Matrix Effect in Bio-Analysis of Illicit Drugs with LC-MS/MS: Influence of Ionization Type, Sample Preparation, and Biofluid

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The purpose of the present work was to evaluate the synergistic effect of ionization type, sample preparation technique, and bio-fluid on the presence of matrix effect in quantitative liquid chromatography (LC)-MS/MS analysis of illicit drugs by post-column infusion experiments with morphine (10-μg/mL solution). Three bio-fluids (urine, oral fluid, and plasma) were pretreated with four sample preparation procedures [direct injection, dilution, protein precipitation, solid-phase extraction (SPE)] and analyzed by both LC-electrospray ionization (ESI)-MS/MS and LC-atmospheric pressure chemical ionization (APCI)-MS/MS. Our results indicated that both ionization types showed matrix effect, but ESI was more susceptible than APCI. Sample preparation could reduce (clean up) or magnify (pre-concentrate) matrix effect. Residual matrix components were specific to each bio-fluid and interfered at different time points in the chromatogram. We evaluated matrix effect in an early stage of method development and combined optimal ionization type and sample preparation technique for each bio-fluid. Simple dilution of urine was sufficient to allow for the analysis of the analytes of interest by LC-APCI-MS/MS. Acetonitrile protein precipitation provided both sample clean up and concentration for oral fluid analysis, while SPE was necessary for extensive clean up of plasma prior to LC-APCI-MS/MS. (J Am Soc Mass Spectrom 2003, 14, 1290–1294) © 2003 American Society for Mass Spectrometry

Over the last decade, liquid chromatography (LC) combined with mass spectrometry (MS) has become a powerful analytical tool [1–5]. A breakthrough was the introduction of two atmospheric pressure ionization (API) interfaces, electrospray ionization (ESI) [6, 7] and atmospheric pressure chemical ionization (APCI) [8]. Today, LC-MS has evolved into a technique characterized by sensitivity, selectivity, and specificity, allowing for the analysis of trace amounts of target analytes in complex mixtures. Based on these characteristics, one would expect that sample preparation prior to analysis could be minimized or even eliminated. Simplification of sample preparation would reduce time-consuming method development and sample analysis time during routine application of the method.

One limitation associated with LC-MS analysis is its susceptibility to matrix effect [9, 10]. Matrix effect is defined as the effect of co-eluting residual matrix components on the ionization of the target analyte. Typically, suppression or enhancement of analyte response is accompanied by diminished precision and accuracy of subsequent measurements [11–13]. Matrix effect thus limits the utility of LC-MS for quantitative analysis.

In an effort to reduce sample preparation time for hundreds of biological samples collected in clinical studies at NIDA, we aimed to simplify sample preparation prior to LC-MS/MS analysis. However, we recognized that the presence of residual matrix components after sample pretreatment could seriously jeopardize the precision and accuracy of the quantitative analyses. The purpose of the present work was the evaluation of matrix effects due to the combination of bio-fluid, sample preparation technique, and ionization

Published online September 18, 2003

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1044-0305/03/$30.00
doi:10.1016/S1044-0305(03)00574-9

Received April 9, 2003
Revised July 29, 2003
Accepted July 29, 2003

type. Urine and oral fluid were pretreated by four common sample preparation techniques—direct injection, dilution, protein precipitation, and solid-phase extraction (SPE)—and analyzed by LC-MS/MS. Due to the high protein content of plasma and the subsequent high "clogging" risk for the analytical column, direct injection and dilution were not evaluated for this bio-fluid. All bio-fluid extracts were analyzed by both LC-ESI-MS and LC-APCI-MS. Matrix effect was evaluated by post-column infusion of morphine during LC-MS analysis of blank pretreated bio-fluid samples. The information obtained from this study was used to pair the optimal sample preparation technique and ionization type for the quantitation of illicit drugs and opioid therapeutic medications in a given bio-fluid.

Experimental

Morphine was purchased from Cerilliant (Austin, TX). A 10-μg/mL morphine working solution was prepared by dilution with methanol/acetonitrile (50:50). All reagents were of ACS grade or higher, with solvents of HPLC grade or higher. Blank human plasma was obtained from the Johns Hopkins Bayview Medical Center blood bank (Baltimore, MD). Drug-free oral fluid and urine were obtained from healthy volunteers. Oral fluid was collected with a Salivette device with neutral cotton wool (Sarstedt, Nümbrecht, Germany). All samples were stored at −20 °C until use.

Four different sample preparation procedures were investigated: direct injection, dilution, protein precipitation and SPE. Prior to direct injection of 100 μL of bio-fluid onto the LC-MS system, 400 μL of a given sample was centrifuged at 510 × *g* for 5 min. In the case of sample dilution, 200 μL of sample was combined with 200 μL of a mixture of 10 mM aqueous ammonium formate (pH 4.5) and acetonitrile (97:3), vortexed, centrifuged (5 min at 510 × *g*), and 100 μL injected. Protein precipitation was performed by addition of 600 μL of acetonitrile to 200 μL of sample, vortexing for 30 s, and centrifugation at 15,996 × *g* for 10 min. The supernatant was evaporated to dryness under nitrogen at 45 °C. The dried sample was reconstituted in 200 μL of 10 mM aqueous ammonium formate (pH 4.5) and acetonitrile (97:3), and 50 μL injected. SPE was performed with 200-mg Clean-Screen DAU columns (United Chemical Technologies Inc., Bristol, PA). The SPE procedure for the analysis of cocaine, opiates, and metabolites in biofluids has been published previously [14]. Briefly, 1 mL of sample was combined with 3 mL 2M sodium acetate buffer (pH 4.0). After conditioning the column, the sample was applied. Subsequently, the column was washed with 2 mL of water, 1.5 mL of 0.2M hydrochloric acid, and 2 mL of methanol. The column was dried under vacuum and eluted with 4 mL of elution solvent [methylene chloride:isopropanol:ammonium hydroxide (30%) (40:10:1)]. The eluate was dried under nitrogen, reconstituted in 200 μL of 10 mM ammonium formate in water (pH 4.5) and acetonitrile (97:3), and 50 μL injected.

All LC-MS experiments were carried out on an LCQ Deca XP Ion Trap Mass Spectrometer, equipped with an orthogonal APCI or ESI source, and interfaced to a Surveyor HPLC system (ThermoFinnigan, San Jose, CA). A chromatographic method was developed using a Synergi Polar RP column (150 × 2.0 mm, 4 μm), fitted with an identically packed guard column (4 × 2.0 mm) (Phenomenex, Torrance, CA). The column oven was maintained at 25 °C. The following gradient elution with 10 mM aqueous ammonium formate, 0.001% formic acid (pH = 4.5) as "A" and acetonitrile as "B" was used at a flow rate of 300 μL/min: 0–13 min: 5% B → 26% B; 13–22 min: 26% B → 90% B; 22–24 min: 90% B → 90% B; 24–27 min: 90% B → 5% B, and 27–35 min: 5% B. All MS/MS data for morphine were collected in positive ion mode by selected reaction monitoring (SRM) of the transition *m/z* 286.3 → *m/z* 268.3, 229.1, 211.2. The optimized parameter settings for ESI were: spray voltage, 4.0 kV; sheath gas, 50; auxiliary gas, 20 (both high-purity nitrogen). The APCI parameter settings were: corona discharge needle voltage, 4.5 kV; vaporizer temperature, 450 °C; sheath gas (high-purity nitrogen), 70; and no auxiliary gas. Transfer capillary temperature and electron multiplier voltage were set at 220 °C and 850 eV, respectively, for both ESI and APCI.

Matrix effect was evaluated by the experimental technique developed by Bonfiglio et al. [15]. A blank pretreated bio-fluid sample was injected during continuous post-column infusion of a 10-μg/mL morphine solution at a flow rate of 5 μL/min. The affected area of the chromatographic run was determined by comparing the SRM chromatographic profiles for morphine obtained with an injection of the blank pretreated bio-fluid and an equivalent injection of mobile phase. Each experiment was performed in duplicate.

Results

Matrix effect was evaluated during method development of the simultaneous analysis of 25 opioids, cocaine and metabolites in three different bio-fluids. All analytes of interest eluted within 26 min with the gradient specified [16]. An example of the SRM profiles of morphine obtained during LC-ESI-MS/MS analysis of oral fluid pretreated by acetonitrile protein precipitation (Figure 1b) is compared to that obtained by injection of an equal volume of mobile phase (Figure 1a). The differences in the RICs illustrate signal suppression of 70% from 1–2 min, 15% from 2–8 min, and 50% from 17.5–22 min. Conversely, the same experiment using APCI showed no signal suppression (Figure 2).

Table 1 provides an overview of the results obtained with the continuous post-column infusion experiments. The different time intervals of the chromatographic analysis affected by matrix effect, as well as, the extent of the effect (%) are documented for each combination of bio-fluid, sample preparation technique, and ioniza-

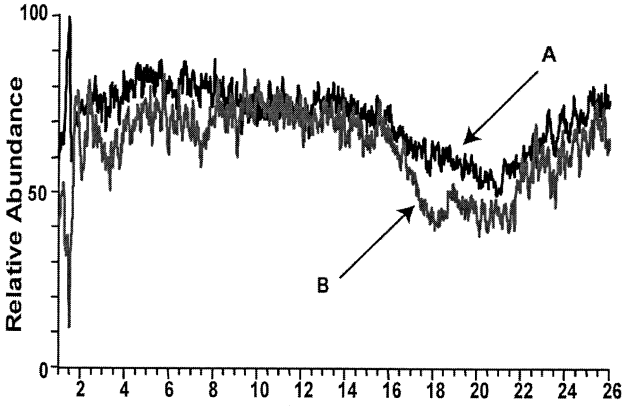

Figure 1. Post-column infusion chromatogram of morphine for LC-ESI-MS/MS analysis of (**A**) blank mobile phase injection and (**B**) oral fluid pretreated by acetonitrile protein precipitation.

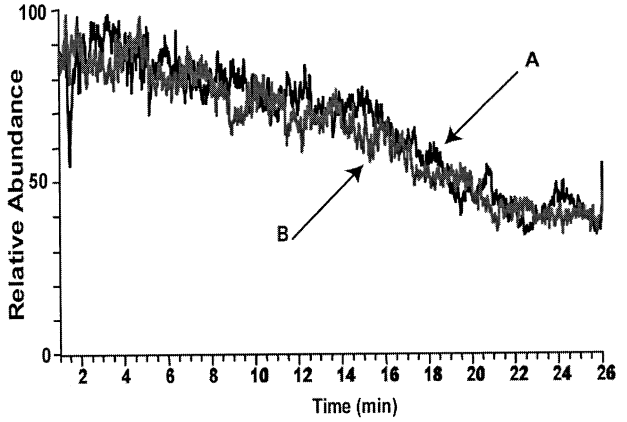

Figure 2. Post-column infusion chromatogram of morphine for LC-APCI-MS/MS analysis of (**A**) blank mobile phase injection and (**B**) oral fluid pretreated by acetonitrile protein precipitation.

tion type. For reporting purposes, matrix effect was expressed as the percentage of suppression or enhancement. The authors are fully aware of the variable nature of matrix effect. Therefore, the percent matrix effect is a relative indicator of the degree of suppression or enhancement.

Discussion

Post-column infusion of morphine provided rapid visualization of the effect of interfering species on the ionization of the target analyte, indicating areas of the LC gradient affected by matrix effect. Although morphine had a retention time of 7.6 min with the stated HPLC gradient, it was considered a good indicator of ion suppression or enhancement across the entire chromatographic run [19]. Post-column infusion of morphine allowed us to assess the efficiency with which four common sample preparation techniques removed matrix components from three bio-fluids and to study the effect of residual matrix components on the ionization of the target . Due to the analyte dependent nature of matrix effect, caution needs to be taken when correlating these results to other analytes of interest.

Matrix effect was observed with both ionization types, but was more prevalent with ESI than APCI. ESI was clearly affected by components with a wide polarity range, making this type of ionization highly susceptible to matrix effect. APCI proved less susceptible to matrix effect than ESI [11, 17, 18], especially in the presence of hydrophobic interferences. Suppression was only observed in the beginning of the chromatograms for all three matrices. This is likely due to the

Table 1. Results of post-column infusion experiments, showing time (minutes) and extent (−%: suppression; +%: enhancement) of the observed matrix effects

	Electrospray Ionization		
	Urine	Oral fluid	Plasma[b]
Direct injection	1–2 min: −90% 8–9.5 min: −20%	1–2 min: −70%	
Dilution	1–2 min: −60–95%	ND[a]	
Protein precipitation	1–2 min: −85% 2–8 min: −15% 18–18.5 min: −20%	1–2 min: −70% 17.5–22 min: −50%	1–2.5 min: −75% 20–22 min: −65% 22.5–23 min: +150%
Solid-phase Extraction	1–2 min: −40% 8 min: −30%	1–2 min: −10% 12–20 min: −10–15%	1–2 min: −35% 20–22 min: −50%
	Atmospheric pressure chemical ionization		
	Urine	Oral fluid	Plasma[b]
Direct injection	1–2.5 min: −97.5% 6–7 min: −50%	ND[a]	
Dilution	1–2 min: −70%	ND[a]	
Protein precipitation	1–2 min: −99% 5.5 min: −40% 6–6.5 min: −50%	ND[a]	1–2.5 min: −60%
Solid-phase extraction	1–3 min: −20%	1–3 min: −20%	ND[a]

[a]Matrix effect not detected.
[b]direct injection and dilution were not evaluated for plasma.

different ionization process, with APCI based on gas-phase reactions and ESI mainly on liquid-phase reactions [10, 19].

Large differences in matrix effect were also observed between sample preparation techniques. In general, acetonitrile protein precipitation in combination with LC-ESI-MS/MS had the greatest matrix effect, with major suppression areas in the beginning and end of the chromatographic separation. Due to the non-selective nature of protein precipitation as a sample pretreatment, clean up of the bio-fluid is minimal. The combination of large amounts of residual matrix components and ESI clearly demonstrated matrix effects. These findings agree with results previously reported in the literature [15].

SPE, often proposed as a solution for matrix effect, also had suppression when used in conjunction with LC-ESI-MS/MS. Although matrix clean up is more extensive with SPE, the pre-concentration step increased the concentration of target analytes but also increased the concentration of interfering substances from the bio-fluid and/or sample preparation. We concluded that the SPE pre-concentration step magnified matrix effect. Additionally, we noted that direct injection and dilution, two sample preparation methods without a pre-concentration step, had much less suppression in LC-ESI-MS/MS. However, the drawback of omitting a pre-concentration step from the sample preparation procedures is a loss in sensitivity and an increase in limits of detection.

The presence of matrix effect also proved to be dependent on the bio-fluid analyzed. Matrix components, characteristic to each bio-fluid, interfered at different times and to a varying extent throughout the analysis. The major interferences in urine proved to be hydrophilic residual components, most likely inorganic salts. Oral fluid had more interferences than urine, mainly in ESI, and residual matrix components were of a hydrophilic and hydrophobic nature, including proteins, amino acids, and especially mucin. Finally, for plasma, residual matrix components also had a wide polarity range, but their concentrations were apparently higher than those of oral fluid.

Conclusions

Our results indicated that matrix effect was dependent on ionization type, sample preparation, and bio-fluid type. When matrix effect resulted in severe ion suppression or enhancement of the target analyte by co-eluting residual components, it was located in isolated regions of the chromatogram. Although matrix effect was observed for both ionization types, ESI was especially susceptible, while APCI proved to be less vulnerable. Sample preparation had a clear influence on matrix effect. Although a more extensive clean up generally yielded cleaner extracts, pre-concentration of matrix components during sample preparation magnified matrix effect. However, pre-concentration is often necessary to obtain adequate sensitivity. Each bio-fluid was characterized by endogenous matrix components that caused different matrix effects.

In conclusion, LC-APCI-MS/MS is the ionization of choice for quantitative analysis of multiple illicit and therapeutic drugs. APCI allowed for simplification of sample preparation prior to analysis without jeopardizing the quality of quantitative data. The selection of sample pretreatment for each bio-fluid was dependent upon expected analyte concentrations and required detection limits. For urine, the expected high concentration of metabolites allowed for a simple dilution of sample prior to analysis. In the case of oral fluid, expected analyte concentrations were much lower and pre-concentration was needed. Acetonitrile protein precipitation provided sufficient pre-concentration and protein removal for quantitative analysis of the analytes of interest in oral fluid. Finally, matrix suppression data indicated that solid-phase extraction was required prior to LC-APCI-MS/MS analysis of the analytes of interest in plasma.

Acknowledgments

RD gratefully acknowledges the travel grant V 4.037.01N from the F.S.R.-Flanders (Belgium). This research was supported by NIDA Intramural Research Program funds.

References

1. Krstulovic, A. M.; Lee, C. R.; Firmin, S.; Jacquet, G.; Van Dau, C. N.; Tessier, D. Applications of LC-MS Methodology in the Development of Pharmaceuticals. *Lc Gc Europe* **2002,** *15,* 31.
2. Simal-Gandara, J.; Damant, A. P.; Castle, L. The Use of LC-MS in Studies of Migration from Food Contact Materials: A Review of Present Applications and Future Possibilities. *Crit. Rev. Anal. Chem.* **2002,** *32,* 47–78.
3. Marquet, P. Progress of Liquid Chromatography-Mass Spectrometry in Clinical and Forensic Toxicology. *Therapeut. Drug Monit.* **2002,** *24,* 255–276.
4. Thomas, R. Recent developments in LC-MS-MS for the Identification and Measurement of Nanoscale Amounts of Proteins and Peptides. *Spectroscopy* **2001,** *16,* 28.
5. Hogendoorn, E.; van Zoonen, P. Recent and Future Developments of Liquid Chromatography in Pesticide Trace Analysis. *J. Chromatogr. A* **2000,** *892,* 435–453.
6. Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. Electrospray Ionization—Principles and Practice. *Mass Spectrom. Rev.* **1990,** *9,* 37–70.
7. Bruins, A. P. Mechanistic Aspects of Electrospray Ionization. *J. Chromatogr. A* **1998,** *794,* 345–357.
8. Thomson, B. A.; Danylewychmay, L.; Henion, J. D. LC MS MS with an Atmospheric-Pressure Chemical Ionization Source. *Am. Chem. Soc.* **1983,** *186,* 19 (abstracts).
9. Buhrman, D. L.; Price, P. I.; Rudewicz, P. J. Quantitation of SR 27417 in Human Plasma Using Electrospray Lquid Chromatography Tandem Mass Spectrometry: A Study of Ion Suppression. *J. Am. Soc. Mass Spectrom.* **1996,** *7,* 1099–1105.
10. King, R.; Bonfiglio, R.; Fernandez-Metzler, C.; Miller-Stein, C.; Olah, T. Mechanistic Investigation of Ionization Suppression in Electrospray Ionization. *J. Am. Soc. Mass Spectrom.* **2000,** *11,* 942–950.

11. Fu, I.; Woolf, E. J.; Matuszewski, B. K. Effect of the Sample Matrix on the Determination of Indinavir in Human Urine by HPLC with Turbo Ion Spray Tandem Mass Spectrometric Detection. *J. Pharmaceut. Biomed. Anal.* **1998,** *18,* 347–357.
12. King, R.; Barrish, A.; Bonfiglio, R.; McLoughlin, D.; Merkle, K.; Miller-Stein, C.; Olah, T. *Proceedings of the 46th ASMS Conference on Mass Spectrometry and Allied Topics*; Orlando, FL, 1998.
13. Mei, H.; Hsieh, Y.; Nardo, C.; Xu, X.; Wang, S.; Ng, N.; Korfmacher, W. A. Investigation of Matrix Effects in Bioanalytical High-Performance Liquid Chromatography/Tandem Mass Spectrometric Assays: Application to Drug Discovery. *Rapid Commun. Mass Spectrom.* **2003,** *17,* 97–103.
14. Cone, E. J.; Hillsgrove, M.; Darwin, W. D. Simultaneous Measurement of Cocaine, Cocaethylene, Their Metabolites, and Crack Pyrolysis Products by Gas-Chromatography Mass-Spectrometry. *Clin. Chem.* **1994,** *40,* 1299–1305.
15. Bonfiglio, R.; King, R. C.; Olah, T. V.; Merkle, K. The Effects of Sample Preparation Methods on the Variability of the Electrospray Ionization Response for Model Drug Compounds. *Rapid Commun. Mass Spectrom.* **1999,** *13,* 1175–1185.
16. Dams, R.; Murphy, C. M.; Choo, R. E.; Lambert, W. E.; De Leenheer, A. P.; Huestis, M. A. LC-APCI-MS/MS Analysis of Multiple Illicit Drugs, Methadone, and Their Metabolites in Oral Fluid Following Protein Precipitation. *Anal. Chem.* **2003,** *75,* 798–804.
17. Smeraglia, J.; Baldrey, S. F.; Watson, D. Matrix Effects and Selectivity Issues in LC-MS-MS. *Chromatographia* **2002 ,** *55,* S95–S99.
18. Hsieh, Y. S.; Chintala, M.; Mei, H.; Agans, J.; Brisson, J. M.; Ng, K.; Korfmacher, W. A. Quantitative Screening and Matrix Effect Studies of Drug Discovery Compounds in Monkey Plasma Using Fast-Gradient Liquid Chromatography/Tandem Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2001,** *15,* 2481–2487.
19. Kebarle, P.; Tang, L. From Ions in Solution to Ions in the Gas-Phase—the Mechanism of Electrospray Mass-Spectrometry. *Anal. Chem.* **1993,** *65,* A972–A986.