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Determination of SCH 211803 by nanoelectrospray infusion mass spectrometry: evaluation of matrix effect and comparison with liquid chromatography–tandem mass spectrometry

Jiwen Chen [a], Liyu Yang [a,1], James T. Kapron [b,2], Li Ma [a], Ellan Pace [b], Colleen K. Van Pelt [b], Patrick J. Rudewicz [a,*]

[a] *Schering-Plough Research Institute, Kenilworth, NJ 07033, USA*
[b] *Advion BioSciences, Ithaca, NY 14850, USA*

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Abstract

A high throughput assay for SCH 211803, an M2 muscarinic receptor antagonist in human plasma using nanoelectrospray infusion tandem mass spectrometry is described. Sample processing consisted of protein precipitation followed by solid phase extraction using octadecasilyl resin-filled pipette tips on a liquid handling robotic system. The sample extracts were infused directly to the mass spectrometer using a nanoelectrospray interface in a silicon chip format. SCH 211803 was quantified in plasma over the concentration range of 1–1000 ng/mL. In comparison with a liquid chromatography–tandem mass spectrometry assay, the nanoelectrospray method has comparable accuracy, precision and limit of quantitation, with a nine-fold improvement in sample throughput. Using the nanoelectrospray assay, ion suppression was evaluated and found to be 15%. This represented a four-fold reduction in matrix suppression when compared to a conventional electrospray source operating in the flow injection analysis mode at a flow rate common for LC–MS/MS analysis.

Keywords: Matrix effects; Nanoelectrospray infusion; SCH 211803

1. Introduction

Due to its sensitivity, specificity and speed of analysis, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become the technique of choice for obtaining pharmacokinetic data for small molecule compounds in drug development. With technological advances in the pharmaceutical industry, increasing numbers of new chemical entities (NCEs) are entering clinical trials each year. An increase in the number of NCEs demands higher sample throughput in pharmaceutical bioanalytical laboratories. Most of the efforts in sample throughput improvement have focused on reducing the LC–MS/MS cycle time per sample, through techniques such as the multiplexed mass spectrometer interface [1], staggered LC injection [2,3] or ultra-fast LC separation [4,5]. On the other hand, method development for a LC–MS/MS assay, rather than sample analysis itself, can become the bottleneck. Biological matrices are complex and optimizations are needed for sample extraction, HPLC separation and mass spectrometry conditions. As a result it is not uncommon for a chemist to spend several weeks developing a bioanalytical method before performing the method validation.

The development of nanoelectrospray infusion mass spectrometry [6,7] eliminates chromatography in bioanalysis, thereby reducing the time and effort required for method development. In this setup, a robotic system aliquots a small amount of sample using a conductive tip. The sample-filled tip subsequently aligns with a silicon chip nozzle for infusion to the mass spectrometer at a flow rate less than 1 μL/min.

* Corresponding author. Present Address: Boehringer Ingelheim Pharmaceuticals Inc., 900 Ridgebury Road, Ridgefield, CT 06877, USA. Tel.: +1 203 791 6336; fax: +1 203 791 6003.
E-mail address: prudewic@rdg.boehringer-ingelheim.com (P.J. Rudewicz).
[1] Present address: Biogen Idec, Cambridge, MA 02142, USA.
[2] Present address: PRACS Institute, Fargo, ND 58104, USA.

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Each chip contains an array of nozzles with 8 μm internal diameter machined with high mechanical precision by processes that are standard in the semiconductor industry. Nanoelectrospray initiates when the spray delivery pressure and high voltage are applied through the sample tip.

In the absence of chromatographic separation, matrix effect may become a concern and should be explored. Matrix effect is a well-documented phenomenon in LC–MS/MS bioanalysis [8,9]. Co-eluting matrix components may suppress or enhance analyte signal in a number of ways. For example, in the electrospray ionization mode it can be due to changes in surface tension of the droplet or competition for the charge [8,10], whereas in the atmospheric pressure chemical ionization mode it is often caused by co-precipitation of the analyte with non-volatile materials or gas phase charge transfer reactions. Matrix effects are often variable. They may change from one study subject to the next, or it may be more pronounced for the study samples than the calibration standards. The variability of matrix effects could therefore significantly compromise quality of the analytical data. An even more serious problem arises when the calibration standard and quality control (QC) samples are prepared from the same plasma pool. If the matrix effect from study subject plasma is substantial and variable, the results for study samples might be erroneous even though, measured concentration values of the QC samples fall within the acceptance criteria.

In an LC–MS/MS bioanalysis experiment, flow rates of a few hundred microliters per minute into the electrospray source are common. Using a nanosplitting device, it was demonstrated that when the flow rate was reduced to the nanoliter per minute range, matrix suppression could be reduced substantially [11]. This was attributed to higher desolvation and ionization efficiency under nanoelectrospray conditions. Therefore, it is reasonable to speculate that ion suppression may be reduced in a nanoelectrospray source when compared to a conventional electrospray ion source. On the other hand, with nanoelectrosray infusion, chromatography is not employed; consequently, matrix suppression of ionization may be much greater relative to LC–MS/MS where chromatography is often used to separate the analyte from the bulk of the matrix components. As a result, with nanoelectrospray, prudent sample clean up before analysis is necessary.

In this study, we describe the analysis of SCH 211803, a novel M2 muscarinic receptor antagonist by nanoelectrospray infusion. To help evaluate matrix effect, each QC sample was prepared from a different lot of human plasma. In addition, QC samples from four different animal matrices were included. In the absence of HPLC separation, the extent of ion suppression was evaluated in both a nanoelectrospray source and using flow injection analysis in a conventional electrospray source. SCH 211803 was also analyzed using a validated LC–MS/MS assay. The relative merits of the nanoelectrospray infusion versus the LC–MS/MS method are discussed.

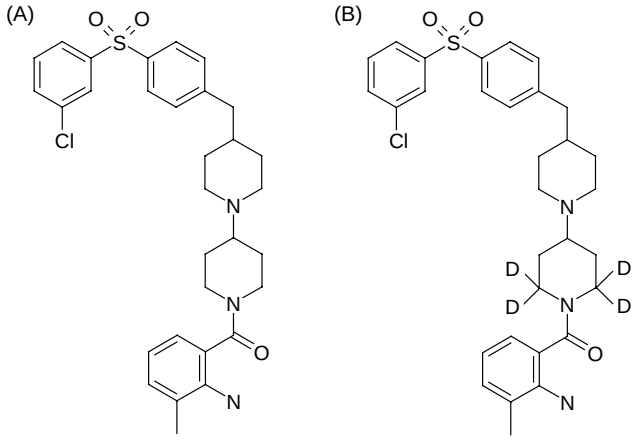

Fig. 1. Chemical structures of (A) SCH 211803 and (B) d4-SCH 211803.

2. Experimental

2.1. Materials and reagents

SCH 211803 and its isotopically labeled internal standard, d4-SCH 211803 (Fig. 1) were synthesized at Schering-Plough Research Institute (Kenilworth, NJ, USA). Water was prepared using an A10 Mill-Q water purification system (Millipore, Bedford, MA, USA). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Sample preparation

Analyte stock solution containing SCH 211803 and d4-SCH 211803 at 100 μg/mL each were prepared in methanol. Spiking solutions were subsequently made from these stock solutions by diluting the stock solution into 50:50 water:methanol. Calibration standards (STD) were prepared at seven concentrations: 1, 5, 10, 50, 100, 500, and 1000 ng/mL. QC samples were prepared at three concentrations: 3, 400, and 800 ng/mL. STD and QC samples were prepared by adding 50 μL of spiking solution to 950 μL of blank plasma. Internal standard working solution at 2 μg/mL was prepared by diluting the d4-SCH 211803 stock solution into 80:20 water:methanol.

2.3. Sample extraction

To 1000 μL of sample, 50 μL of internal standard working solution was added. Subsequently, 50 μL of sample was aliquoted into a Coster cluster tube (Fisher Scientific, Pittsburgh, PA) and mixed with 150 μL of acetonitrile with 0.2% formic acid. The sample was vortexed for 30 s and centrifuged at 10,000 × *g* for 10 min. Eighty microliters of the supernatant from each sample were aliquoted into a 96-well collection plate (Fisher Scientific, Pittsburgh, PA, USA). For LC–MS/MS analysis, no further sample extraction was performed.

For nanoelectrospray infusion analysis, the aliquots were dried down to less than 5 μL and reconstituted in 20 μL of 0.1% trifluroacetic acid in water. A C18 ZipTip (Millipore, Bedford, MA, USA) extraction was performed on a Multiprobe robotic liquid handling system (Packard, Meriden, CT, USA). Each ZipTip was conditioned with 10 μL of methanol, followed by 10 μL of 0.1% trifluroacetic acid in water. Twenty microliters of the sample was slowly pipetted into the ZipTip. The ZipTip was subsequently washed twice with 10 μL of 0.5% formic acid in water, before the analyte was eluted twice with 10 μL of elution solution (75:25:0.1 methanol:water:formic acid) into a microtiter plate. The recovery of the ZipTip extraction was approximately 40%.

2.4. Sample preparation for testing matrix effect

To prepare post-extraction spiked samples, after extraction of three samples of blank human plasma with ZipTips, 15 μL of the extract was withdrawn and added to 2 μL of SCH 211803 at 1000 ng/mL in 50:50 water:methanol. To prepare matrix free samples, 15 μL of 75:25:0.1 methanol:water:formic acid was added to 2 μL of SCH 211803 at 1000 ng/mL in 50:50 water:methanol. Matrix effect was calculated from the difference of mean response between the matrix free sample and the post-extraction spiked sample divided by the mean response of the matrix free sample.

2.5. Nanoelectrospray infusion mass spectrometry

The mass spectrometer used in this study was an API 3000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Ont., Canada). The source housing was fitted with a NanoMate 100 automated nanoelectrospray system under the control of the ChipSoft Version 4.7.1 software (Advion BioSciences, Ithaca, NY, USA). A stream of nitrogen at 0.4 psi maintained infusion of the sample to the mass spectrometer at approximately 150 nL/min, and the nanoelectrospray was initiated by applying a voltage of 1.55 kV on the sample tip.

The mass spectrometer was operated in positive ion multiple reaction monitoring (MRM) mode at unit mass resolution in both Q1 and Q3 (0.7 ± 0.2 amu full width half-maximum). The MRM transitions for SCH 211803 and d4-SCH 211803 were $566 \rightarrow 134$ and $572 \rightarrow 134$, respectively. To minimize the isotope contribution of SCH 211803 to d4-SCH 211803 during MRM analysis, the ^{37}Cl isotope was selected as the parent ion for d4-SCH 211803. Dwell time was 250 ms for the analyte and 150 ms for the internal standard. Total data acquisition was 0.3 min and the onset of the spray occurred at 0.06 min after start of the acquisition. Analyst Version 1.2 was used for data acquisition and processing.

For most samples, average peak height was determined by averaging signal intensity from scan number 10 (at 0.06 min) to scan number 29 (at 0.18 min). For a few samples, where there was a delay of the onset of the spray, averaging started from the second scan with non-zero intensity after 0.06 min.

2.6. Liquid chromatography–mass spectrometry

Sample analyses were performed using an API 3000 triple quadrupole mass spectrometer with a CTC HTS PAL autosampler (LEAP Technologies, Carrboro, NC, USA) and a set of LC-10ADvp pumps (Shimadzu, Columbia, MD, USA). The Turbo-ionspray source conditions in the mass spectrometer were optimized for the flow rate used, whereas the analyzer parameters were identical to those used for the nanoelectrospray infusion analysis. The HPLC was operated at 300 μL/min using gradient elution on a Betasil 2 mm × 100 mm C18 column (Keystone Scientific, Bellefonte, PA, USA). Mobile phase A was 0.2% formic acid in water and mobile phase B consisted of 0.2% formic acid in 50:50 methanol:acetonitrile. Mobile phase B was changed from 55% to 70% in 3 min, then ramped to 100% in 0.5 min, which was held for 2 min before returning to the initial condition (55%). After each injection, the autosampler was washed with 0.1% trifluroacetic acid in acetonitrile followed by 0.1% formic acid in 80:20 water:methanol. This LC–MS/MS method is a variation of a previously published method for the quantitation of SCH 211803 in rat and monkey plasma [12].

For flow injection analysis, the system setup was identical to LC–MS analysis, but without a column. The flow rate was 300 μL/min and the mobile phase was 0.1% formic acid in 75:25 methanol:water.

3. Results and discussions

3.1. Nanoelectrospray assay: precision and accuracy

A one run method validation run for SCH 211803 was performed over the concentration range of 1–1000 ng/mL. The batch sequence contained duplicate calibration standards at seven concentrations. The internal standard was d4-SCH 211803. Peak area ratios (analyte/internal standard) for calibration standards were plotted against concentration and fit to a linear regression with 1 per concentration weighting (Fig. 2A). Detector response was linear over the entire calibration range. The correlation coefficient was 0.9996 and there was no detectable decrease of the response factor (peak area ratio divided by the analyte concentration) at higher concentrations.

The performance of the assay was evaluated by assessing the accuracy and precision for QC samples at 3, 400 and 800 ng/mL. It should be noted that each QC sample was prepared from a different source of human plasma to more closely represent the matrix variation that one might encounter in sample analysis for clinical studies. The accuracy (percent deviation from theoretical) ranged from −5.0% to

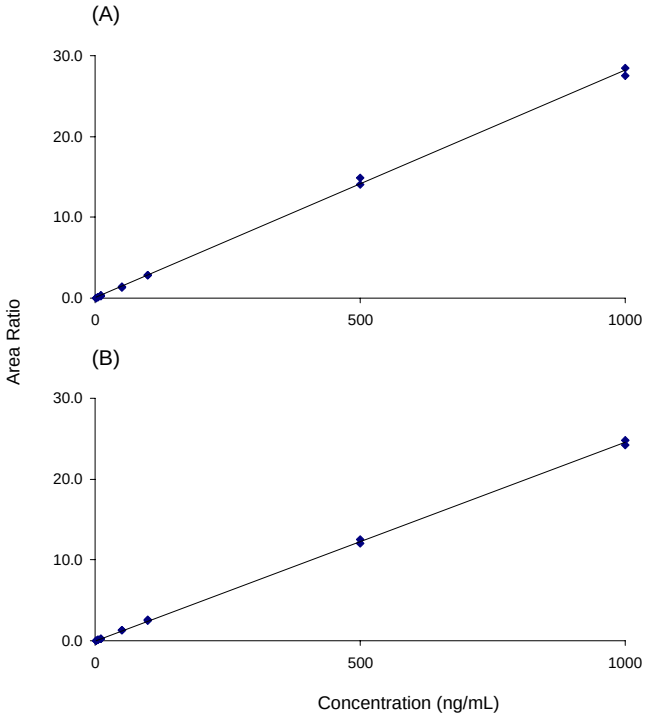

Fig. 2. Calibration curves for (A) nanoelectrospray infusion assay and (B) LC–MS assay.

1.6% whereas the precision (CV) ranged from 1.6% to 9.8% (Table 1). The accuracy and precision were within current FDA-recommended acceptance criteria of 15% at the low, medium, and high QC levels.

Table 1
Accuracy and precision of the QC samples analyzed by nanoelectrospray infusion

	QC low 3.0 ng/mL	QC medium 400 ng/mL	QC high 800 ng/mL
Human			
Mean percentage difference	−5.0	1.6	−1.2
CV	9.8	1.6	2.9
Monkey			
Mean percentage difference	n.d.	1.7	n.d.
CV	–	1.4	–
Rat			
Mean percentage difference	n.d.	−1.4	n.d.
CV	–	4.7	–
Rabbit			
Mean percentage difference	n.d.	0.8	n.d.
CV	–	2.6	–
Dog			
Mean percentage difference	n.d.	3.3	n.d.
CV	–	10.4	–

At each concentration level samples were analyzed in triplicate. n.d.: not determined.

To further test the ruggedness of the assay, medium QC samples in triplicate from four different animal matrices (monkey, rat, rabbit, and dog) were processed along with human plasma samples. It appeared that one could quantify these animal QC samples reasonably well with calibration standards prepared from human plasma. Results indicated that accuracy ranged from −1.4% to 3.3% and precision ranged from 1.4% to 10.4% and (Table 1). Moreover, instrument response (as measured by the average peak height) for the human QC samples did not differ significantly from animal QC samples, suggesting that matrix effect did not vary substantially among plasma from each of the five species.

System carryover was assessed by comparing the instrument response of the blank plasma sample before the first calibration curve (Fig. 3A) to another blank plasma sample placed immediately after the calibration standard with the highest concentration (Fig. 3B). Instrument response for the two samples were fairly comparable to each other, indicating that system carryover was negligible. Unlike LC–MS/MS analyses, in nanoelectrospray infusion, each sample was aliquoted by a unique, disposable pipette and sprayed through a unique, disposable nozzle, making carryover from one sample to the next during mass spectrometry analysis unlikely. This is an advantage of nanoelectrospray infusion for quantitative analysis. It has been reported in the literature that with LC–MS/MS, LLOQs are often several orders of magnitude higher than the limit of detection, mainly because of the presence of substantial carryover [13].

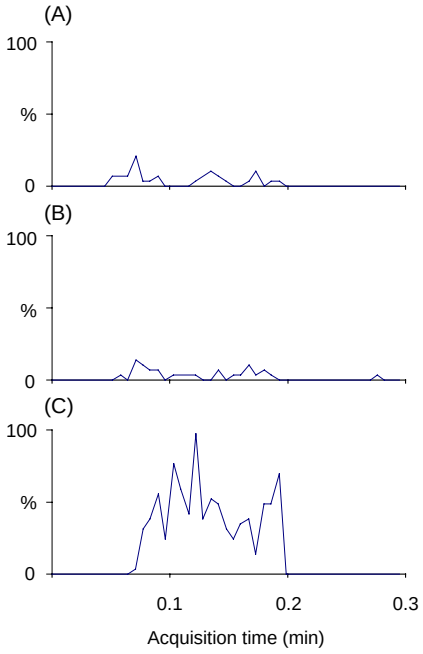

Fig. 3. Ion current profiles for SCH 211803 obtained by nanoelectrospray infusion analysis, with the intensity axis normalized to the same scale. (A) Plasma blank before the calibration standards. (B) Plasma blank immediately after the calibration standard with the highest concentration. (C) LLOQ sample. The ion current profiles were not smoothed to reflect true signal-to-noise ratio.

In the nanoelectrospray assay, the ion current profile of the LLOQ sample had adequate signal to noise ratio (Fig. 3C). Data collection was triggered immediately after the start of the infusion; however, onset of the nanoelectrospray did not occur until 0.06 min into data acquisition, due to the delay in the sample reaching the end of the pipette tip. This delay was caused by an air gap of 1–2 μL which was used to reduce the possibility of sample-to-sample cross-contamination. Sample was delivered to the chip for 0.12 min. Average peak area of the blank plasma from four different sources was approximately 11% (ranging from 9% to 17%) of the mean LLOQ area, indicating that the assay met the current regulatory requirement for selectivity (equal to 20% of the signal of the LLOQ). In LC–MS/MS analysis, detection of the LLOQ sample is not adversely affected by the presence of endogenous compounds having the same ion transition, as long as the interference is adequately separated from the analyte.

3.2. Matrix effect comparison

To determine the effect of sample matrix on the ionization process, SCH 211803 was spiked into extracted blank plasma and matrix-free solvents in triplicate and analyzed. Using nanoelectrospray infusion, matrix suppression of analyte signal was 15%. Flow injection analysis (of the samples previously analyzed using nanoelectrospray) was also performed to determine the extent of matrix effect using a conventional electrospray source operating at a flow rate of 300 μL/min. Matrix suppression under this condition was approximately 60%. These results indicated that ion suppression was significantly reduced in the nanoelectrospray source relative to a flow injection analysis into a conventional electrospray ion source. It should be noted that the success of direct infusion relies on an ionization source with low matrix suppression, such as the nanelectrospray source described here. In an LC–MS/MS experiment, chromatography is often used to separate the analyte from the the bulk of the sample matrix thereby reducing ion suppression.

3.3. Comparison with LC–MS/MS

SCH 211803 was also analyzed using an LC–MS/MS assay and the same injection sequence employed with the nanoelectrospray infusion. Sample extraction for the LC–MS/MS method had an identical protein precipitation step as in the nanoelectrospray infusion assay, but samples were not subjected to further solid phase extraction. Additional sample clean up was not necessary with the LC–MS/MS assay as the LC column provides separation of the analyte from the salts in the sample extract. Quantitation results (Table 2) were comparable to those obtained with nanoelectrospray analysis.

Run time per sample in the LC–MS/MS assay was 6 min. By contrast, in the nanoelectrospray assay the data acquisition time was 18 s and total cycle time per sample was 39 s. As a result, sample analysis of a 96-well plate could be completed in an hour. This represents a nine-fold throughput improvement over the LC–MS/MS method.

Table 2
Accuracy and precision of the QC samples analyzed by LC–MS/MS

	QC low 3.0 ng/mL	QC medium 400 ng/mL	QC high 800 ng/mL
Human			
Mean percentage difference	10.8	−3.4	−4.4
CV	4.3	1.3	2.6
Monkey			
Mean percentage difference	n.d.	−0.8	n.d.
CV	–	1.2	–
Rat			
Mean percentage difference	n.d.	−1.1	n.d.
CV	–	2.3	–
Rabbit			
Mean percentage difference	n.d.	−3.3	n.d.
CV	–	2.1	–
Dog			
Mean percentage difference	n.d.	−0.7	n.d.
CV	–	2.9	–

At each concentration level samples were analyzed in triplicate. n.d.: not determined.

Comparison of the plasma blanks analyzed by LC–MS/MS before (Fig. 4A) and after (Fig. 4B) the calibration curve indicated that system carryover was minimal. Nevertheless, this is an advantage of using nanoelectrospray for quantitative bioanalysis. During the development of the LC–MS/MS method for the quantitation of SCH 211803, carryover exceeding 20% of the LLOQ was observed when a 3 min gradient elution was used, despite an extensive post-injection wash of the autosampler. The main source of carryover was found to come from the HPLC column. As a result, a relatively long HPLC gradient profile with a 6 min run time was required to address the carryover issue while keeping the chromatographic peak reasonably narrow.

In reversed-phase chromatography, when the analyte is injected in a weak elution solvent, it is concentrated on the head of the column. On the other hand, various band broadening processes occurring during the separation tend to dilute the sample. As a result, the overall effect is generally dilution of the sample in the LC step. For example, in this assay 10 μL of the sample was injected on to the column. Baseline width of the chromatographic peak was around 0.15–0.2 min, depending on the sample concentration. The chromatographic peak volume was therefore at least 45 μL, resulting in a four-fold dilution in average concentration, and approximately two-fold dilution at the peak apex.

In contrast, there is no chromatographic sample dilution in nanoelectrospray infusion. Therefore, when inherent sensitivity is compared using matrix-free samples without

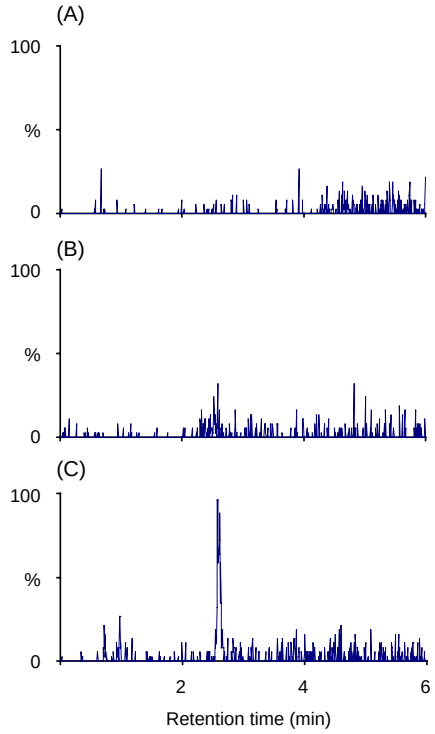

Fig. 4. Extracted ion chromatograms for SCH 211803 obtained by LC–MS/MS analysis, with the intensity axis normalized to the same scale. (A) Plasma blank before the calibration standards. (B) Plasma blank immediately after the calibration standard with the highest concentration. (C) LLOQ sample. The chromatograms were not smoothed to reflect true signal-to-noise ratio.

extraction, the nanoelectrospray setup might be expected to achieve a lower detection limit than LC–MS/MS. In practice, however, careful optimization of the sample extraction and LC step could yield a significant pre-concentration of the sample before analysis, thereby allowing a substantially lower limit of detection in the LC–MS/MS assay. Comparison of the LLOQ sample from a generic nanoeletrospray assay (Fig. 3C) and the LC–MS/MS method with no sample pre-concentration during extraction (Fig. 4C) indicated that signal to noise ratio was similar.

It is in the area of selectivity that nanoelectrospray infusion compares the least favorably to LC–MS/MS. Endogenous compounds in the matrix or metabolites having the same mass transition as the analyte could compromise data quality in nanoelectrospray, whereas in LC–MS/MS the interfering compound can typically be separated by optimizing LC conditions. Although interference could also be eliminated by judicious sample extraction in nanoelectrospray infusion analysis, LC tends to offer a much higher separation power than that obtainable with sample clean-up. An alternative strategy for reducing interference from labile metabolites which may convert to the parent drug molecule in the electrospray source (such as glucuronide, *N*-oxide and sulfate) is to use a lower than normal declustering potential in the first vacuum region of the mass spectrometer immediately after the ion source [14]. However, this approach usually results in lower analyte signal intensity or higher background due to insufficient desolvation of solvated ions and does not always completely eliminate the interfering fragmentation process. Perhaps future work could couple the nanoelectrospray source with an ion mobility device [15] so that the analyte can be effectively separated from the interference in gas phase inside the mass spectrometer.

4. Conclusions

The application of nanoelectrospray infusion to the quantitative analysis of SCH 211803 is demonstrated. Results demonstrated that ion suppression in the absence of HPLC separation did not compromise accurate quantitation. The nanoelectrospray infusion assay reported here provides a viable alternative to conventional LC–MS/MS analysis.

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