Highly Sensitive LC–MS–MS Analysis of a Pharmaceutical Compound in Human Plasma Using Monolithic Phase-Based On-line Extraction

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Abstract

Monolithic columns have been used in recent years for fast chromatographic separation due to their high permeability and low backpressure. We have explored the potential of monolithic material as sample preparation tool in bioanalytical applications. By taking advantage of monolithic columns' online concentration capability, we have developed a highly sensitive liquid chromatography–tandem mass spectrometry assay for quantitative determination of a pharmaceutical compound in human plasma. The assay was fully validated to satisfy the requirements of precision and accuracy, selectivity, matrix effect, and reproducibility. A linear dynamic range from 0.011 ng/mL to 12.3 ng/mL was established as the calibration standard. The percentage of bias for quality control samples was between –9.9% and –2.5%. Coefficient of variation, a measurement of precision, was within 9.9%. On-line extraction with monolithic support provided adequate sample cleanup and on-line concentration of the analyte. The assay exhibited good tolerance to matrix effect and has been applied successfully to a clinical study. The incurred sample analysis showed that original and repeat values were within ±10.1% for all assay samples.

Introduction

High-throughput determination of pharmaceutical compounds in biological matrices in low picogram-per-mililiter range presents significant challenges to bioanalytical scientists. Endogenous components from biological matrices at this quantitation level can cause serious interference to an analytical method. Extraction from a relatively large matrix volume to achieve a very low limit of quantitation may also bring difficulties in assay automation. Although low-flow liquid chromatography mass spectrometry (LC–MS) analysis in nanospray or microspray mode provides a considerable sensitivity increase for the qualitative analysis of macromolecules such as proteins and peptides (1), such conditions may not be transferred to routine quantitative small molecule analysis. Other techniques for sensitivity improvement may have a limited range of applications, such as hydrophilic interaction chromatography for polar compound analysis (2–3).

The recent advances in monolithic chromatography have attracted considerable interest in high-throughput applications (4–6). When coupled with tandem MS, the technique provides fast and sensitive determination of analytes, especially pharmaceutical compounds, in complex matrices such as biological fluids (6). Monolithic phases have been reported for applications in direct injection of biological samples (7–8). Because of their high permeability, the extraction of biological samples can be performed with a high flow-rate without generating high backpressure. The flow-rate can be 5–10 times higher than that generally used conventional supports. The separation efficiency is less dependent on the flow-rate of monolithic columns, which leads to short run-times while maintaining separation efficiency. Plumb et al. have demonstrated that monolithic supports can tolerate several milliliters of plasma without significant performance degradation (8). An on-line solid-phase extraction (SPE) method using a monolithic-based weak cation-exchange column has been reported for the simultaneous determination of alpha1-adrenergic receptor antagonists in human plasma (9). Monolithic packed 96-tips have also been evaluated for extraction and quantification of pindolol and metoprolol in human plasma samples with an LC–MS–MS technique (10).

We previously described automated approaches using on-line extraction with monolithic sorbent for pharmaceutical component analysis in biological matrices by LC–MS–MS (11–13). A short monolithic C_{18} cartridge was used as extraction support for high-speed loading and washing of biological samples. The approach was applied to high-throughput quantitative LC–MS–MS analysis of pharmaceutical compounds in animal studies and clinical studies under Good Laboratory Practice regulations (11–12). Rugged performance was demonstrated from approximately two thousand samples analyzed (12). The on-line extraction approach using a monolithic support was also demonstrated in simultaneous LC–MS–MS quantitation of a pharmaceutical compound and a hydroxylated metabolite in urine (13). In this article, we extended the usage of monolithic cartridges for the highly sensitive determination of concentrations of a pharmaceutical compound with a lower limit of quantitation (LLOQ) of approximately 10 pg/mL in human plasma.

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Experimental

Chemicals

Acetonitrile, methanol, and acetic acid were purchased from EM Science (Gibbstown, NJ). Water was produced by a Millipore (Bedford, MA) Milli-Q unit. Compound A and its internal standard (IS), d_5-labled Compound A, were obtained from Abbott Laboratories (Abbott Park, IL). The chemical structure of Compound A is not disclosed for proprietary reasons. Normal human plasma with potassium EDTA as anti-coagulant was purchased from Biological Specialties Corporation (Colmar, PA).

Calibration standard and quality control samples

Stock solution and working solutions were made in DMSO. Calibration standard and quality control (QC) samples were prepared from separate weightings. Calibration standard levels 1 to 10, at concentrations of 0.0111, 0.0223, 0.0445, 0.193, 0.386, 0.772, 1.54, 3.09, 6.17, and 12.3 ng/mL for Compound A were prepared by adding the appropriate volume of stock solution, working solution, or higher level standard solution into a 25-mL class A volumetric flask and diluting to the mark with normal human plasma with potassium EDTA as anti-coagulant. Standards were then aliquoted into 4-mL polypropylene tubes and stored in a freezer maintained at approximately –20ºC. QC samples were prepared in essentially the same manner at concentrations of 0.0292, 1.20, and 10.2 ng/mL.

Sample preparation

Samples were thawed at room temperature, sonicated, and mixed to ensure homogeneity. All steps of sample preparation were handled in automated fashion. Sample transfer steps were accomplished by a liquid handler with positive displacement capability (Hamilton Lab AT 2 Plus, Reno, NV). Each plasma sample (0.30 mL) was loaded into the appropriate well of a clean 2.0-mL polypropylene 96-well plate.

After 0.040 mL of working IS solution at 100 ng/mL in 50% methanol was transferred to each well, 0.86 mL of acetonitrile was added. The plate was briefly sonicated and vortexed. After being centrifuged at approximately 3000 rpm for 5 min, 0.90 mL of supernatant was transferred from each well to a clean 96-well plate and dried under nitrogen. The plate was reconstituted with 0.30 mL of 20:80:0.02 (v/v/v) MeOH–H_2O–acetic acid, and 0.070 mL of the resulting solution was injected into LC–MS–MS system equipped with on-line extraction setup.

LC–MS–MS instrumentation

The on-line SPE system has been described previously (11–13). A Chromolith RP-18e 10 × 4.6 mm cartridge (Merck KGaA, Darmstadt, Germany, ordered through VWR International) was used as the extraction column. Briefly, an Agilent 1100 pump (Hewlett-Packard, Waldbronn, Germany) with a two-way solvent selector (Parker Instrumentation, Fairfield, NJ) was used to deliver a high flow through the extraction column to load and wash the sample and subsequently to flush and equilibrate the extraction column. A Shimadzu LC-10ADvp pump (Shimadzu, Columbia, MD) was used to deliver the mobile phase. The mobile phase was used to elute the analytes from the extraction column and to perform separation on the analytical column, as well as to wash both the syringe and the autosampler injector. A Shimadzu SIL-HTC autosampler/controller was used to inject samples. A Valco ten-port valve (Valco Instruments, Houston, TX) was used to control on-line extraction and liquid flow to the MS.

An Agilent Zorbax SB-C18 column (2.1 × 100 mm, 3.5 micron) was used as the analytical column. An isocratic high-performance liquid chromatography method was employed for separation. The mobile phase composition was approximately 80:20:0.5 (v/v/v) methanol–H_2O–acetic acid. The flow rate for this program was set to 0.25 mL/min. The analytical column was maintained at room temperature.

LC–MS–MS detection was performed using an API 5000 (Applied Biosystems, Toronto, ON) triple-quadrupole MS with an electrospray ionization source operated in the positive ion mode. The computer control system was Analyst version 1.3.2. The selected reaction monitoring detection channels for analytes are as follows: *m/z* 349.3 to 173.2 for Compound A and *m/z* 354.3 to 178.2 for d_5-labled Compound A. There are no known metabolites of Compound A that would interfere with the quantitative analysis of Compound A at the transition channels monitored.

System operation for on-line extraction procedure

The plasma extract sample was loaded onto the extraction column using solvent A, 40:60:0.1 (v/v/v) methanol–H_2O–acetic acid, at a flow-rate of 2 mL/min. After loading, the valve was switched to the elution position, which positioned extraction and analytical columns in tandem in the flow path of the separation pump. The separation pump, running an isocratic flow of the mobile phase, elutes analytes to the MS. After the elution step, the switching valve was switched back to the original position. The extraction pump delivered solvent B, 95:5:0.1 (v/v/v) methanol–H_2O–acetic acid, at a flow-rate of 2 mL/min to flush the extraction column. For the rest of the run cycle, the extraction pump delivered solvent A at 2 mL/min to re-equilibrate the extraction column for the next sample. The run-time for the assay of one sample was approximately 4 min. An example of the time program of the Agilent 1100 pump for the on-line extraction is listed in Table I.

The Shimadzu SIL-HT_C autosampler/controller sent the signal to inject the sample and to start the program on the Agilent 1100 pump. It also sent out a signal to the MS to start the data acquisition. For overnight operation, a contact-closure signal was sent from the HT_C autosampler/controller to the

Table I. An Example of a Timing Program for On-Line Extraction

Time (min)	Event
0.00	Sample loaded onto extraction column; flow-rate 2 mL/min
0.70	Valve switched; SPE cartridge in tandem with an analytical column
0.72	Solvent selector switched; deliver washing solvent
2.40	Valve switched; wash SPE cartridge
3.00	Solvent selector switched; conditioning SPE cartridge
3.50	Program ends; flow-rate 2 mL/min

Agilent 1100 pump to shut down the pump at the end of sample analysis.

Calibration curves and quantitation of samples

Analyst version 1.3.2 was used for the data acquisition, peak area integration, regression analysis, and quantitation. For each analytical batch, a calibration curve was derived from the peak area ratios (analyte/IS) using weighted linear least-squares regression of the area ratio versus the concentration of the standards. A weighting of $1/x^2$ (where x is the concentration of a given standard) was used for curve fitting. The regression equation for the calibration curve was used to back-calculate the measured concentration at each standard level, and the results were compared with the theoretical concentration to obtain the accuracy, expressed as a percentage of the theoretical value, for each standard level measured.

Results and Discussion

The demand for a highly sensitive analytical method for the determination of compound A in human plasma was driven by the need for drug development in clinical studies to provide pharmacokinetic evaluation at low dosages. Our previous work with on-line extraction using monolithic material has shown that such approaches can be generic for method development, as long as the compound of interest can be retained on the monolithic support. With a few adjustments made in plasma sample volume, reconstitution solution volume of the plasma extract, and injection volume, it was found that the on-line approach was applicable for low picogram-per-mililiter level determination of compound A in human plasma. As a result, method development was completed in less than a week's time.

The method has been subsequently validated under Food and Drug Administration (FDA) guidelines for the industry (14). Precision and accuracy were demonstrated by four consecutive analytical batches. Each batch contained a single set of calibration standards, six replicates of QCs at three concentration levels, six replicates of LLOQ evaluation samples, and six replicates of upper limit of quantitation (ULOQ) evaluation samples. Each batch also contained other test samples such as a system suitability sample.

Statistical data of calibration curve parameters computed from four consecutive analytical curves are listed in Table II. The correlation coefficients of 4 calibration curves were all ≥ 0.993. The standards showed a linear range of 0.0111–12.3 ng/mL for Compound A using weighted ($1/x^2$) least-square linear regression. The precision and accuracy data for LLOQ, ULOQ, and QC samples are summarized in Table III. The data show that this method is consistent and reliable with low % coefficient of variation (CV) and %bias values. The accuracy (%bias) at the LLOQ was 4.5% and the precision (%CV) at the LLOQ was 12.1%. The inter-day %bias and %CV of the QC samples were within ± 9.9% and ≤ 9.9%, respectively.

To achieve an LLOQ at low pictogram-per-mililiter level for Compound A, a plasma volume of 0.30 mL was used to provide an absolute amount of the analyte for on-column analysis. One major concern was whether the approach would provide sufficient sample cleanup due to the relatively large plasma volume used. A selectivity test was performed using 6 lots of blank plasma samples. The acceptance criteria was that quantifiable peak areas of each lot of blank plasma extracted without IS must be less than 20% of the peak area of the LLOQ sample extracted without IS. No interference was found from any lot of blank plasma samples, which demonstrated the specificity of the method. Figure 1 shows the chromatograms from a blank sample extracted without IS.

Representative chromatograms of an LLOQ sample are shown in Figure 2. The estimated signal-to-noise ratio at LLOQ was greater than 12:1, which demonstrated that sufficient sensitivity was achieved. For the current on-line extraction method, only 70 µL out of approximately 300 µL of reconstitution solution was loaded onto the on-line extraction column for LC–MS–MS analysis. It is projected that sensitivity can be easily improved with a higher injection volume if needed. The peak shape of the LLOQ appeared to be broad; however, this was mainly attributed to the high background as noted in both Figures 1 and 2. In this application, an API 5000 MS was used for method validation and further sample analysis. The performance of the API 5000 was compared to that of an API 4000 instrument during method development. Although analyte signal of the given plasma sample from the API 5000 was approximately four times of that from the API 4000, the background intensity also more than doubled in the transition channel monitored.

One phenomenon influencing MS-based bioanalytical assays is matrix effect (15–17). Matrix effect is the suppression or enhancement of ionization of analytes by the presence of matrix components in the biological samples. The FDA sug-

Table II. Summary of Calibration Curves Obtained for Compound A*

Conc. (ng/mL)	STD 1 0.0111	STD 2 0.0223	STD 3 0.0445	STD 4 0.193	STD 5 0.386	STD 6 0.772	STD 7 1.54	STD 8 3.09	STD 9 6.17	STD 10 12.3	Coefficient of determination (r^2)
Mean	0.0116	0.0207	0.0407	0.193	0.395	0.783	1.56	3.13	6.32	12.1	0.996
SD	0.000279	0.00142	0.00267	0.00850	0.0143	0.0170	0.0374	0.0593	0.183	0.305	0.001
%CV	2.4	6.9	6.6	4.4	3.6	2.2	2.4	1.9	2.9	2.5	—
%Bias	4.5	–7.2	–8.5	0.0	2.3	1.4	1.3	1.3	2.4	–1.6	—
n	4	4	4	4	4	4	4	4	4	4	4

* Mean values in the table are average of the back-calculated concentrations from the standard curve.

gested that quantitative measurement of matrix effects is needed to provide useful information in validation of MS-based bioanalytical methods (18). The matrix effects are generally due to the influence of co-eluting compounds such as endogenous phospholipids or formulation vehicle during the actual analyte ionization process, well before the analyte ions enter the high vacuum of the mass analyzer. The matrix effect, if present, is typically more severe for analytical assays dealing with very low LOQ, such as those used to support early stage of clinical studies.

Table III. Inter-Day Accuracy and Precision of LLOQ, QC, and ULOQ Samples*

Concentration (ng/mL)	LLOQ 0.0111	Low QC 0.0292	Mid QC 1.20	High QC 10.2	ULOQ 12.3
Mean	0.0116	0.0263	1.17	9.49	12.1
Inter-run SD	0.0014	0.0026	0.0387	0.274	0.375
Inter-run %CV	12.1	9.9	3.3	2.9	3.1
Inter-run %Bias	4.5	–9.9	–2.5	–7.0	–1.6
n	24	24	24	24	24

*Mean values in the table are average of the back-calculated concentrations from the standard curve.

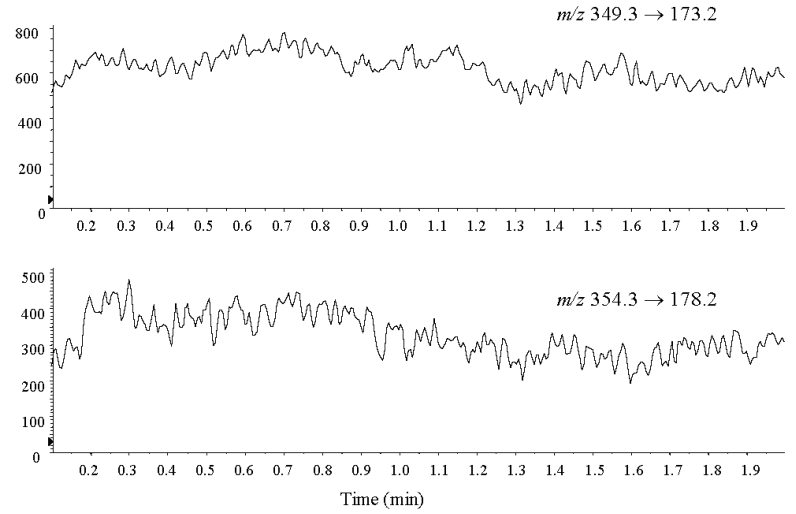

Figure 1. Representative ion chromatograms of an extracted blank sample without IS.

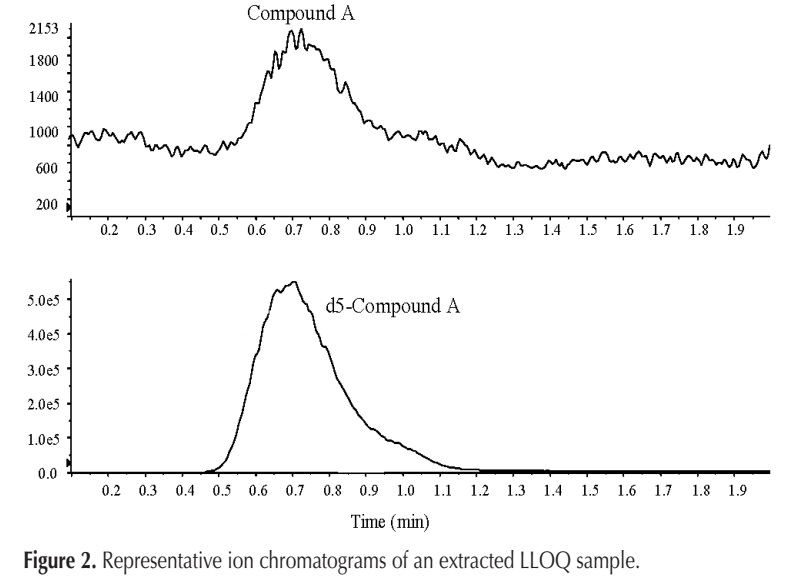

Figure 2. Representative ion chromatograms of an extracted LLOQ sample.

In this method, Compound A was well separated from early eluting peaks of plasma extracts. To demonstrate that the assay performance is independent from the sample matrix, matrix effect evaluation samples, similar in concentration to the low QC, were prepared using six different lots of human plasma, plus the human plasma pool used to prepare calibration standards and QCs for the validation. None of the six evaluation lots were used for the makeup of the human plasma pool. After analysis, the mean found concentration for the human plasma pool matrix effect sample was used as the theoretical concentration for the other six lots. The acceptance criteria were that the mean bias for each lot of matrix effect evaluation sample must be within ± 15% of the theoretical value. All six testing lots met acceptance criteria. The accuracy (%bias) of these matrix effect evaluation samples ranged from 3.8% to 11.2%, and the precision (%CV) was ≤ 9.2%. The results, as summarized in Table IV, demonstrated that matrix effect for the assay was well within the measurement errors.

The extraction recovery was determined by comparing the response factors (area/on-column amount) of extracted QC samples with those of neat solutions at similar concentrations. An average of 43% extraction recovery was determined. Typically, on-line SPE with a monolithic support approach offers high recovery, as we have demonstrated in previous publications (11–13). In the sample preparation of this method, the supernatant from the protein precipitation step was evaporated and reconstituted prior to on-line SPE due to the relatively larger plasma sample volume used. The lower than expected recovery was most likely due to loss of material in the drying process as a part of the sample preparation. System carry-over was evaluated by comparing the analyte response from a blank sample with that of a ULOQ sample that was in a previous injection. The percentage of the carry-over was found at 0.043% when calculated from the peak areas. The on-line system was found to be very rugged for the analysis of plasma samples after a simple protein-precipitation treatment. Because of its highly porous nature, the backpressure generated on the extraction column was very low (approximately 10 bar) at the flow-rate of 2 mL/min. The backpressure on the extraction column and the analytical separation column remained the same after at least 200 injections of samples.

The method was successfully applied to a clinical study designed to evaluate the efficacy of

Table IV. A Summary of Accuracy and Precision Results of Matrix Effect Evaluation Samples Prepared in 6 Different Lots of Human Plasma*

Lot #	A	B	C	D	E	F	Overall
Mean	0.0360	0.0378	0.0366	0.0366	0.0374	0.0353	0.0366
SD	0.0033	0.0024	0.0025	0.0028	0.0029	0.0020	0.0005
%CV	9.2	6.4	6.8	7.8	7.7	5.7	1.2
%Bias	5.9	11.2	7.7	7.5	10.1	3.8	7.7
n	6	6	6	6	6	6	36

* Mean values in the table are average of the back-calculated concentrations in ng/mL from the standard curve. Theoretical concentration of the evaluation sample was 0.0340 ng/mL.

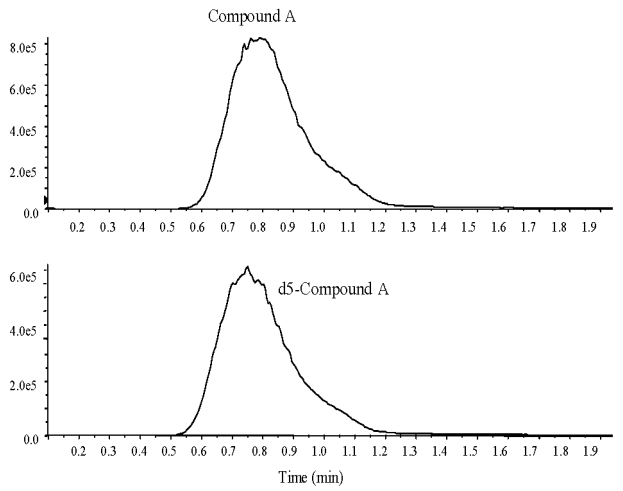

Figure 3. Representative ion chromatograms of a plasma sample from a dosed subject in a clinical study.

compound A. Selected samples near the maximum plasma concentration and at the terminal elimination phase of dosed subjects in the study were used for incurred sample repeat analysis. The results showed that original and repeat values were within ± 10.1% for all assay samples. Figure 3 shows the chromatograms of a plasma sample from a dosed subject in the study.

Conclusions and Future Perspectives

In conclusion, an on-line extraction approach using monolithic phase-based extraction support has been extended for highly sensitive LC–MS–MS quantitation of a pharmaceutical compound in human plasma samples. On-line extraction not only provided the concentration factor needed for on-column analysis of low picogram-per-milliliter samples, but also provided adequate sample cleanup from the biological matrix by taking advantage of high speed loading, extraction, and washing on the monolithic support. Currently, the commercial availability of monolithic materials is limited in both type and size. A silica-based 2 mm i.d. monolithic column by Merck KGaA has been recently introduced for small molecule analysis. This new addition, and other sorbents in different bonding or dimensions coming to the market, will further expand the capability of the monolithic-based approach in high-throughput bioanalysis.

References

1. W.J. Griffiths. Nanospray mass spectrometry in protein and peptide chemistry. *EXS* **88:** 69–79 (2000).
2. W. Naidong. Bioanalytical liquid chromatography tandem mass spectrometry methods on underivatized silica columns with aqueous/ organic mobile phases. *J. Chromatogr. B* **796:** 209–24 (2003).
3. Y. Hsieh. Potential of HILIC-MS in quantitative bioanalysis of drugs and drug metabolites. *J. Sep. Sci.* **31:** 1481–1491 (2008).
4. F. Svec and C.G. Huber. Monolithic materials: promises, challenges, achievements. *Anal. Chem.* **78:** 2101–2107 (2006).
5. F. Svec and N. Tanaka. Monolithic separation media: where are they heading? *J. Sep. Sci.* **27:** 745 (2004).
6. R.N. Xu, L. Fan, M. Rieser, and T.A. El-Shourbagy. Recent advances in high-throughput quantitative bioanalysis by LC-MS/MS. *J. Pharm. Biomed. Anal.* **44:** 342–355 (2007).
7. Y. Hsieh, G. Wang, Y. Wang, S. Chackalamannil, and W.A. Korfmacher. Direct plasma analysis of drug compounds using monolithic column liquid chromatography and tandem mass spectrometry. *Anal. Chem.* **75:** 1812–1818 (2003).
8. R. Plumb, G. Dear, D. Mallett, and J. Ayrton. Direct analysis of pharmaceutical compounds in human plasma with chromatographic resolution using an alkyl-bonded silica rod column. *Rapid Commun. Mass Spectrom.* **15:** 986–993 (2001).
9. X. Wei, J. Yin, G. Yang, C. He, and Y. Chen. On-line solid-phase extraction with a monolithic weak cation-exchange column and simultaneous screening of alpha1-adrenergic receptor antagonists in human plasma. *J. Sep. Sci.* **30:** 2851–2857 (2007).
10. M. Abdel-Rehim, C. Persson, Z. Altun, and L. Blomberg. Evaluation of monolithic packed 96-tips and liquid chromatography-tandem mass spectrometry for extraction and quantification of pindolol and metoprolol in human plasma samples. *J. Chromatogr. A* **1196:** 23–7 (2008).
11. R.N. Xu, L. Fan, G.E. Kim, and T.A. El-Shourbagy. A monolithic-phase based on-line extraction approach for determination of pharmaceutical components in human plasma by HPLC-MS/MS and a comparison with liquid-liquid extraction. *J. Pharm. Biomed. Anal.* **40:** 728–736 (2006).
12. R.N. Xu, G. Kim, B. Boyd, J. Polzin, M. Rieser, and T.A. El-Shourbagy. High-throughput quantitative LC-MS/MS assays by on-line extraction using monolithic support. *LC-GC* **25:** 396–405 (2007).
13. R.N. Xu, B. Boyd, M. Rieser, and T.A. El-Shourbagy. Simultaneous LC-MS/MS quantitation of a highly hydrophobic pharmaceutical compound and its metabolite in urine using online monolithic phase-based extraction. *J. Sep. Sci.* **30:** 2943–2949 (2007).
14. Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, May 2001.
15. D.L. Buhrman, P.I. Price, and P.J. Rudewicz. Quantitation of SR 27417 in human plasma using electrospray liquid chromatography-tandem mass spectrometry: a study of ion suppression. *J. Am. Soc. Mass Spectrom.* **7:** 1099–1105 (1996).
16. R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, and T. Olah. Mechanistic investigation of ionization suppression in electrospray ionization. *J. Am. Soc. Mass Spectrom.* **11:** 942–950 (2000).
17. B.K. Matuszewski, M.L. Constanzer, and C.M. Chavez-Eng. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal. Chem.* **75:** 3019–3030 (2003).
18. C.T. Viswanathan, S. Bansal, B. Booth, A.J. DeStefano, M.J. Rose, J. Sailstad, V.P. Shah, J.P. Skelly, P.G. Swann, and R. Weiner. Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. *Pharm. Res.* **24:** 1962–73 (2007).

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