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Research Article

Overcoming Column-to-Column Retention Time Variability in LC-MS/MS Quantification of Paroxetine in Human Plasma

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ABSTRACT

A simple, selective, and reliable bioanalytical method was developed for the quantitation of paroxetine in human plasma by LC-MS/MS. Paroxetine, a unique small molecule with a single secondary amine, posed significant challenges in the development of reproducible chromatography. Substantial retention time shift was observed for paroxetine using acidic mobile phases with either new or used columns. Buffer strength played an essential role in reducing the observed column-to-column retention time variation. A 96-well plate-based liquid-liquid extraction was developed. The method was validated and used to support clinical studies for the measurement of paroxetine in human plasma in the range of 0.250-50.0 ng/mL using paroxetine-d6 as its internal standard. This article discusses factors impacting the retention time of analytes in liquid chromatography.

Keywords: Paroxetine; Reversed-phase chromatography; Automated liquid-liquid extraction (LLE); Column to column retention time variation

INTRODUCTION

Paroxetine is an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class [1]. It is used to treat major depressive disorder, obsessive-compulsive disorder, social anxiety disorder, panic disorder, posttraumatic stress disorder, generalized anxiety disorder and premenstrual dysphoric disorder [2,3]. Quantitative measurement of paroxetine in biological matrices, e.g., human plasma, is essential for dosage adjustment. High performance liquid chromatography (HPLC) methods have been reported for the determination of paroxetine using different types of detection, including ultraviolet spectrophotometry [4,5], fluorescence [6-8], electrochemical detection [9], and mass spectrometry [10-12].

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is widely considered to be the technology of choice for bioanalysis of small molecule drugs in biological matrices to support drug discovery and development [13,14]. In LC-MS/MS based bioanalysis, chromatography and extraction play equally important roles in separating analytes of interest from co-extracted matrix components [15,16]. As such, reproducible chromatography is critical for reliable quantitation of drugs and/or their metabolites using LC-MS based assays.

Quantitation of paroxetine in human plasma using HILIC-MS/ MS [11] and RP-LC-MS/MS [17] were reported. Here we describe efforts to troubleshoot and re-develop a robust reversed phase (RP) chromatography to quantitate paroxetine in human plasma. When an outdated LC-MS/ MS method in the authors' lab was brought to the current regulatory standards for the quantitation of paroxetine in human plasma, significant variation in paroxetine's retention time was observed. The method employed a RP chromatography. Therefore, efforts were spent to re-develop a RP chromatography, which could provide consistent chromatographic performance for paroxetine. Ammonium formate was used as a modifier in mobile phase A to reduce column-to-column, retention time variability. Reproducibility in paroxetine retention time was achieved using a C18 column under neutral, buffered mobile phase conditions, and gradient elution. The method was successfully validated and applied to the analysis of paroxetine in human plasma samples containing sodium heparin as the anticoagulant.

MATERIALS AND METHODS

Chemicals and Reagents

Reagents and sources were as follows: Paroxetine was purchased from USP; paroxetine-d6 was purchased from TLC Pharma (Ontario, Canada); acetonitrile (LC-MS grade, ≥ 99.9%) and methanol (LC-MS grade, ≥ 99.9%) from Fluka; ammonium formate (AR ACS grade, 99+%min) and ethyl acetate from Sigma-Aldrich (HPLC plus grade; St Louis, MO, USA); formic acid (ACS grade; >98%) from EMD (New Jersey, USA); acetic acid, (>98%) from Baker JT (Center Valley, PA, USA); and filtered water was obtained from a Milli-Q filtration system (Millipore, Bedford, MA, USA).

Solution Preparation

The ammonium buffer and the mobile phases (A and B) were prepared as follows. For 1.0 M ammonium formate buffer, 31.5 g of ammonium formate was dissolved in 500 mL water. Mobile phase A (MPA) was water/1.0 M ammonium formate (1000:20, v/v), while mobile phase B (MPB) was 100% acetonitrile for all experiments. Stock solutions of paroxetine and paroxetine-d6 were both prepared at a concentration of 100 µg/mL in methanol. Stock solutions were stored at -25 ± 5°C.

Preparation of calibration standards and quality control samples (QCs)

Different lots of human plasma (sodium heparin) were pooled prior to use. The pooled plasma was used for the preparation of calibration standards and quality controls (QCs). Calibration standards were prepared to cover an analytical range of 0.250 to 50.0 ng/mL at 0.250, 0.450, 0.750, 2.00, 6.00, 18.0, 40.0 and 50.0 ng/mL for paroxetine in plasma. QCs were prepared at final concentrations of 0.250 (LLOQ, lower limit of quantitation), 0.500 (LQC, low concentration QC), 20.0 (MQC, medium concentration QC) and 37.5 ng/mL (HQC, high concentration QC) for paroxetine in plasma. Each QC pool was divided into small aliquots for daily use and stored at -25 ± 5°C.

Mass Spectrometry

Mass spectrometric detection was performed on an API 4000 triple quadrupole mass spectrometer (AB Sciex, Ontario, Canada) equipped with a Turbo V Ionspray source operating in the positive ion mode. Data acquisition was performed with Analyst 1.6.2 software (AB Sciex, Ontario, Canada). The mass spectrometer was operated in the multiple-reaction monitoring (MRM) mode using the transitions 330.2→192.0 (m/z) for paroxetine (top panel of Figure 1) and 336.2→198.2 (m/z) for paroxetine-d6 (bottom panel of Figure 1). The instrumental parameters were optimized, with nebulizer, heater and curtain gas flow rates at 60, 65 and 30 arbitrary units, respectively; ionspray needle voltage 2000 V; source temperature 600°C; collision gas flow (N2) 10 units; declustering potential 75 V; and collision energy 30 V.

Chromatography Development

A significant challenge was encountered during the chromatography development primarily due to abnormal column-to-column retention time shifting of paroxetine. In the beginning of the chromatography development, a XBridge C18 column (50 × 2.1 mm, 5 µm, Waters) was used. An acidic mobile phase was employed with 0.1% formic acid in water for MPA. Unless otherwise noted, pure acetonitrile was used as MPB. Substantial retention time variability was observed when the reproducibility of the chromatography was evaluated on multiple XBridge C18 columns. Figure 2 shows chromatograms of paroxetine under isocratic elution of 35% B and a flow rate of 800 µL/min using five new columns (solid lines) and three randomly selected, used columns (dashed lines). The five new columns were not previously used. For all subsequent experiments, these five new columns were tested under various conditions and continuously termed "new". The retention times of paroxetine on the five new columns were 0.589, 0.711, 0.719, 0.577, and 1.570 minutes; versus 0.968, 1.020, and 1.140 minutes from the three used columns, respectively. The range between the longest retention time and the shortest retention time of the eight columns was 0.993 minutes. Used columns (0.968 →1.140 minutes) led to less variation in paroxetine's retention time than new columns (0.577 →1.570 minutes). This may be due to better equilibration of the used columns compared to the new ones.

To solve the problem of retention time variation, multiple different columns were evaluated, including Hypersil C8 (Thermo Scientific), Kinetex C18 (core shell, Phenomenex), Fortis C18 (Fortis Technologies), Zorbax AQ C18 (Agilent), Onyx monolithic C18 (Merck), and XBridge phenyl (Waters). The results showed that all of these columns demonstrated the same issue of huge retention time variations (results not shown) under the same isocratic chromatographic conditions (35% B at 800 µL/min) and the same acidic MPA as those shown in Figure 2. The results indicated that the variation in retention time was unlikely column-related because all seven different types of column from five manufacturers behaved similarly. Further, pump performance was ruled out as the cause since the pumps were operated under isocratic conditions (65%A/35% B). Thus, efforts were then focused on mobile phase conditions, and the XBridge C18 column (50 mm × 2.1 mm, 5 µm, Waters) was employed for all subsequent experiments.

Another weak acid, acetic acid, was evaluated as MPA-0.1% acetic acid in water. No improvement was observed with respect to paroxetine's retention time variation, when acetic acid was used. In fact, acetic acid worsened the column-to-column retention time variation. Figure 3 shows chromatograms for paroxetine under isocratic elution (35% B) at a flow rate of 800 µL/min using the same 5 new columns (solid lines) and 3 used columns (dashed lines). The retention times for paroxetine on the eight columns were 0.651, 1.260, 1.450, 0.568, 4.520, 3.280, 3.630, and 4.860 minutes, respectively. The range between the longest retention time and the shortest retention time was 4.29 minutes. A 7.4-fold or 740% difference in retention time is clearly unacceptable for any analysis, let alone for regulated bioanalysis! The used columns provided more consistent paroxetine retention times and peak widths than the new columns, probably as a result of being well equilibrated. The earliest eluted peak (new column) with a RT of 0.568 minutes had a peak width of 2.44 seconds, while the peak at 4.520 minutes (new column) had a peak width of 22.4 seconds. Compared to formic acid, acetic acid resulted in greater column-to-column variability in paroxetine's retention time. It was unclear as to the underlying reasons for this difference. Since the only difference was the acid modifier of mobile phase A, it was likely related to the interaction between the acid, and paroxetine and/or the column stationary phase.

Since the use of acidic mobile phase A did not reduce column-to- column retention time variation, neutral mobile phase A with different buffer strengths, (i.e., 1.0 mM, 10 mM, and 20 mM ammonium formate) was evaluated. MPA containing 1.0 mM ammonium formate in water was evaluated first. Chromatography was run under isocratic elution with 40% B and a flow rate of 800 µL/min. Figure 4 shows chromatograms for paroxetine using the same 5 new columns (solid lines) and 3 used columns (dashed lines). The retention times of paroxetine on the eight columns were 0.479, 0.821, 0.675, 0.495 1.71, 0.921, 0.983, and 1.12 minutes, respectively. The range between the longest retention time and the shortest retention time was 1.23 minutes. Compared to the use of 0.1% acetic acid MPA, the use of 1.0 mM ammonium formate significantly reduced paroxetine's retention time variation. Nevertheless, there was no improvement over the use of 0.1% formic acid. The strength of ammonium formate in MPA was increased from 1.0 to 10.0 mM. The same eight columns were evaluated under isocratic 40% B with a flow rate of 800 µL/min. Retention times for paroxetine on the eight columns were 0.590, 0.873, 0.614, 0.553, 0.768, 1.05, 1.05, and 0.717 minutes, respectively (data not shown). The range of retention time was 0.497 minute. Using a higher concentration of buffer helped to reduce the retention time variation for paroxetine.

Buffer strength of ammonium formate was further increased to 20.0 mM in water for MPA. Isocratic chromatography, with 40% B and a flow rate of 800 µL/min, was employed. The same five new columns were tested. The retention times of paroxetine were 0.697, 0.829, 0.729, 0.642, 0.734, and 0.830 minutes, respectively. The retention time range of the five columns was 0.193 minutes. The same three used and twelve additional, randomly selected used columns were evaluated for retention time reproducibility. The retention times of paroxetine, from the 15 used columns, were 0.697, 0.835, 0.764, 0.660, 0.709, 0.790, 0.871, 0.693, 0.672, 0.760, 0.809, 0.691, 0.665, 0.904, and 1.030 minutes, respectively. Figure 5 depicts the paroxetine chromatograms from all twenty columns tested. The use of 20 mM ammonium formate in MPA improved column performance by reducing paroxetine's retention time variation, resulting in a %CV of 12.8% (n=20), compared with a %CV of 25.8% (n=8) for the 10 mM ammonium formate containing MPA. However, the retention time difference among the 20 columns was 0.388 minutes, which was still considerably large compared to an average retention time of 0.766 minutes. This level of variation was not deemed suitable for regulated bioanalysis, where consistent peak integration is imperative. Considerable variation in retention potentially requires integration of paroxetine chromatographic peaks using different parameters. Although it is doable, the change in integration parameters should be minimized or avoided for compliance reasons.

Gradient elution was attempted while keeping intact all other conditions used to generate the data shown in Figure 5. The gradient was as follows: 0.0 to 0.2 minutes, 25% B; 0.2 to 0.4 minutes, 25-50% B; 0.4-1.1 minutes, 50% B; 1.1

to 1.2 minutes, 95% B; 1.2 to 2.0 minutes, 95% B; 2.0 to 2.1 minutes, 25% B; 2.1 to 3.0 minutes, 25% B. Figure 6 shows the paroxetine chromatograms from 20 columns. The previously evaluated eight columns (five new columns and 3 used columns) were tested first. The retention times of paroxetine were 0.783, 0.812, 0.787, 0.766, 0.791, 0.813, 0.817, and 0.800 minutes, respectively. The retention range was 0.051 minutes, demonstrating a much-improved consistency in paroxetine retention time. An additional 12 columns were then evaluated. For the 12 columns, the retention times of paroxetine were 0.771, 0.782, 0.798, 0.926, 0.771, 0.777, 0.794, 0.814, 0.779, 0.777, 0.832, and 0.906 minutes, respectively. The retention time range from the 20 columns was 0.160 minutes. The %CV of paroxetine's retention time was 5.23% (n=20), which was reduced by more than half compared to a %CV of 12.8% (n=20) for the isocratic elution using the same mobile phases. The results showed that the chromatography employing 20 mM ammonium formate and gradient elution can effectively control and minimize paroxetine retention time variation. Therefore, the chromatographic conditions were finalized for commencing method validation.

Sample Preparation

Protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE) are the three most commonly used sample preparation techniques to isolate small molecules from biological matrices [18]. Protein precipitation is generally not the method of choice, especially for high sensitivity assays (i.e., pg/mL to sub pg/ mL) where severe matrix effects can be encountered from matrix components that are co-extracted and or co-eluted. In practice, protein precipitation is normally avoided for human clinical bioanalysis applications. In comparison, LLE and SPE normally provide cleaner extracts than PPT [15,18]. Paroxetine is a basic compound, making cation exchange SPE one option to extract it from human plasma using an in-house method. Results showed that the mean recovery was consistently ~61%, but with relatively high, ~50%, matrix suppression. LLE, with the use of a mixture of ethyl acetate/hexane (50:50; v/v), has been employed to extract paroxetine from human plasma [17]. Samples were treated with sodium hydroxide, which likely facilitated the formation of neutral paroxetine and in turn increased its recovery [17]. In contrast to the manual/individual vial based LLE, a simple, automated 96-well plate based LLE, using a NIMBUS 96-channel liquid handler, was developed. Ethyl acetate was used as the solvent to extract paroxetine and its internal standard from human plasma. In short, 50-µL aliquot of human plasma samples and 25-µL working internal standard solution were added to a 96-well plate. Samples were mixed. The NIMBUS liquid handler transferred 1000-µL of ethyl acetate and performed sample mixing. A portion of the supernatant was transferred to a new 96-well plate after centrifugation. Sample extracts were completely dried and reconstituted with 200-µL of reconstitution solution. Recovery was consistent for paroxetine and its internal standard with a mean recovery about 80%. The LLE procedures were effective in removing unwanted matrix components resulting in minimal matrix effects (i.e., matrix factor ~0.96). The current 96-well plate based LLE was simple and easy to implement compared to either vial-based LLE [17] or SPE extractions.

Method Validation

Validation of the developed bioanalytical method was performed according to the US FDA Bioanalytical Method Validation Guidance. This included evaluating selectivity, linearity, precision and accuracy, recovery, matrix effect, and stability [19].

Selectivity

The selectivity of the assay was evaluated by analyzing six different lots of blank human plasma samples with and without the internal standard, paroxetine-d6. There was no measurable response at the retention times of paroxetine or paroxetine-d6. In other words, the matrix-derived response is less than 20% of the LLOQ of paroxetine in all six matrix blank samples. Likewise, the matrix-derived response is less than 5% of the paroxetine-d6 from all matrix blank samples.

Sensitivity

The LLOQ was defined as the lowest concentration on the calibration curve of paroxetine measured with acceptable precision and accuracy (i.e., %CV ≤ 20% and RE within ± 20%). For reliable quantitation, the minimal sensitivity at the LLOQ needs to have a signal-to-noise ratio or S/N, greater than 5:1, preferably at least 10:1. For the current method, the typical S/N ratio at the LLOQ for paroxetine was >20:1, ensuring adequate sensitivity for the dependable measurement of paroxetine at low concentrations.

Calibration Curve

Eight calibration standards were analyzed in duplicate over the nominal concentration range of 0.250 to 50.0 ng/mL. A linear, $1/x^2$ weighted least-squares regression algorithm was used to plot the peak area ratios of paroxetine to paroxetine-d6 versus paroxetine's concentrations. The average correlation coefficients of the calibration. The distribution between phases is determined by the self- disproportionation (SDE) of enantiomers, that of helical structured supramolecular associations and their interactions in the solution are controlled

used to plot the peak area ratios of paroxetine to paroxetine-d6 versus paroxetine's concentrations. The average correlation coefficients of the calibration curves from three core precision and accuracy runs were >0.990 (data not shown).

Precision and Accuracy

For paroxetine, precision (expressed as CV in %) and accuracy (expressed as relative error, RE, in %) were calculated for the four QC concentrations (i.e., LLOQ, LQC, MQC and HQC). At the LLOQ (0.250 ng/mL), the inter-assay precision and accuracy were 12.6% and 4.90%, respectively (n=18). The inter-assay precision for three other QC levels (0.500, 20.0 and 37.5 ng/mL) was between 3.97% and 11.5% (n=18). The accuracy for the same three levels of QCs was between -0.984% and 1.25% (n=18). The results are a good indication of the reliability of the LC-MS/MS method for the measurement of paroxetine in human plasma samples.

of 5.31% (n=8) and its averaged IS-MF was 0.974 with a CV of 4.56% (n=8). At HQC level, the averaged MF for paroxetine was 0.925 with a CV of 3.77% (n=8) and its averaged IS-MF was 0.977 with a CV of 1.70% (n=8). These data demonstrate

RESULTS AND DISCUSSION

A simple and effective LC-MS/MS method was developed for the quantitation of paroxetine in human plasma samples. Significant column-to-column retention time variation was observed for paroxetine. Although the root cause for the variation could not be positively identified, it is possible that the unique structure of paroxetine can play a role in the observed phenomenon. In the authors' laboratories, hundreds of bioanalytical methods have been successfully developed and validated to support various small molecule candidates during drug development. A significant portion of the molecules, we have worked on to date, are basic. Abnormal retention time shifts, which were observed for paroxetine, were rarely encountered. Even though paroxetine is basic in nature, it has a single functional group, i.e., a secondary amine. As such, changing the pH of the mobile phases can have a drastic effect on the peak shape and retention time of an analyte when it has at least one ionizable functional group. In the case of paroxetine, the secondary amine in the piperidine ring is an ionizable group. Paroxetine's retention can be sensitive to the mobile phase pH [20], depending on

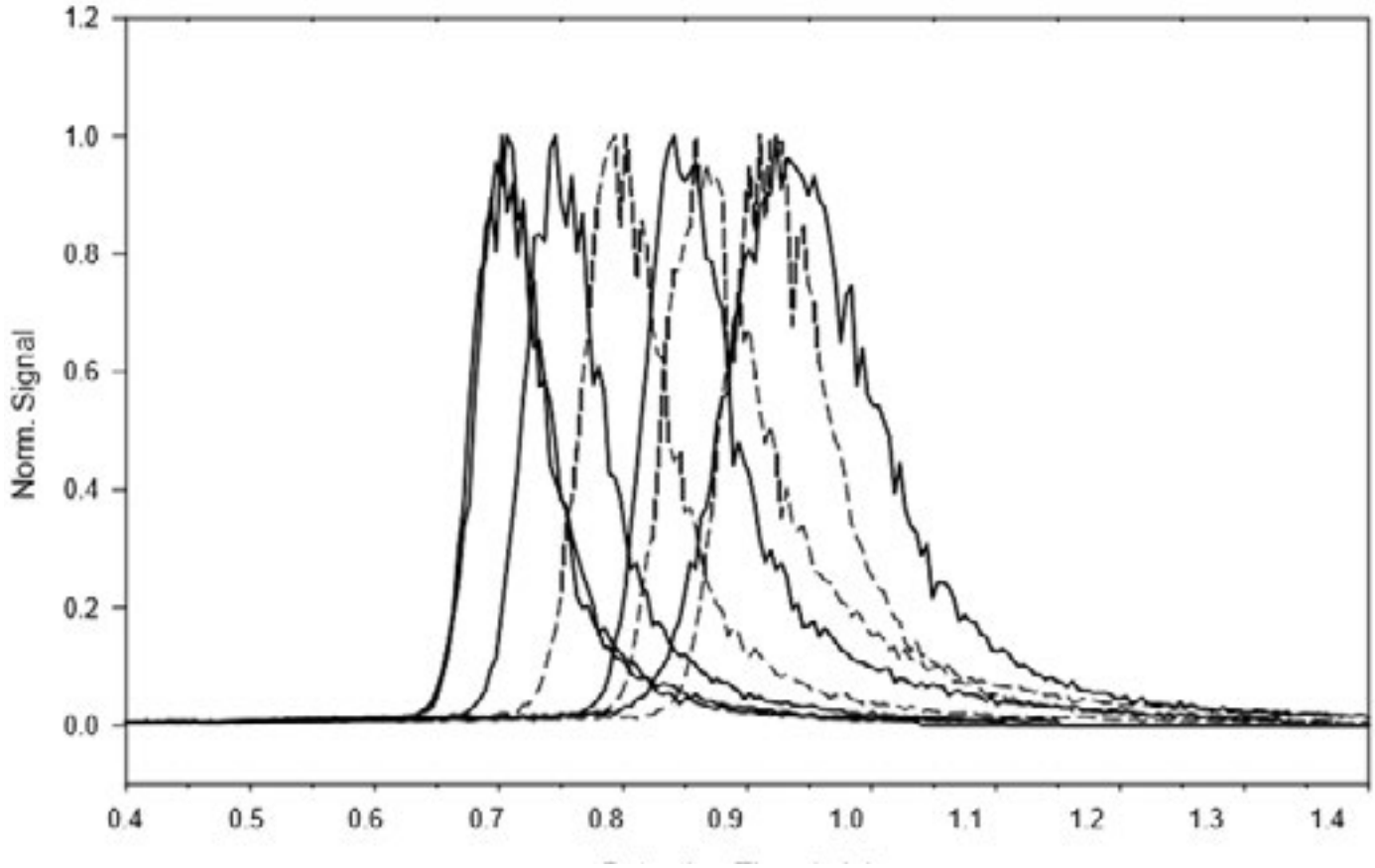

Figure 7: Chromatograms of paroxetine from five new columns (solid lines) and three used columns (dotted lines) using 0.1% ammonium hydroxide in mobile phase A. Significant peak broadening and tailing were observed.

the pKa of a compound and the mobile phase pH is not linear, such that there are no clear rules for the effect of pH on retention [20]. (Figures 1) illustrate how the change in mobile phase pH impacted paroxetine's retention time. A change of 0.6 units in MPA pH, from 2.6 for 0.1% formic acid to 3.2 for 0.1% acetic acid, resulted in a drastic increase in the variability of the retention times observed from 0.993 mins to 4.29 mins.

To minimize pH-related variability, the addition of a buffer to MPA proved to be effective. Sufficient buffer capacity significantly reduced the variation in paroxetine's retention time. It seems that the variation that was observed for paroxetine's retention time was inevitable due to the substantial difference between its pKa (~11) and the pH of the acidic mobile phases (≤ pH 3.2). In other words, paroxetine's retention was very susceptible to small environmental changes, under acidic conditions, which could be introduced via the use of multiple new and used columns. A RP chromatography was used under acidic mobile phases for bioanalysis of paroxetine in human plasma [17]. Retention time variation was not reported for paroxetine but its peak width (baseline) was broad, almost one-minute wide with a retention time of 1.6 minutes [17]. It is possible that potential variation in paroxetine's retention was mitigated by the extremely broad peaks [17]. The current chromatographic limitations were overcome via the use of mobile phase A with sufficient buffering capacity (20 mM ammonium formate) and by utilizing gradient elution. It should be noted that chromatographic peaks that were broad and exhibited tailing were observed for paroxetine under basic mobile phase conditions such as 0.1% ammonium hydroxide in water. In contrast, most small molecule drug candidates have multiple functional groups such as a primary, secondary, or tertiary amine, carboxylic acid, amide, ester, alcohol, or sulfate. The presence of multiple functional groups likely makes the retention of these molecules less susceptible to variability as a result of changes in mobile phase pH. This may explain why significant retention time shifts are not frequently observed for most basic compounds.

CONCLUSION

The method was fully validated in accordance to the US FDA Method Validation Guidance and was successfully applied to measure paroxetine in human plasma samples from in-life studies. This case study illustrates the importance of chromatography development to support regulated bioanalysis, in which consistent column performance is fundamental to achieve critical compliance.

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