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ARTICLE *in* RAPID COMMUNICATIONS IN MASS SPECTROMETRY · AUGUST 2005

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Rapid Commun. Mass Spectrom. 2005; **19**: 2144–2150
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/rcm.2037

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High-throughput biological sample analysis using on-line turbulent flow extraction combined with monolithic column liquid chromatography/tandem mass spectrometry

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Received 3 February 2005; Revised 27 May 2005; Accepted 28 May 2005

A high-throughput liquid chromatography/tandem mass spectrometry (LC/MS/MS) method, which combines on-line sample extraction through turbulent flow chromatography with a monolithic column separation, has been developed for direct injection analysis of drugs and metabolites in human plasma samples. By coupling a monolithic column into the system as the analytical column, the method enables running 'dual-column' extraction and chromatography at higher flow rates, thus significantly reducing the time required for the transfer and mixing of extracted fraction onto the separation column as well as the time for gradient separation. A strategy of assessing and reducing the matrix suppression effect on the on-line extraction LC/MS/MS has also been discussed. Experiments for evaluating the resolution, peak shape, sensitivity, speed, and matrix effect were conducted with dextromethorphan and its metabolite dextrorphan as model compounds in human plasma matrix. It was demonstrated that the total run time for this assay with a baseline separation of two analytes is less than 1.5 min. Copyright © 2005 John Wiley & Sons, Ltd.

One of the major challenges in bioanalytical chemistry has been the development of high-throughput assays to provide drug metabolism and pharmacokinetics (DMPK) data in accelerating the process of drug discovery and development.[1,2] In many cases, the timeline for the analysis of a set of *in vitro* and *in vivo* study samples has changed from weeks to hours.[3] This has created demands for fast assays as well as short method development timelines. In recent years, liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) has become the method of choice for the quantitative analysis of drugs and metabolites in biological samples.[3–6] The merits of high sensitivity and selectivity of MS/MS methodologies have allowed the use of fast high-performance liquid chromatography (HPLC) (e.g., with short columns) to shorten the analysis time. However, certain sample preparation steps prior to LC/MS/MS analysis are still essential in order to remove protein and/or endogenous interference species from matrix. As demonstrated by many investigators,[5,7–9] a selective sample pre-treatment is often a critical step in fast LC/MS/MS assays in developing high-throughput methods.[3,10–13]

With the improvements in instrumentation and column technology, on-line sample extraction that is coupled to LC/MS/MS has developed as an important tool for facilitating direct injection analysis of biological samples in the past few years.[14–19] For example, turbulent flow chromatography in which extraction columns are packed with retaining particles of large size (e.g., 50 μm) accomplishes an orthogonal two-dimensional separation on a single column.[18,20,21] Turbulent flow achieves an effective fractionation by molecular weight, thus removing proteinaceous materials in biological matrix. The selective extraction of desired compounds is further achieved by the stationary phase of turbulent flow chromatography. As compared to the off-line automated solid-phase extraction (SPE) (e.g., with 96-well or 384-well format),[10] on-line extraction provides more flexibility for troubleshooting and method development. In addition, it lowers the cost and reduces variability by using the same column for all samples.

Turbulent flow on-line extraction can be performed with two different configurations. In the single mobile phase extraction, samples are loaded onto an extraction column, and extracted directly with the mobile phase of strong solvent (e.g., high organic content for reversed-phase extraction) onto the analytical column. This approach provides a very high sample throughput and low sample carry-over between injections. However, the peak capacity in the chromatographic separation with this method can be low due to the use of solvents of strong elution strength for both extraction and separation. Single mobile phase extraction has thus certain limitations for the applications where drugs, the metabolites and/or endogenous species need to be separated from each other to eliminate interference in mass spectrometric

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detection.[12,13] In a dual-column configuration (See details in the Experimental section), samples are extracted with the solvent that is delivered by the turbulent flow pump. In this step, the extraction solvent is usually set at a low flow rate in order to maintain a focusing effect (see discussion below). To minimize the change-over time needed for switching from loading solvent (usually a weak one) to extraction solvent at relatively low flow rate, the method with a loop configuration was implemented in our laboratory (see Fig. 2(b)). In this set-up, the extraction solvent is previously stored in the loop and can be quickly delivered to the extraction column. The extracts are transferred through a tee to the analytical column into which the analytical pump delivers a weak mobile phase at a higher flow rate relative to the extraction flow. Maintaining a relatively high ratio of two flows is essential in order to dilute the (strong) extraction solvent and thus focus the extracted compounds on the analytical column. A poor focusing may lead to broad or even split peaks.[22] Once the sample is transferred, the compounds are eluted and separated with a gradient or step elution on the analytical column. During the analytical separation, the extraction loop is being filled with the extraction solvent by the extraction pump for the next extraction. Dual-column extraction LC/MS/MS generally provides better chromatographic separation and good peak shape as compared to the single mobile phase method. In general, the extraction flow rate in the mixing step needs to be minimized in order to uphold a desired focusing effect, which along with a gradient elution on the analytical column can certainly reduce the analytical throughput.

In this paper, we describe a method for improving sample throughput with dual-column extraction LC/MS/MS. A monolithic column is employed as the analytical column, thus allowing both gradient separation and the mixing of extraction solvent at a flow rate much higher than that with a conventional analytical column. Evaluation of this approach was conducted with human plasma samples.

EXPERIMENTAL

Equipment

All experiments were performed on a Cohesive 2300 HTLC system (Cohesive Technologies, Franklin, MA, USA) coupled with an API 365 except that the method validation for the determination of dextrorphan and dextromethorphan in human plasma was conducted on an API 3000 tandem mass spectrometer (Sciex, Concord, Canada). A Cohesive Cyclone C18 (50 × 1.0 mm, 50 μm) column was used for the sample extraction. Two analytical columns, a Zorbax XBD C18 analytical column (20 × 4.6 mm, 3.5 um) (Agilent, Palo Alto, CA, USA) and a Chromolith Speed ROD RP-18e (50 × 4.6 mm) (Merck KgaA, Darmstadt, Germany), were used for the evaluation.

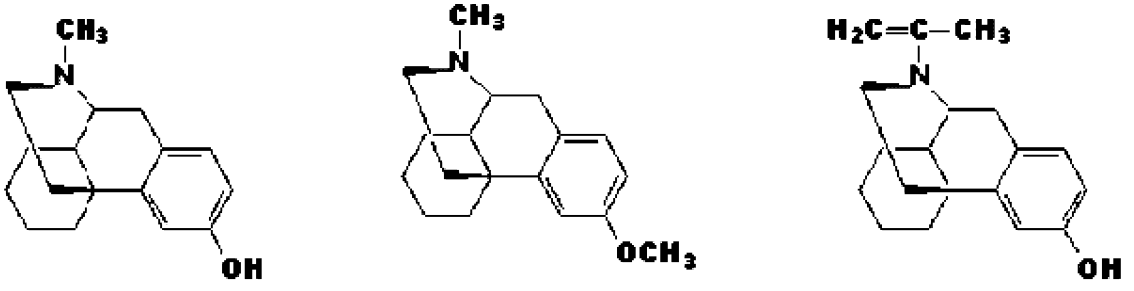
Chemicals and sample preparation

Dextrorphan, dextromethorphan, and the internal standard levallorphan (Fig. 1) were purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC-grade water, methanol and formic acid were purchased from Fisher Scientific (St. Louis, MO, USA), and trifluoroacetic acid (TFA) was from Sigma. Stock and working solutions were prepared with methanol and stored at 2–8°C. Plasma samples were prepared by spiking appropriate amounts of working solutions into blank K_3-EDTA human plasma (Biochemd, Winchester, VA, USA), which was previously screened to be free of interference. Aliquots of 100 μL of the spiked plasma samples were diluted with 100 μL of 0.1% TFA, and centrifuged at 3000 rpm for 10 min using a HJ-6 centrifuge (Beckman, Fullerton, CA, USA) prior to injection.

Method and procedure

In single mobile phase extraction experiments, the samples were loaded through pump 1 with 0.1% TFA at a flow rate of 5 mL/min into the extraction column, and cleaned for 15–30 s. The compounds were then extracted with the mobile phase delivered by pump 2 at a flow rate of 1 mL/min into the analytical column. The compositions of mobile phase are stated in the respective sections. Afterwards, the extraction column was further cleaned using methanol with 0.1% formic acid, and eventually equilibrated with the loading solvent for subsequent injections.

Dual-column extraction experiments were performed through four steps including loading samples, transfer of extracts, gradient elution and post-extraction cycle (Fig. 2). A loop of 300 μL was connected in valve A as shown in Fig. 2. Prior to extraction, the loop was filled with 0.1% formic acid in methanol, which used as extraction solvent. The solvents delivered through pump 2 for gradient elution were 0.1%



Dextrorphan Dextromethorphan Levallorphan

Figure 1. Chemical structures of the compounds studied.

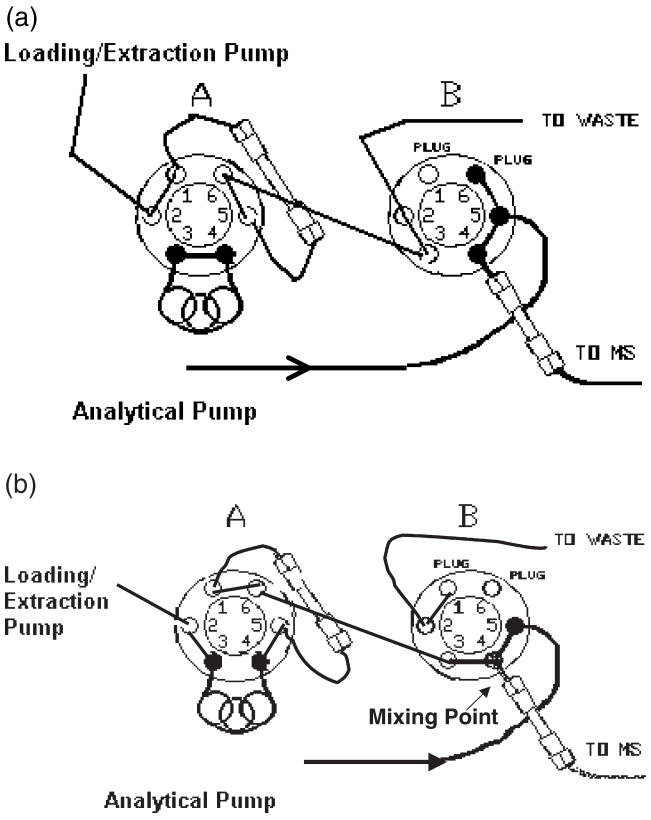


Figure 2. Configuration of a typical dual-column on-line extraction LC/MS system: (a) loading samples and (b) eluting, mixing and analyzing extracted compound(s).

formic acid in water and 0.1% formic acid in methanol. The detailed parameters including extraction flow and gradient profiles are given along with the data presented in the Results and Discussion section. For the assessment of matrix suppression effect in mass spectrometric detection, a neat working solution at a concentration of 200 ng/mL was introduced into the source by post-column infusion at a flow rate of 10 μL/min with injections of extracted plasma or reagent blank.

On-line extraction LC was coupled with the API mass spectrometer through a Turbo-ionspray interface (Sciex, Concord, Canada). The compounds were detected using multiple reaction monitoring (MRM) in the positive ion mode at a source temperature of 450°C. The ion transition channels are 258 → 157 for dextrorphan, 272 → 215 for dextromethorphan, and 284/157 for levallorphan. The ionspray capillary voltage was set at 3500 V for all experiments. Nitrogen was used as the nebulizer gas at a setting of 10. Focusing potential, declustering voltage and collision energy were optimized for each of the compounds.

RESULTS AND DISCUSSION

Single mobile phase vs. dual-column extraction

Figure 3 shows the chromatogram of a plasma sample analyzed using the single mobile phase extraction LC/MS/MS. The compounds were extracted from the Cyclone C-18 column with a mobile phase composed of methanol/water/formic acid (85:15:0.1, v/v/v) at a flow rate of 1.0 mL/min, and eluted at the same flow from the Zorbax XBD C-18 column running isocratic mobile phase.

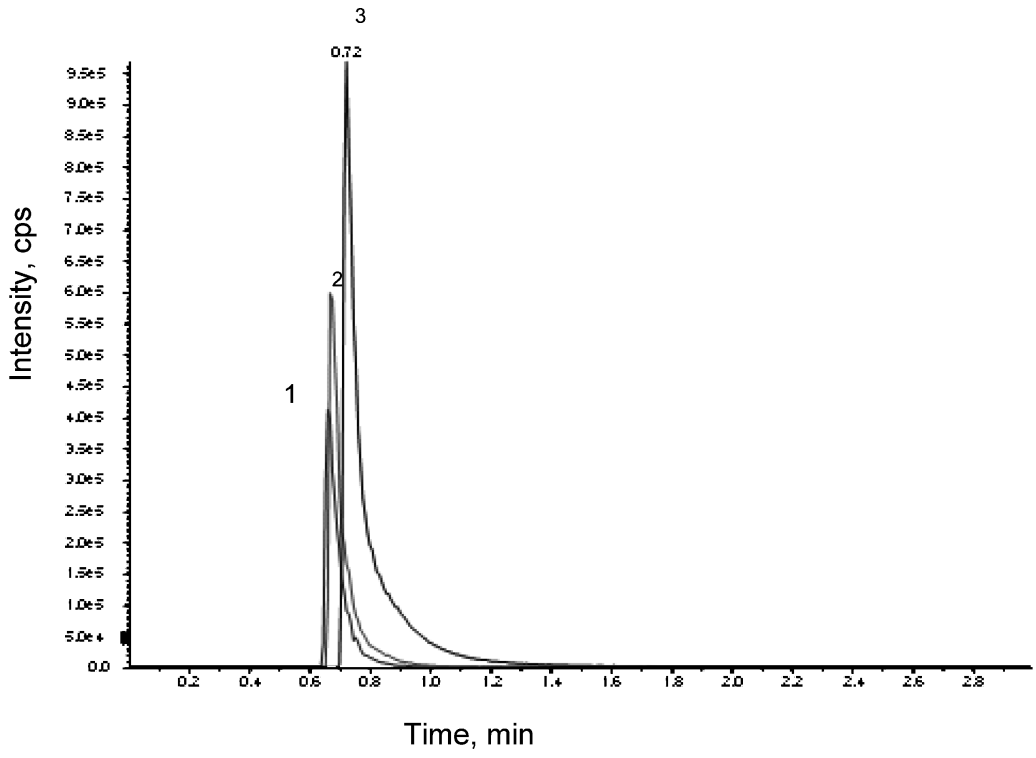


Figure 3. Chromatogram of dextrorphan (1), levallorphan (2) and dextromethorphan (3) using single mobile phase on-line extraction LC/MS/MS. Loading solvent: 0.1% TFA in water; flow rate: 5.0 mL/min. LC mobile phase: 0.1% formic acid in methanol: water (85:15, v/v); flow rate: 1.0 mL/min.

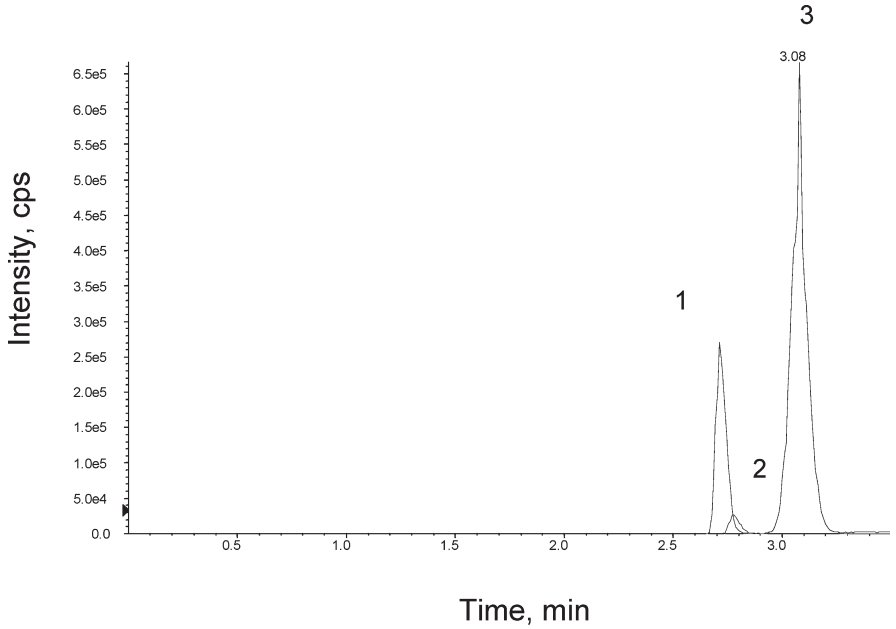


Figure 4. Chromatogram of dextrorphan (1), levallorphan (2) and dextromethorphan (3) in human plasma using dual-column on-line extraction LC/MS/MS. Extraction column: Cyclone C-18, 50 × 1 mm, 50 μm. Analytical column: Zobax XBD, 20 × 4.6 mm, 3.5 μm. Loading solvent: 0.1% TFA in water. Eluting solvent: 300 μL of 0.1% formic acid in methanol. Transfer flow rate: 0.2 mL/min (90 s in total). Analytical flow rate: 1.5 mL/min. Solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in methanol; gradient after mixing: 0 % to 50% B in 30 s, then to 100% B in 15 s and hold for 75 s. During the analysis step, the extraction loop on valve A is refilled with 0.1% formic acid in methanol for the next sample.

Dextrorphan and dextromethorphan were eluted around 0.7 min, indicating weak retention on the column. The tailing peaks could be due to poor chromatographic efficiency of the extraction column run under a non-turbulent flow condition (in this case, 1 mL/min).

A significant improvement in both resolution and peak shape was achieved with the dual-column approach, as evident in the chromatogram shown in Fig. 4. The same Zorbax column was employed as the analytical column. Dextrorphan and dextromethorphan were baseline separated, and the peak shape was symmetric. The run time was less than 3.5 min, 2 min longer than the single mobile phase approach. In the dual-column extraction, the speed of a dual-column assay is mainly limited by two steps: mixing and gradient elution. In the mixing step, the extract needs to be transferred onto the analytical column at a low flow rate relative to the flow of analytical mobile phase, and the analytical mobile phase needs to remain weaker in order to maintain a desired focusing effect. In this case, with 300 μL of the total extraction volume as optimized, the time needed for transfer was 90 s at a flow rate of 0.2 mL/min.

Dual-column extraction LC/MS/MS using a monolithic column

Monolithic HPLC columns are packed with monolithic silica sorbent in rod form with both macropores and mesopores, a combined structure that enables fast mass transfer at lower back pressure. In particular, the column efficiency does not decrease significantly when the operating flow rate is increased since the van Deemter curves of monolithic columns are much flatter than traditional HPLC columns that are packed with small silica particles.[23] In this study, the flow rate of analytical mobile phase was increased to 3.0 mL/min with the Chromolith column, resulting in a significant decrease in run time (Fig. 5) with relatively low back pressure (approximately 40 bar with 0.1% formic acid). Figure 5 shows that the run time was decreased from 3.5 to 1.5 min using a monolithic column at 3.0 mL/min to replace the regular C18 column at 1.0 mL/min. This increase in the analytical flow allowed the increase of the extraction flow accordingly during the mixing without distorting the focusing effect. For example, the transfer/mixing time was decreased to 30 s at extraction flow of 0.6 mL/min from the previous 90 s with the Zorbax column. In addition, higher analytical flow also decreased the time needed for gradient elution and column equilibration.

Running on-line extraction at higher flow rates may reduce the signal sensitivity to some extent, depending on several factors including influences of flow rate on MS response, theoretical plate of the column, and retention time (especially for gradient elution).[24,25] For example, the theoretical plate height of a monolithic column at 5 mL/min is approximately 1.5 times higher than that of the Zorbax column run at 1 mL/min (data not shown). Therefore, the peak concentration at the high flow will be approximately 1.2 times lower, assuming the same retention capacities (k′) under the

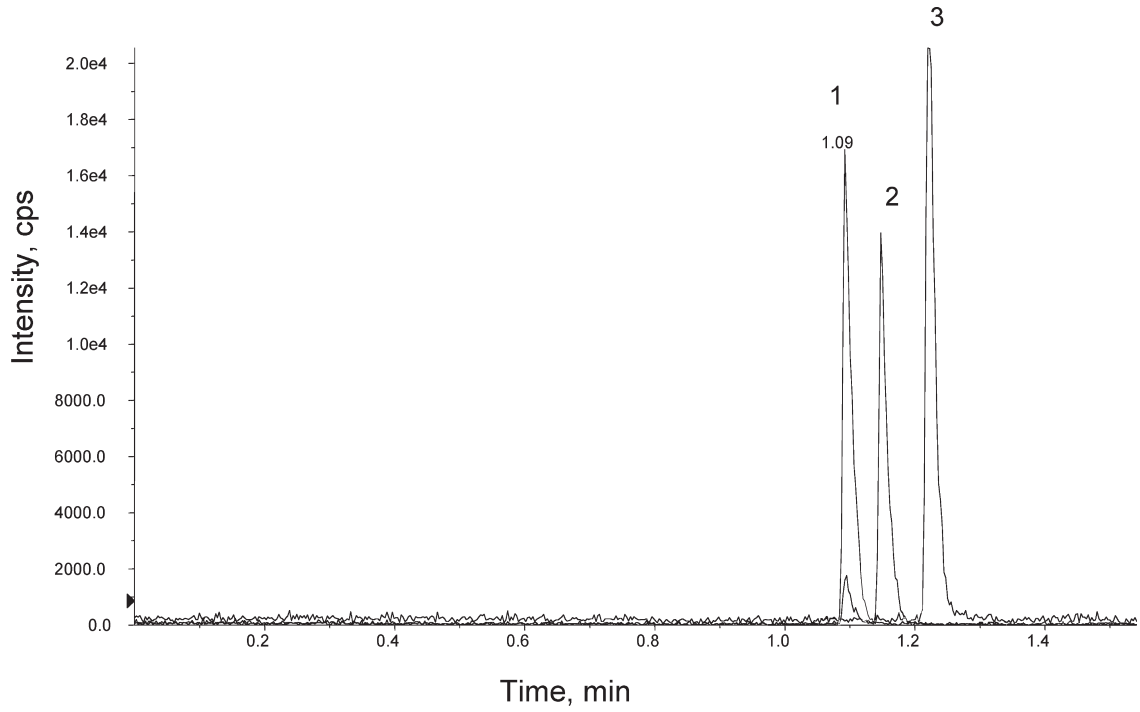


Figure 5. Chromatogram of dextrorphan (1), levallorphan (2) and dextromethorphan (3) in human plasma using dual-column on-line extraction LC/MS/MS. Extraction column: Cyclone C-18, 50 × 1 mm, 50 μm. Analytical column: Chromolith Speed ROD RP-18e, 50 × 4.6 mm. Loading solvent: 0.1% TFA in water. Eluting solvent: 300 μL of 0.1% formic acid in methanol. Transfer flow rate: 0.6 mL/min (30 s in total). Analytical flow rate: 3.0 mL/min. Solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in methanol; gradient after mixing: 0% to 50% B in 30 s, then to 100% B in 15 s and hold for 75 s. During the analysis step, the extraction loop on valve A is refilled with 0.1% formic acid in methanol for the next sample.

same isocratic condition. In practical applications, the post-column split that is usually necessary in order to accommodate the flow limitation in the MS interface must be optimized by varying the split ratio.[24] In this work, the HPLC flow was split into the mass spectrometer at a flow rate of 0.4 mL/min.

Assessment of matrix suppression effect

Matrix suppression effect has been an issue that may significantly affect the sensitivity and integrity of biological sample analysis.[5,7–9] In electrospray ionization, matrix components of high concentration levels may reduce the efficiency of ionization of the co-eluting analyte.[25,26] To assess the suppression effect, the analyte solution is usually post-column infused into the ion source while a blank matrix extract is injected through a column to pinpoint the location of matrix peaks. In on-line extraction, we proposed to inject the neat solution that is prepared in water or water/organic mixture as a reference point to diagnose the suppression.[27]

Figure 6 shows the chromatograms obtained with a neat solution (Fig. 6(a)) and plasma sample (Fig. 6(b)), respectively, under a condition with a fast gradient. Matrix suppression was observed for the last eluting peak. This was demonstrated by the decreased peak height. The post-column infusion method was used to further confirm the suppression (Fig. 6(c)). The gradient elution profile was then modified to separate the suppression from the analytes of interest and the chromatogram is presented in Fig. 5.

Practical applications

Theoretically, all off-line reverse-phase SPE extraction methods can be adapted to using the turbulent flow on-line extraction. The application boundaries can be further extended to polar and ionic species with the recent development of ion-exchange turbulent flow columns. Along this line, our laboratory has successfully developed a number of assays, including methods for the determination of lisinopril,[17] gabapentin,[19] glyburide and moxifloxacin.[27]

An assay was recently validated for the determination of dextrorphan and dextromethorphan in human plasma using the method as described here combining on-line extraction with the high-speed monolithic column. Standard curves were produced on three different days in pooled human plasma over the range of 5–2500 ng/mL. The response was linear and the coefficients of determination (r^2) for both compounds were greater than 0.996. Intra- ($n = 6$) and inter-day ($n = 18$) precision and accuracy calculated from quality control (QC) samples are tabulated in Table 1. The same method was also validated for the analysis of dextrorphan and dextromethorphan in human urine.

Optimization of extraction parameters, including the selection of extraction column, loading, pre-wash and extraction solvent to selectively remove interference species from sample matrix, is among the key factors for the successful development and improvement of on-line extraction methods. For analytical HPLC, applying a weak initial solvent to dilute the extraction solvent is critical to main-

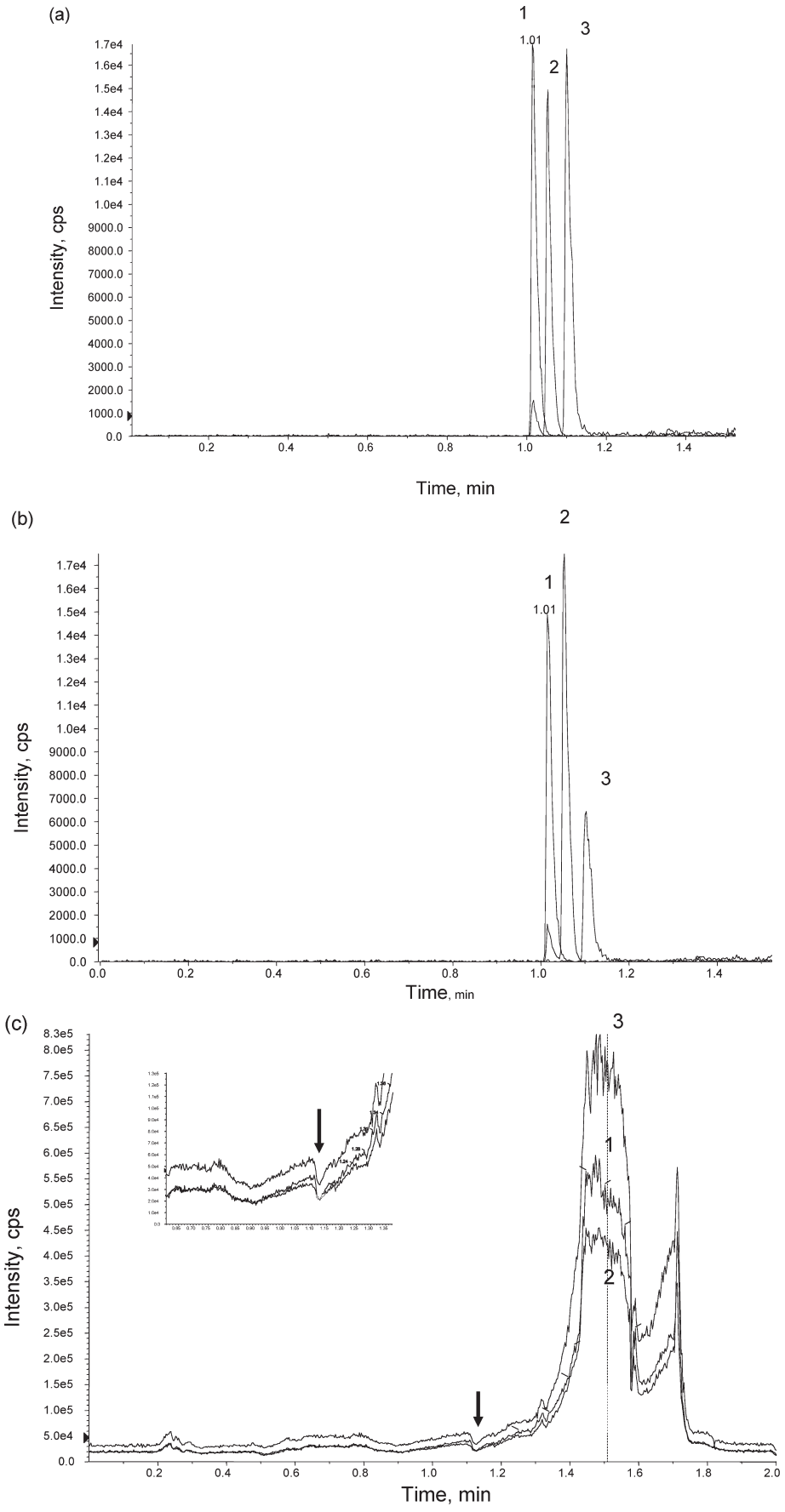


Figure 6. Comparison of chromatograms between neat solution (a) and plasma sample (b) for evaluation of matrix suppression effect. (c) Profile of post-column infusion to pinpoint the suppression. Extraction and LC/MS conditions are the same as described in Fig. 5, except the first step of gradient after mixing is in 15 s instead of in 30 s.

Table 1. Intra- and inter-day accuracy and precision data for QC samples

	Intra-day (n = 6)					Inter-day (n = 18)		
Dextromethorphan (ng/mL)	5.00	15.0	350	1750	8750	15.0	350	1750
Mean (ng/mL)	5.25	15.2	387	1712	8383	14.7	374	1748
RSD (%)	2.12	1.70	1.98	2.48	2.98	5.71	6.51	4.28
RE (%)	5.04	0.98	10.66	−2.19	−4.20	−2.03	−0.23	−0.11
Dextrorphan (ng/mL)	5.00	15.0	350	1750	8750	15.0	350	1750
Mean (ng/mL)	4.81	14.1	363	1749	8774	14.0	356	1749
RSD (%)	3.10	2.97	1.72	2.68	5.69	5.45	4.91	3.58
RE (%)	−3.90	−5.90	3.72	−0.07	0.27	−6.57	−5.01	−0.04

RSD: relative standard deviation; RE: relative error.

tainimg the desired focusing effect. However, it is not uncommon that the gradient elution, especially starting with a relatively weak solvent, can lead to serious carry-over issues that might limit the curve ranges of assays. On the other hand, the use of the monolithic column can significantly reduce the level of carry-over, thanks to the frit-free column structure and to the high flow rate that enables rapid flushing of the analytical column.

SUMMARY AND CONCLUSIONS

With high-speed columns, the total analysis time (extraction and separation) can be significantly reduced thanks to the feasibility of high-flow extraction/LC analysis. Improved chromatographic resolution is achieved through dual-column extraction at high flow rate. Matrix suppression effect can be readily assessed with the on-line extraction LC/MS/MS system. This work demonstrated that the combination of a high-flow analytical column with dual-column extraction provides an approach enabling high-throughput analysis.

Acknowledgements

We would like to thank Cohesive Technologies for their generous support with the HTLC 2300 system. The invaluable comments and suggestions of a reviewer for improving the manuscript are highly appreciated.

REFERENCES

1. White RE. *Annu. Rev. Pharmcol. Toxicol*. 2000; **40**: 133.
2. Zweigenbaum J, Henion JD. *Anal. Chem*. 2000; **72**: 2446.
3. King RC, Miller-Stein CR, Magiera DJ, Brann J. *Rapid Commun. Mass Spectrom*. 2002; **16**: 43.
4. Bruins AP, Covey TR, Henion JD. *Anal. Chem*. 1987; **59**: 2642.
5. Matuszewski BK, Constanzer ML, Chevez-Eng CM. *Anal. Chem*. 1998; **70**: 882.
6. Jemal M, Xia Y-Q. *Rapid Commun. Mass Spectrom*. 1999; **13**: 97.
7. King RC, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, Olah T. *J. Am. Soc. Mass Spectrom*. 2000; **11**: 942.
8. Matuszewski BK, Constanzer ML, Chavez-Eng CM. *Anal. Chem*. 2003; **75**: 3019.
9. Zhou S, Larson M, Weng N, Jiang X. *Proc. 49th ASMS Conf. Mass Spectrometry and Allied Topics*, Orlando, FL, June 2–6, 2002.
10. Rule G, Henion J. *J. Am. Soc Mass Spectrom*. 1999; **10**: 1322.
11. Jemal M. *Biomed. Chromatogr*. 2000; **14**: 422.
12. Plumb R, Dear G, Mallett D, Ayrton J. *Rapid Commun. Mass Spectrom*. 2001; **15**: 986.
13. Hsieh Y, Wang G, Wang Y, Chackalamannil S, Korfmacher WA. *Anal. Chem*. 2003; **75**: 1812.
14. Ayrton J, Dear GJ, Leavens WJ, Mallett DN, Plumb RS. *Rapid Commun. Mass Spectrom*. 1997; **11**: 1953.
15. Zhou S, Larson M, Weng N, Jiang X. *AAPS Annual Meeting and Exposition*, Denver, CO, October 21–25, 2001.
16. Powell ML, Jemal M. *Am. Pharm. Rev* 2001; **4**: 63.
17. Zhou S, Larson M, Jiang X, Weng N. *Proc. 49th ASMS Conf. Mass Spectrometry and Allied Topics, Orlando*, FL, June 2–6, 2002.
18. Herman JL. *Rapid Commun. Mass Spectrom*. 2002; **16**: 421.
19. Zhou S, Larson M, Jiang X, Weng N. *AAPS Annual Meeting and Exposition*, Toronto, Canada, November 10–14, 2002.
20. Herman JL. *Int. J. Mass Specctrom*. 2004; **238**: 107.
21. Herman JL. *Rapid Commun. Mass Spectrom*. 2005; **19**: 696.
22. Zhou S, Larson M, Jiang X, Weng N. *AAPS Annual Meeting and Exposition*, Denver, CO, October 21–25, 2001.
23. Bidlingmaier B, Unger KK, von Doehren N. *J. Chromatogr. A* 1999; **832**: 11.
24. Zhou W, Zhou S, Pelzer M, Mao D, Jiang X, Weng N. *Proc. 52nd ASMS Conf. Mass Spectrometry and Allied Topics*, Nashville, TN, May 31–June 4, 2004.
25. Zhou S. *Proc. 52nd ASMS Conf. Mass Spectrometry and Allied Topics*, Nashville, TN, May 31–June 4, 2004.
26. Kebarle P, Tang L. *Anal. Chem*. 1993; **65**: A972.
27. Zhou S, Oliver E, McCort-Tipton M, Jiang X, Weng N. *AAPS Annual Meeting and Exposition*, Salt Lake City, UT, October 26–30, 2003.