pH and Ion-suppression in Reversed Phase LC/MS

Liming Peng and Tivadar Farkas
Phenomenex, Inc., 411 Madrid Ave.,
Torrance, CA 90501 USA

Introduction

LC/MS/MS has become the most powerful analytical technique in the pharmaceutical industry for the fast analysis of combinatorial libraries, and in DMPK studies, due to its high sensitivity, selectivity, and robustness. While sample preparation for analysis is continuously simplified, and ultra-fast chromatographic separations using short columns are utilized for the quantitative analysis of drugs and their metabolites in complex biological matrices, LC/MS/MS matrix effects have become evident and of great concern for their negative impact on the reproducibility and accuracy of LC/MS/MS assays. To ensure that such effects are minimal, their assessment has become standard practice as part of LC/MS/MS method development and validation.

Signal suppression/enhancement effects have been investigated for several common sample preparation procedures such as liquid-liquid extraction (LLE), protein precipitation (PP), and solid phase extraction (SPE), with chromatographic separations conducted in acidic, neutral or basic mobile phases[1-11]. Recent developments in HPLC column technology allow for the analysis of basic drugs in their uncharged state, in high pH mobile phases, with increased retention, improved peak shapes and ESI$^+$ MS responses[12]. As the elution order of sample components is often different on the same LC column in low and high pH mobile phases, the elution pattern of matrix components co-extracted with the analytes of interest may be different too, thus suggesting the need to investigate matrix effects as a function of pH.

We report on the evaluation of ion suppression/enhancement effects in the analysis of polar and non-polar basic drugs and metabolites by ESI$^+$ LC/MS/MS in low and high pH mobile phases, in biological samples prepared by PP or SPE.

Table 1. Analyte Characteristics

Compounds	MW	MS/MS Transition	pK_a	LogP
Codeine	299.37	300.2 → 152.2, 300.2 → 198.9	8.21	1.19
Hydrocodone	299.37	300.2 → 152.2, 300.2 → 198.9	8.90	2.16
Norcodeine	285.34	286.1 → 152.2, 286.1 → 185.2	9.23	0.69
Morphine-3ß-D-glucuronide (M3G)	461.47	462.1 → 286.2		<< 0.69
Morphine	285.30	286.1 → 152.2, 286.1 → 185.2	8.21	0.89
Meperidine	247.34	248.3 → 220.2	8.59	2.72
Normeperidine	233.31	234.2 → 160.3	–	–
Fluoxetine	309.33	310.0 → 44.0	8.70	4.50
Norfluoxetine	295.26	296.1 → 134.2	–	–

Molecular Structures of Basic Drugs and Metabolites
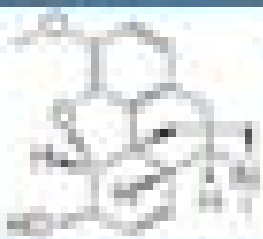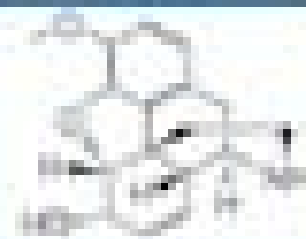
Sample Preparation

Biological Samples

Rat serum (purchased from SeraCare Life Sciences, Inc.)

Sample Preparation by Protein Precipitation

400 µL of rat serum + 1.6 mL acetonitrile; vortexed, then centrifuged for 5 min at 10,000 RPM; transferred supernatant into glass tubes; dried the supernatant under gentle nitrogen flow at 45 ºC; residue re-constituted in 400 µL standard solution or HPLC grade water.

Protein Precipitation SPE

Sample: 400 µL of rat serum + 100 µL of 1 M acetic acid + 500 µL DI water

SPE Sorbent: strata™-X-C (33 µm, 85 Å) 30 mg/ 1 mL tube

Condition: 1 mL methanol followed by 1 mL water

Wash:
1. 1 mL water
2. 1 mL of 0.1 M acetic acid
3. 1 mL methanol

Elute: 1 mL 5 % ammonium hydroxide (28 %) in methanol; evaporate eluate to dryness under gentle nitrogen flow

Reconstitute: 400 µL standard solution or HPLC grade water

Experimental Conditions

Instrumentation

HPLC System: Hewlett-Packard® 1100 series
(*www.agilent.com*)

Pump: G1312A (Binary Pump)

Autosampler: G1329A ALS

MS Detector: API 3000™ LC/MS/MS with
ESI (TurboIonSpray®)
(*www. Appliedbiosystems.com*)

MS Detection

(TurboIonSpray®- ESI, Positive Ion Mode; MRM)
Heater Gas Flow: 7000 cc/min
Heater Temperature: 425 °C

HPLC Conditions

Column: Gemini® 3 µm C18,
50 x 2.0 mm

Flow Rate: 0.5 mL/min (HPLC pump);
40 µL/min (Post-Column Infusion)

Injection Volume: 10 µL

Mobile Phase

- Low pH Mobile Phase:
  A: 0.1 % Formic Acid in Water
  B: 0.1 % Formic Acid in Acetonitrile
- High pH Mobile Phase:
  A: 10 mM NH_4HCO_3, pH 10.0:Acetonitrile (95:5)
 B: 2 mM NH_4HCO_3, pH 10.0 in Acetonitrile: Water (95:5)

Gradient Program

- Low pH Mobile Phase:
  5 to 95 % B in 2 min, hold for 0.5 min; re-equilibrate for 2.5 min
- High pH Mobile Phase:
  5 to 95 % B in 2 min, hold for 1.0 min; re-equilibrate for 2.0 min

Standard Mixture (In Aqueous Solution)

Opiates and Metabolites **125 ng/mL**
Meperidine and Normeperidine **50 ng/mL**
Fluoxetine and Norfluoxetine **50 ng/mL**

Figure 1. LC/MS/MS Response of Basic Compounds in Acid and pH 10 Mobile Phases
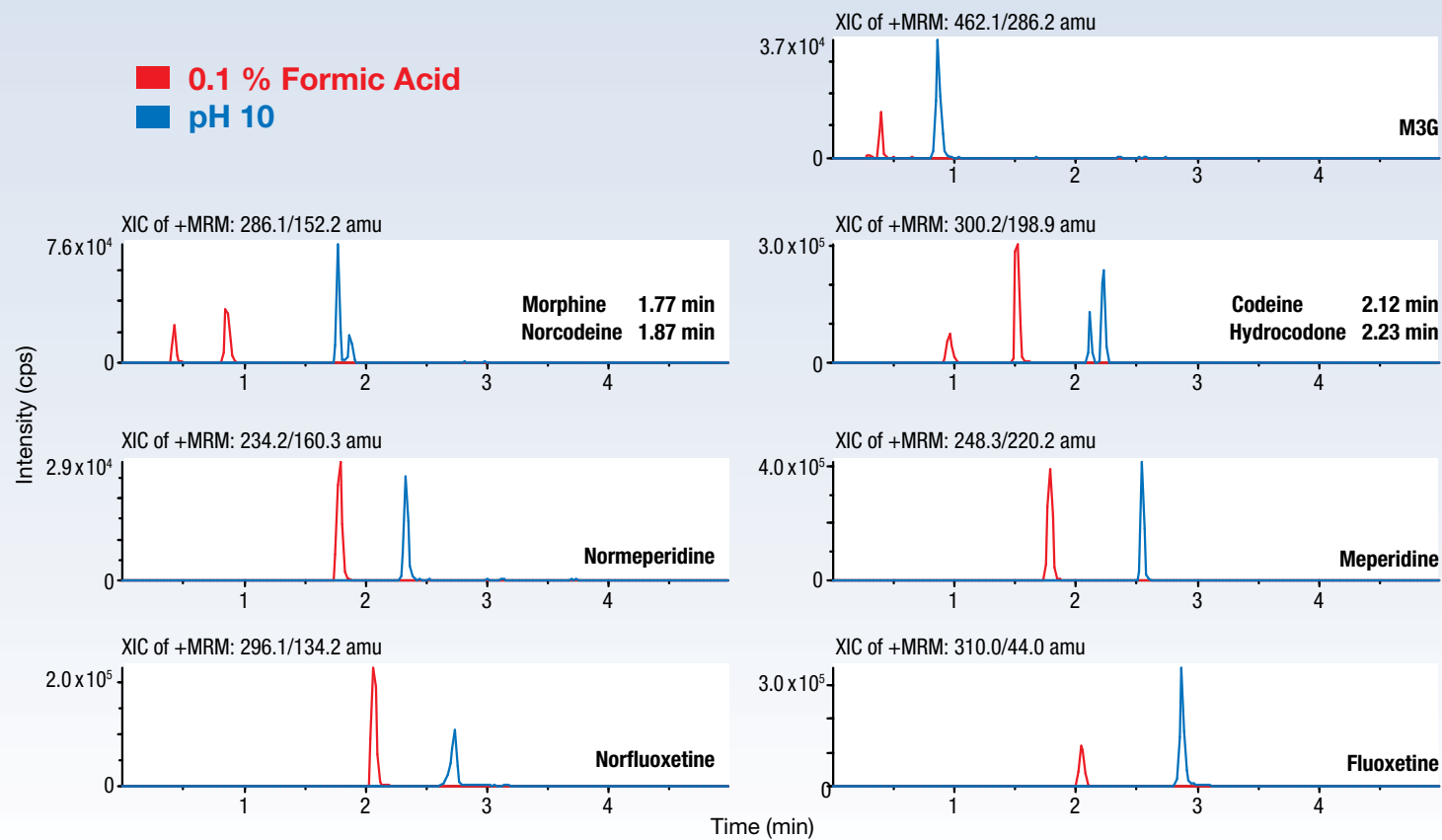
Table 2. Results of Quantitative Matrix Effects

| Compounds	ME in Rat Serum (± %)*			
	SPE*		PP*	
	pH 10	Formic Acid	pH 10	Formic Acid
Codeine	2.31	0.45	23.18	-18.62
Hydrocodone	2.94	-15.94	9.27	-20.61
Norcodeine	-15.56	-1.63	-4.47	-29.12
Morphine-3ß-D-glucuronide (M3G)	-3.88	-45.07	-7.69	-76.63
Morphine	-35.57	-63.75	-3.54	-80.70
Meperidine	-1.14	-49.67	22.26	-46.25
Normeperidine	-5.60	-37.48	77.41	-27.70
Fluoxetine	-56.09	-58.44	-74.75	- 9.58
Norfluoxetine	-39.92	-54.83	41.75	10.91

ME (%)= 100 x B/A - 100; ME > + 15 % - Ion Enhancement; **ME < - 15 %** - Ion Suppression; B - Response in Spiked Biological Matrix; A - Response in Standard Solution; ***SPE:** Solid Phase Extraction; ***PP:** Protein Precipitation
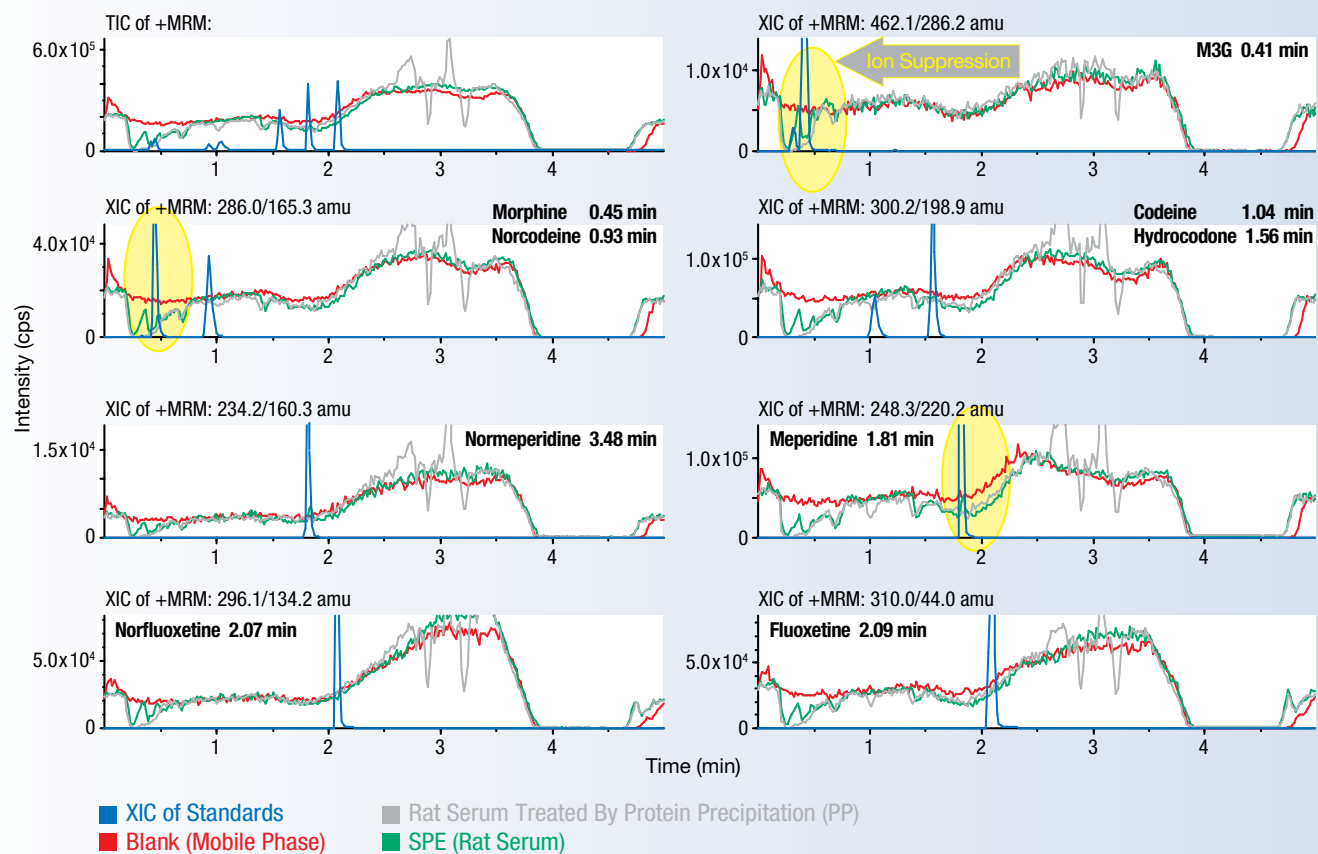
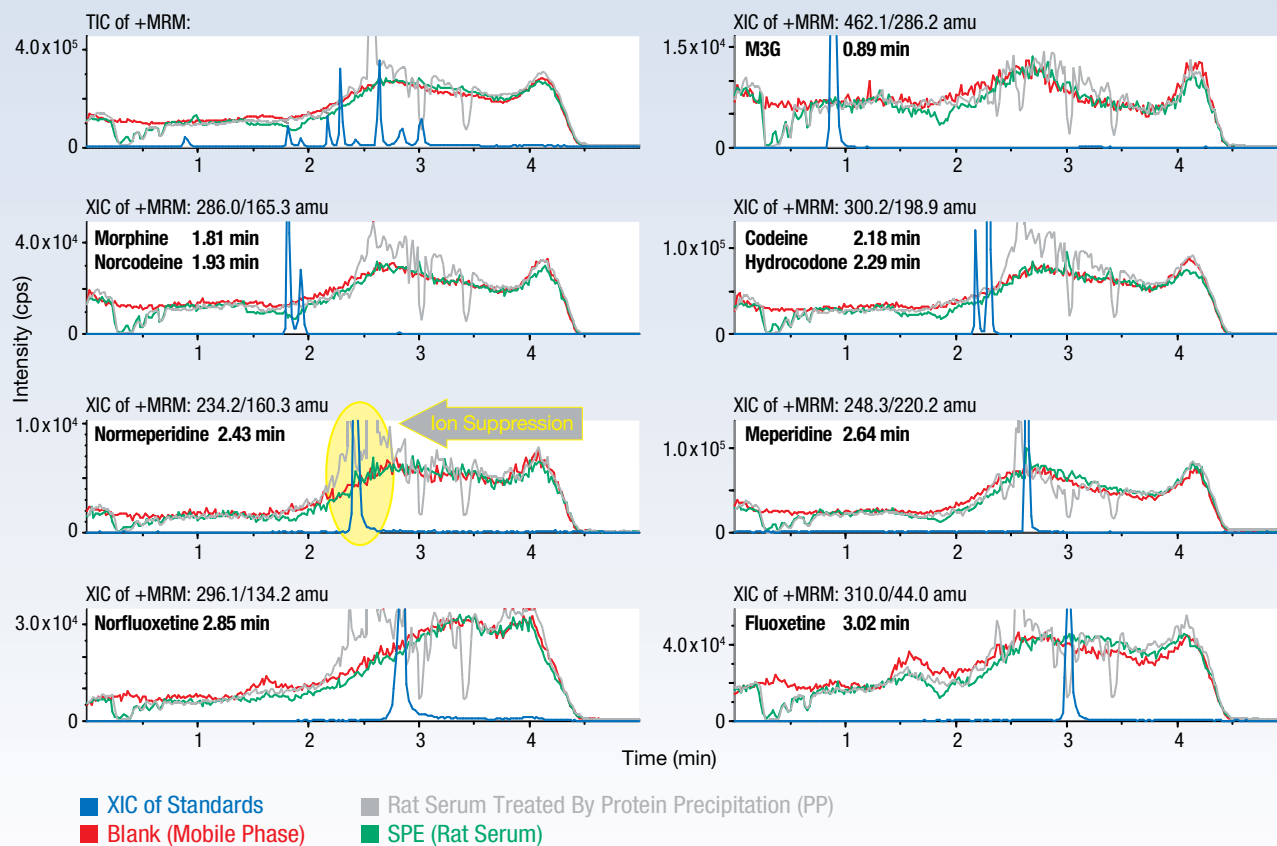**Figure 2.** Comparisons of LC/MS/MS Responses in Biological Matrices in pH 10 Mobile Phase
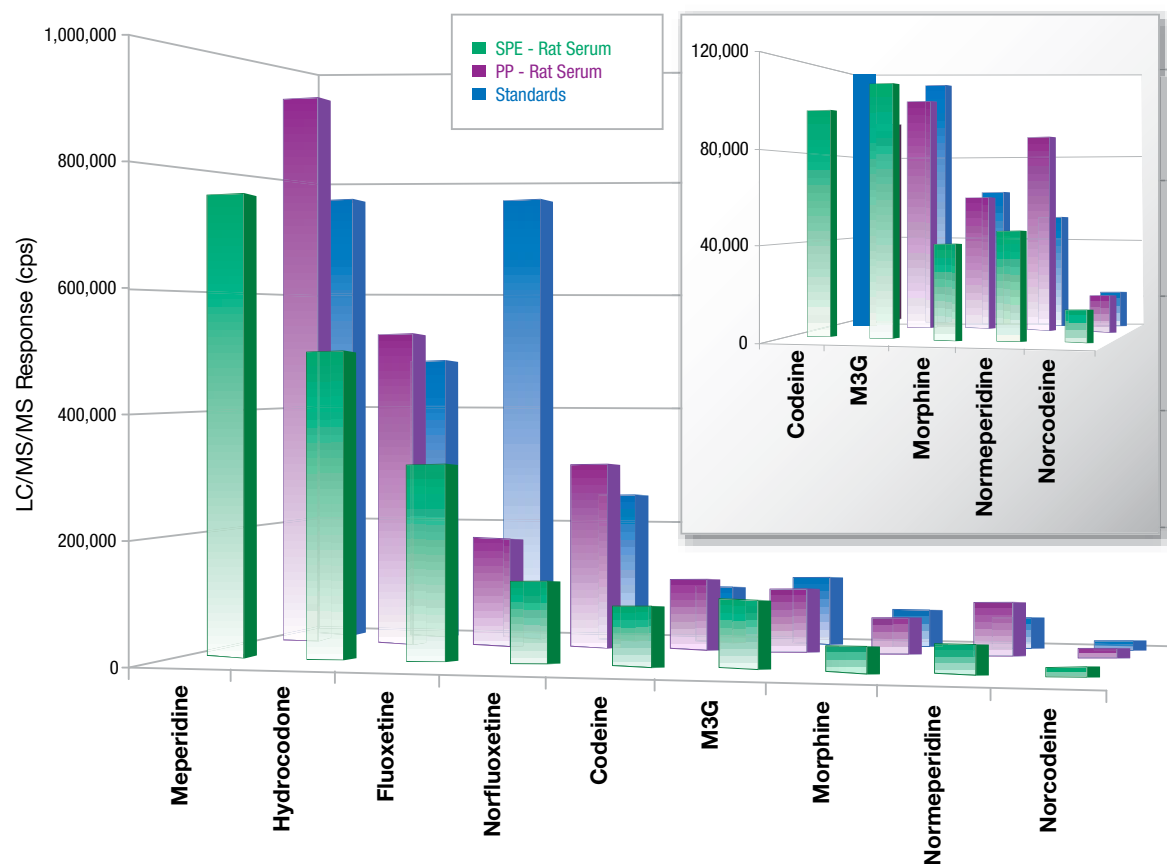
Figure 3. Comparisons of LC/MS/MS Responses in Biological Matrices in Acidic Mobile Phase
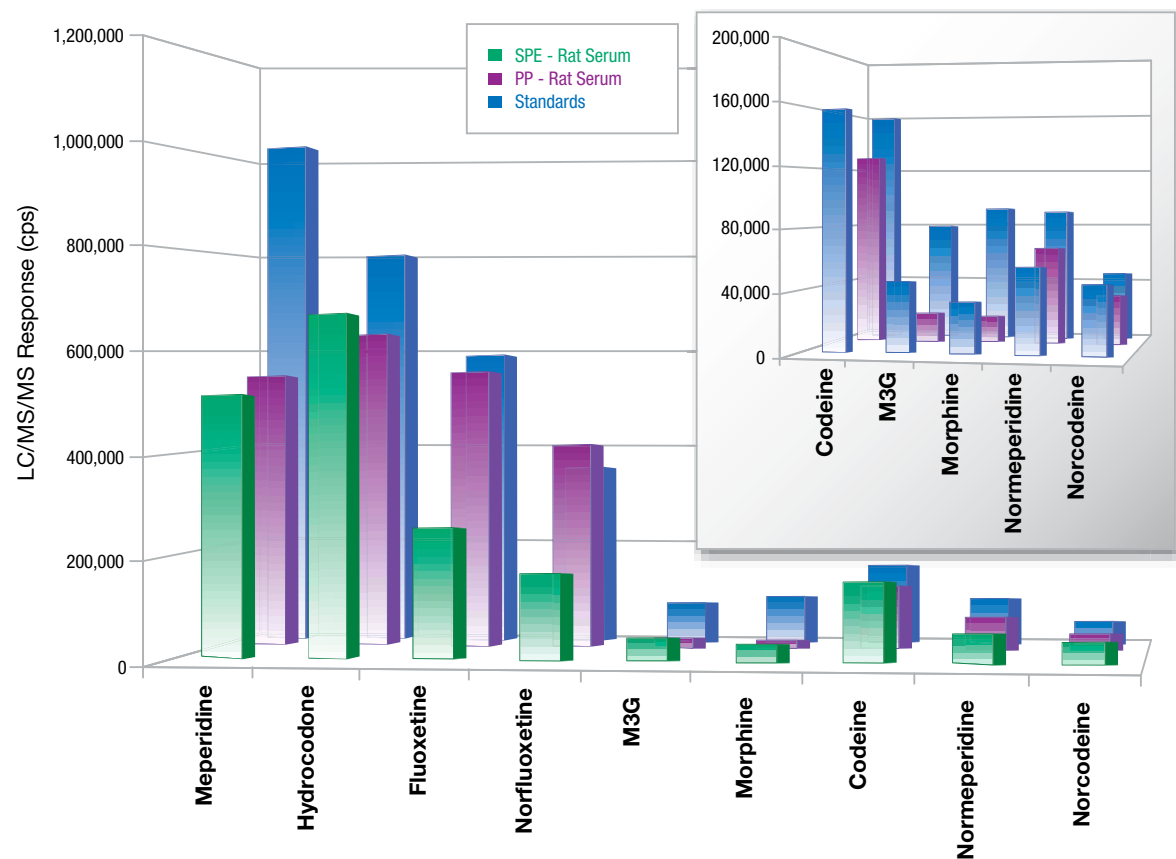
Figure 4. Overlaid EIC for Each Analyte in Mobile Phase Containing 0.1 % Formic Acid in Rat Serum

Figure 5. Overlaid EIC for Each Analyte in Basic Mobile Phase (pH 10) in Rat Serum

Results and Discussion

LC/MS/MS Method and Test Probes

- Chromatographic separations were performed on a Gemini 3 μm C18 50 x 2.0 mm column at a flow rate of 0.5 mL/min, with 0.1 % formic acid (pH 2.7), or 10 mM ammonium bicarbonate (pH 10) aqueous mobile phase, and with acetonitrile as organic modifier, in fast gradient elution mode (2 min linear gradient; 5 min cycle time).

- A wide variety of basic drugs and their metabolites (**Table 1**) were selected as probes for the systematic investigation of ion suppression/enhancement effects across the chromatographic gradient elution.

- Signal intensities for M3G, morphine, fluoxetine, norcodeine and norfluoxetine, in ESI$^+$ LC/MS/MS are varied at different mobile phase pH, while for other basic drugs and metabolites were comparable. In general, the elution of basic compounds, both polar and non-polar, in their uncharged state results in significantly longer retention times, and sharper peak shapes (**Figure 1**).

Biological Matrices and Sample Preparation

- Matrix effects in ESI$^+$ LC/MS/MS were monitored and compared as a function of mobile phase pH (acidic and basic; **Figures 2** and **3**), and sample preparation method (PP and SPE; **Figures 4-5**) for biological matrices relevant to DMPK studies: such as rat serum.

- The signal intensities of basic compounds in samples prepared by SPE were less affected in high pH mobile phase than in acidic mobile phase (except for fluoxetine, LogP 4.5). In general, signal intensities were more affected in samples prepared by PP than SPE, regardless of mobile phase pH (**Figures 2** and **3**, and **Table 2**).

ESI$^+$ LC/MS/MS Traces for Post-Column Infusion Profiles

- The profiles of post-column infusion were collected by injecting bio-matrices pretreated by SPE or PP when continuously infused post-column to a standard mixture solution, at a flow rate of 40 μL/min.

- Areas of ion suppression and enhancement were detected as valleys or hills in the elution profile, compared to a blank (collected by injecting mobile phase while a standard mixture was continuously infused).

- The results show that matrix effects manifested themselves in different time intervals of the chromatographic elution as a function of mobile phase pH. Ion suppression of ESI$^+$ LC/MS/MS signals were observed around the column void time in all cases (**Figures 4-5**). Protein precipitation displays a more significant matrix effect than solid phase extraction in both mobile phases at the end of gradient elution.

- This indicates that matrix effects can be minimized by a judicious selection of the sample preparation method and mobile phase pH. Applying more efficient sample cleanup can eliminate most endogenous components and prevent their negative impact on analyte signal; by varying mobile phase pH, the selectivity of the column can be manipulated resulting in changes in the elution order of basic analytes and matrix components, and also in increased retention for polar compounds which can be eluted away from the early ion suppression region (**Figures 4-5**).

Quantitative Ion Suppression and Enhancement

- Matrix effects on quantitative analysis by ESI$^+$ LC/MS/MS were quantitatively evaluated by comparing analyte responses (peak area) in biological matrices spiked after sample preparation to analyte response in standard solution. Each experiment was performed in triplicate, responses were counted as average peak area. The extent of matrix effect was expressed as a percent change in signal, as calculated based on the equation shown in **Table 2**; matrix effects (%) were documented for rat serum, sample preparation modes, and mobile phases (**Table 2**).

- Matrix effects > ±15 % were considered to have a significant effect on signal intensity. The qualitative results shown in **Table 2** are in agreement with the observations from post-column infusion experiments.

Conclusions

- Matrix effects in the analysis of polar and non-polar basic compounds and metabolites by ESI[+] LC/MS/MS in Bio-matrices – rat serum prepared for analysis by PP and SPE were compared in acidic and high pH mobile phases.

- The matrix affected analytes and metabolites to different degrees. The detection of basic compounds, especially polar bases, was more prone to matrix interferences when analysis was performed in acidic mobile phase.

- The matrix effects varied depending on the type of sample pretreatment. Protein precipitation was less efficient than solid phase extraction in removing interfering matrix components, hence allowing for more pronounced suppression of analyte response in ESI[+] LC/MS/MS.

- Matrix effects can be minimized by choosing optimized conditions for both sample pretreatment, and the chromatographic separation:

    (1) improve the sample cleanup procedure to eliminate matrix interferences (SPE)

    (2) perform the assay under the more efficient chromatographic conditions to separate analytes of interest from endogenous compounds not removed during sample preparation that may affect analyte ionization efficiency (optimize mobile phase pH).

References

1. B. K. Matuszewski, M. L. Constanzer, and C. M. Chavez-Eng *Anal. Chem.* **1998**, 70, 882-889

2. David Temesi and Brian Law *LC-GC* **July 1999**, 17, 626-632

3. B. K. Matuszewski, M. L. Constanzer, and C. M. Chavez-Eng *Anal. Chem.* **2003**, 75, 3019-3030

4. Philip R. Tiller, and Leslie A. Romanyshyn, *Rapid Commun. Mass Spectrom*. **2002**; 16, 92-98

5. Hong Mei, Yunsheng Hsieh, Cymbylene Nardo, Xiaoying Xu et. al. *Rapid Commun. Mass Spectrom*. **2003**; 17, 97-103

6. Michael J. Avery, *Rapid Commun. Mass Spectrom*. **2003**; 17, 197-201

7. Richard King, Ryan Bonfiglio, Carmen Fernandez-Metzler et. al. *J Am Soc Mass Spectrom* **2000**, 11, 942-950

8. Claudia Müller, Patrick Schäfer, Mylène Störtzel et. al. *J. of Chromatography B* **2002**, 773, 47-52

9. C. Mallet, Z. Lu and J. Mazzeo, *Rapid Commun. Mass Spectrom*. 18 (**2004**) 49-58

10. Erin Chambers, Diane M, Diehl and Jeffrey R. Mazzeo AAPS Poster **2005**

11. Kimberly L. Norwood, Edward G. Green, Richard S. Hucker, et. al. ASMS Poster **2006**

12. Liming Peng, Tivadar Farkas, Ismail Rustamov and Lawrence Loo ASMS Poster **2006**