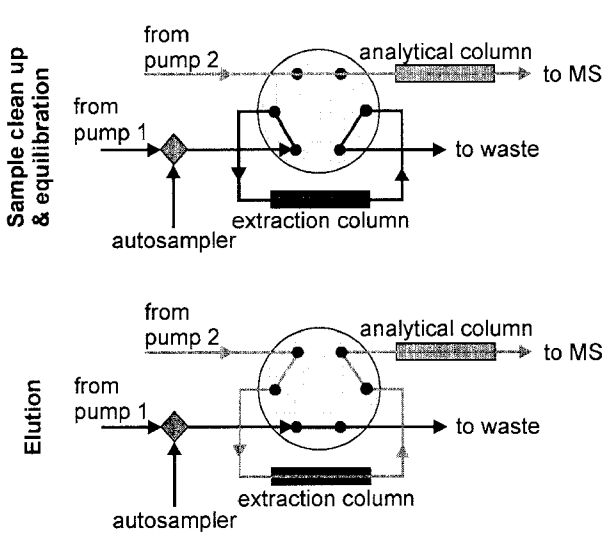

Direct-Injection Procedure	Time	Divert valve position
Sample clean up	0 - 0.3 min	Waste
Elution	0.3 - 5 min	Analytical column & MS
Equilibration	5 - 6 min	Waste

Figure 1. Schematic representation of the HPLC-integrated sample preparation. **(A)** Flow path during sample cleanup and equilibration. **(B)** Flow path during elution.

Experimental

Reagents

Citalopram was a kind gift from Lundbeck (Copenhagen, Denmark). Fluvoxamine was provided by Solvay-Pharma (Suresnes, France). Paroxetin was obtained from SmithKline Beecham (Worthing, UK). Dibenzepin was obtained from Novartis (Basel, Switzerland). HPLC grade acetonitrile and analytical grade concentrated formic acid were obtained from Promochem (Wesel, Germany). A Milli-Q® Plus water purification system (Millipore, Vienna, Austria) was used to obtain purified water for the HPLC solvent.

Materials and Equipment

The LS-MS-MS analyses were performed using a TSP LC system consisting of a vacuum degasser, two P4000 quaternary pumps, an AS3000 autosampler, a six-port switching valve, and a Finnigan LCQ™ ion trap mass spectrometer equipped with an APCI source (Finnigan MAT, USA) run by XCALIBUR 1.2 software.

The on-line extraction was carried out on an Oasis® HLB extraction column (Waters, Austria), 30 µm, 1 × 50 mm internal diameter (i.d.). HPLC separations were performed on a Symmetry C18 (Waters, USA), 5 µm, 3.0 × 150 mm i.d. HPLC column, operated at ambient temperature and protected by a Sentry guard column Symmetry C18 (Waters), 5 µm, 3.9 × 20 mm i.d.

Mass Spectrometric Conditions

Operating conditions for the APCI source used in the positive ionization mode were set to a vaporizer temperature of 450 °C, a heated transfer capillary temperature of 200 °C, a corona discharge intensity of 5 µA, and a sheath gas flow of 80 units (units refer to arbitrary values set by the LCQ software). Nitrogen was used for sample nebulization.

For the generation of MS-MS data, the protonated precursor molecules $[M + H]^+$ of citalopram (*m/z* 325), fluvoxamine (*m/z* 319), paroxetine (*m/z* 330), and dibenzepin (*m/z* 296) were fragmented by helium gas collision in the ion trap at a relative collision energy of 42%. The mass spectra resulting from these fragmentations were acquired in the full scan mode from *m/z* 100 to 400. The most abundant product ions, *m/z* 262 for citalopram, *m/z* 260 for fluvoxamine, *m/z* 192 for paroxetine, and *m/z* 251 for dibenzepin were chosen for selected reaction monitoring (SRM) analysis.

Calibration Standards and Quality Control Samples

Stock solutions of citalopram, fluvoxamine, paroxetine, and dibenzepin (internal standard, IS) were prepared by dissolving 1 mg of the respective analyte in 1 mL of methanol. The stock solutions of citalopram, fluvoxamine, and paroxetine were combined and diluted with 0.1% formic acid to obtain working solutions with concentrations of 1 mg L^{-1} and 10 mg L^{-1}, respectively. The stock solution of the IS was diluted in 0.1% formic acid to yield a final concentration of 10 mg L^{-1}. For citalopram and fluvoxamine seven calibrators with concentrations between 20 and 800 µg L^{-1} were prepared by adding the appropriate amounts of working solution to 1 mL of drug-free human plasma. For paroxetine seven calibrators with concentrations ranging from 10 to 600 µg L^{-1} were prepared accordingly. Three replicate analyses were performed for each calibrator to evaluate linearity. The calibration curves were constructed by linear regression using the ratios of the peak areas of citalopram, fluvoxamine, and paroxetine, respectively, to that of the IS, plotted against the corresponding concentrations.

An analogous dilution procedure was used to make additional working solutions containing citalopram, fluvoxamine, and paroxetine, each at concentrations of 1 mg L^{-1} and 10 mg L^{-1}. These solutions were used to prepare quality control samples from drug-free human plasma. For each quality control sample, 1 mL of plasma was spiked to contain citalopram and fluvoxamine at concentrations of 40, 150, or 500 µg L^{-1}, and paroxetine at concentrations of 30, 150, or 400 µg L^{-1}.

Sample Preparation

Each prepared calibrator or quality control plasma sample was pipetted into a labeled glass tube. The diluted internal standard solution (10 µl; 10 mg L^{-1}) and the appropriate amount of 0.1% formic acid were added to each sample to give a total volume of 2 mL. The tubes were vortex mixed and 400 µL of each sample was transferred into autosampler vials.

HPLC-integrated Extraction and Separation

The direct-injection procedure can be divided into three steps: HPLC-integrated sample cleanup, elution, and equilibration. During sample cleanup, 50 µL of the prepared plasma sample was injected by the autosampler and transferred onto the extraction column with a mobile phase of 0.1% formic acid at a flow rate of 4 mL min^{-1}, which was delivered from pump 1. To avoid contamination of the mass spectrometer with matrix molecules, the effluent was directed to the waste for 0.3 min (Figure 1A). While the analytes of interest were retained on the extraction column, proteins and other large biomolecules were discharged. The valve was then automatically switched to the inject position to couple the extraction column inline with the analytical column and the mass spectrometer (Figure 1B). The analytes were eluted from the extraction column to the analytical column, separated with a binary mobile phase delivered from pump 2, and detected by the mass spectrometer. Each chromatographic separation was carried out at a flow rate of 0.6 mL min^{-1} with a mobile phase of acetonitrile – 0.1% formic acid (28:72, *v/v*). After a total run time of 5 min, the valve was switched back to the waste position. The extraction column was then allowed to re-equilibrate for 1 min before the injection of the next sample, resulting in a total analysis time of 6 min.

Extraction Efficiency

The extraction efficiency was experimentally determined at concentrations of 30, 100, and 500 µg L^{-1}. The absolute extraction recoveries were evaluated by comparing the analyte peak areas obtained from spiked plasma samples (n = 5) to those obtained from the corresponding unextracted reference standards prepared at the same concentrations.

Results

Chromatography and Mass Spectra

All of the compounds investigated gave protonated precursor molecules $[M + H]^+$, dibenzepin (IS) at *m/z* 296, citalopram at *m/z* 325, paroxetine at *m/z* 330, and fluvoxamine at *m/z* 319 in the MS mode. The full scan product ion spectra of the IS, citalopram, paroxetine, and fluvoxamine are depicted in Figure 2. The most intense product ions observed in MS-MS spectra, *m/z* 251 for the IS, *m/z* 262 for citalopram, *m/z* 192 for paroxetine, and *m/z* 260 for fluvoxamine were chosen for SRM analysis. Extracted selected reaction monitoring (SRM) ion chromatograms of the IS, citalopram, paroxetine, and fluvoxamine obtained from spiked plasma are shown in Figure 3. The retention times of the IS, citalopram, paroxetine, and fluvoxamine were 2.09, 2.39, 3.15, and 3.54, min, respectively. The total analysis time was 6 min per sample.

Method Validation

The recoveries for all compounds were greater than 86% at all three concentrations tested. Calibration lines were linear in the range from 20 to 800 µg L^{-1} for citalopram and fluvoxamine and from 10 to 600 µg L^{-1} for paroxetine, all of them with coefficients of determination (r^2 values) ≥ 0.991.

For the lowest calibrators, the deviations of the measured concentrations from the nominal concentrations, determined from six replicate analysis, were less than 15% and the respective coefficients of variation were below 13% for all analytes. Therefore, the lower limits of quantitation (LLOQ) were assigned as 20 µg L^{-1} for citalopram and fluvoxamine and 10 µg L^{-1} for paroxetine. The limit of detection (LOD), defined as the lowest concentration of the analyte that can be detected with a signal-to-noise ratio greater than 7, was established by analysis of plasma samples spiked with decreasing concentrations of each analyte. The LOD was found to be 5 µg L^{-1} for all analytes.

The specificity of the method was evaluated by analyzing drug-free plasma samples from six different plasma pools without supplementation and after the addition of the internal standard. No interferences from endogenous plasma components at the retention times corresponding to the analytes of interest or the internal standard were observed. Representative SRM ion chromatograms of a plasma sample containing only the internal standard in comparison with a plasma sample supplemented with citalopram, paroxetine, and fluvoxamine at their LLOQ and the internal standard are shown in Figure 4.

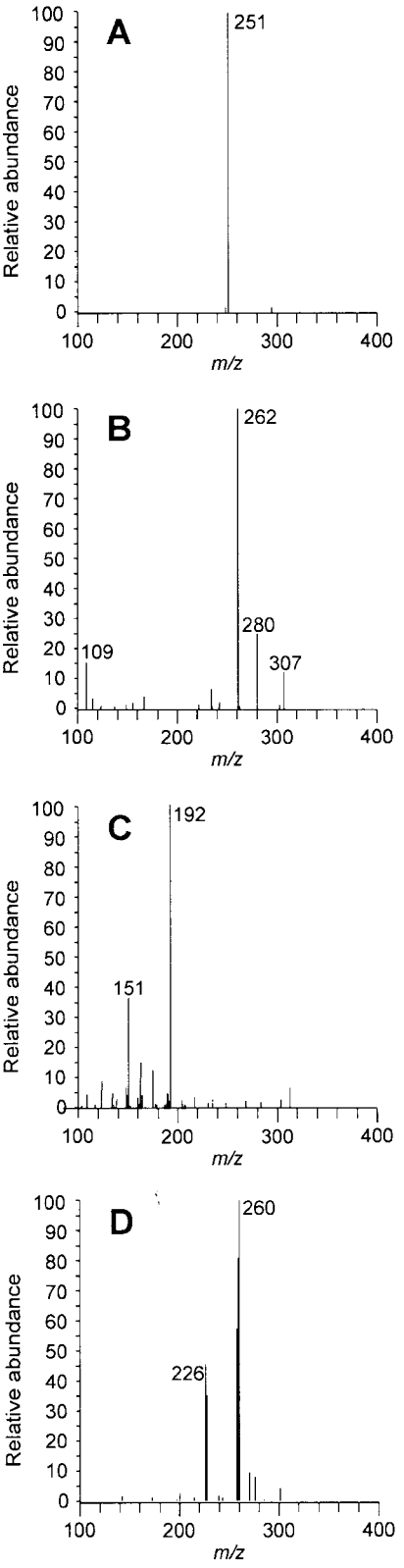

Figure 2. Full scan product ion spectra of **(A)** IS, **(B)** citalopram, **(C)** paroxetine, and **(D)** fluvoxamine.

Accuracy and precision data of the assay are summarized in Table I. To determine intra-assay accuracy and precision,

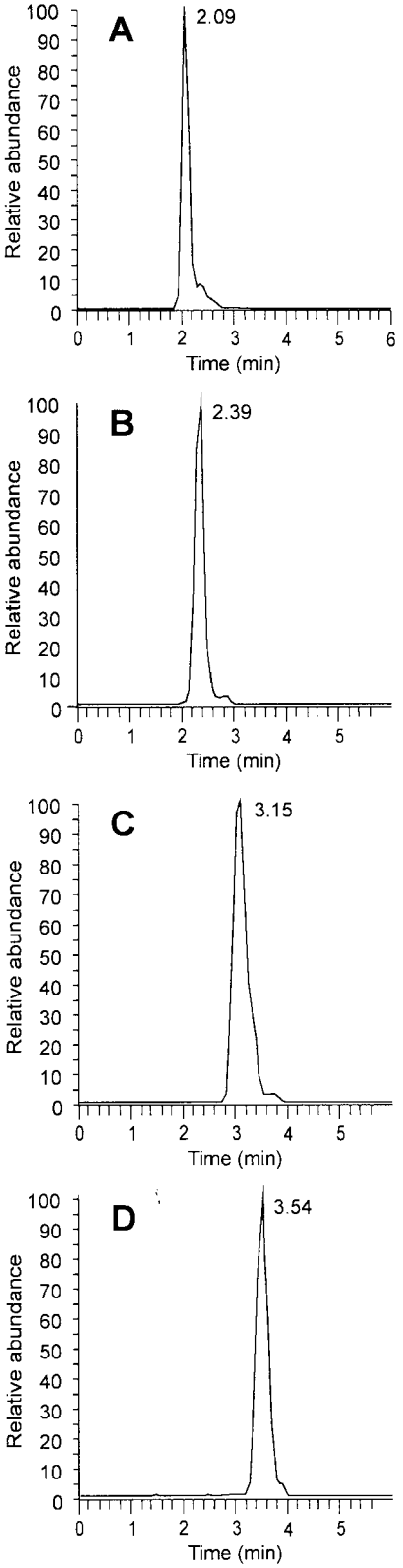

Figure 3. SRM ion chromatograms obtained from human plasma spiked with 100 μg L^{-1} of **(A)** IS, and with 80 μg L^{-1} of **(B)** citalopram, **(C)** paroxetine, and **(D)** fluvoxamine. The product ion traces are *m/z* 251 for IS, *m/z* 262 for citalopram, *m/z* 192 for paroxetine, and *m/z* 260 for fluvoxamine.

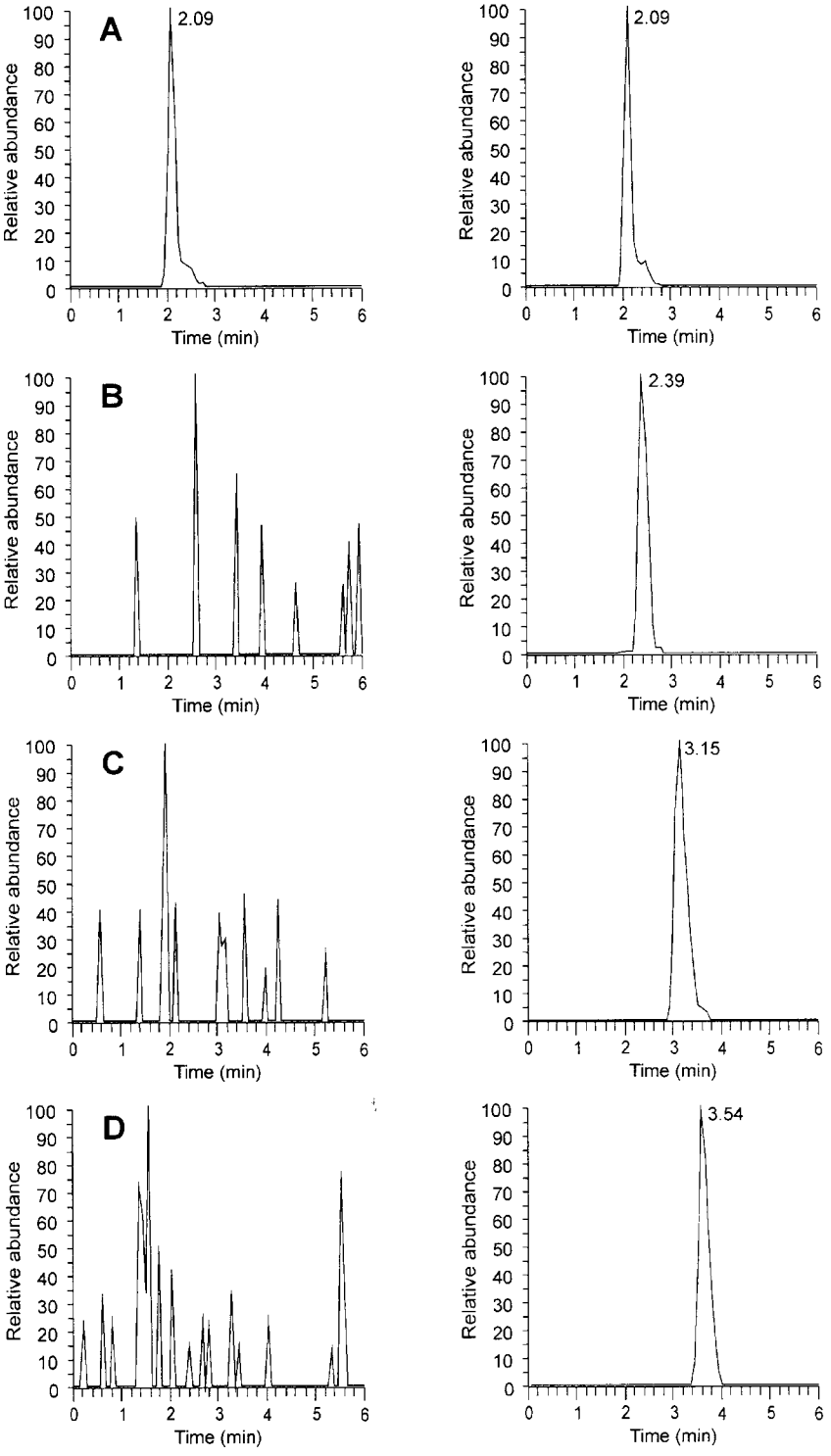

Figure 4. SRM ion chromatograms of **(A)** IS, as well as **(B)** citalopram, **(C)** paroxetine, and **(D)** fluvoxamine in human plasma at 0 μg L^{-1} (left), and at their lower limits of quantitation (right). The concentration of the internal standard was 100 μg L^{-1} in both experiments.

five replicate analyses were performed at the concentrations specified in the Experimental section. Inter-assay accuracy and precision were determined at the same concentrations over a period of 10 days by establishing calibration curves for the analytes on five different days. The three quality control samples were analyzed twice. The intra-assay coefficients of variation (CVs) for all compounds were $\leq$8.4%. All inter-assay CVs were below 11.0%. The accuracies, referred to as % of Target in Table I, were determined by comparing the mean calculated concentration with the spiked target concentration of the quality control samples. The intra- and inter-assay accuracies for all analytes were found to be within 91% and 105% of the target values.

As a part of the method validation, the change in the efficiency of ionization that could be attributable to the sample matrix was estimated by analyzing plasma samples from six different pools supplemented to contain all analytes, as well as the internal standard, at a concentration of 50 µg L^{-1} each. The precisions of absolute peak areas were 8.2% for the IS, 8.9% for citalopram, 9.1% for paroxetine, and 8.8% for fluvoxamine.

Table I. Intra- and inter-assay accuracy and precision data for citalopram, paroxetine, and fluvoxamine.

	Citalopram (µg L^{-1})			Paroxetine (µg L^{-1})			Fluvoxamine (µg L^{-1})		
	40	150	500	30	150	400	40	150	500
Intra-assay*									
Mean	39.3	147	511	28.3	148	388	38.4	145	472
SD	3.04	6.33	25.4	1.89	7.51	29.4	2.31	10.7	39.9
% CV	7.7	4.3	5.0	6.7	5.1	7.6	6.0	7.4	8.4
% of target	98.3	98.0	102.2	94.3	98.7	97.0	96.0	96.7	94.4
Inter-assay*									
Mean	39.7	143	473	28.6	142	375	41.7	137	498
SD	3.58	12.5	40.7	3.11	9.62	31.2	3.55	14.6	45.5
% CV	9.0	8.7	8.6	10.9	6.8	8.3	8.5	10.6	9.1
% of target	99.2	95.3	94.6	95.3	94.7	93.7	104.2	91.3	99.6

* n = 5; SD = standard deviation; % CV = Coefficient of Variation in percent; % of target = Accuracy.

Discussion

The use of a large particle size stationary phase with a high flow of the mobile phase, coupled with the high specificity of tandem mass spectrometry allows for the on-line analysis of selective serotonin reuptake inhibitors from human plasma without prior manual extraction.

The sample cleanup stage was chosen to be 0.3 min, which is equivalent to more than 40 column volumes, to ensure that the analytes are sufficiently separated from the endogenous material. The high flow rate of the mobile phase (4 mL min^{-1}), the small diameter of the column and the large particle size lead to a high linear flow velocity that allows for the separation of the large biomolecules from the analytes of interest. A 100% aqueous formic acid (0.1%) mobile phase was used in order to prevent precipitation of plasma proteins and, hence, avoid both clogging of the column and soiling of other parts of the LC-MS system. Deterioration of the extraction column was noted after approximately 250 injections, resulting in an increased system back pressure and broadened peak shapes. Flow rates and extraction times of less than 3 mL min^{-1} and 0.2 min, respectively, led to increased back pressure of the extraction column after only about 60–90 injections, presumably because proteins were not sufficiently removed from the column. During the elution step, the small analyte molecules were eluted by changing the mobile phase form 0% organic solvent up to 28% acetonitrile. In order to direct the entire effluent and, hence, the whole amount of analyte to the ion trap without splitting of the flow, the flow rate of pump 2 was reduced to 0.6 mL min^{-1}.

The processes that occur in the ion trap detector can be broken down into the following steps: ionization of the molecules, storage of the ions formed in the ion source, selection of ions of a single mass-to-charge ratio (precursor ions) and ejection of all other ions, collision induced dissociation of the precursor ions, and detection of the product ions formed. Compared to single stage LC-MS this technique produces a higher signal to noise ratio, which is hardly affected by the matrix. The product ion chromatograms and mass spectra obtained are significantly less influenced by analytical background noise than those generated by LC-MS. However, undetected co-eluted endogenous matrix components can cause changes in the efficiency of formation of the desired protonated parent molecules $[M + H]^+$. This matrix effect is especially dependent on the quality of the sample cleanup and might lead to fluctuations in the analyte or internal standard peak areas, which can considerably affect the reproducibility and accuracy of the assay [27]. The extent of variability of the MS-MS response has been assessed by analyzing plasma samples from six different pools supplemented to contain all analytes, as well as the internal standard. The precisions of absolute peak areas indicated that the extraction efficiencies of all analytes and the internal standard are similar regardless of the source of the plasma. These data, as well as the accuracy and precision data of the assay, do not indicate any significant matrix effect and, hence, no further assessment of this effect has been conducted.

Conclusion

A direct injection HPLC-APCI-MS-MS method has been developed for the quantitative determination of citalopram, fluvoxamine, and paroxetine in human plasma. The HPLC-integrated sample extraction based on the use of a high flow rate on a large particle stationary phase eliminates time-consuming steps encountered in traditional manual SPE or LLE techniques and, therefore, offers large savings in total analysis time. Coupling of tandem mass spectrometry with LC via an atmospheric pressure ionization interface enhances the specificity and sensitivity of drug identification, which is crucial in applications from biological matrices.

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