Improved Results for LC/MS of Basic Compounds Using High pH Mobile Phase on a Gemini® C18 Column

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Introduction

There is considerable interest in conducting reversed phase HPLC separations at high pH, well above the pK_a values of basic compounds [1-3]. When the pH of the mobile phase is two pH units higher than the pK_a values of basic analytes, the uncharged species are better retained on reversed phase stationary phases. The results: increased retention times of polar basic analytes without using ion-pairing reagents, more reproducible retention, superior peak shapes, alternative column selectivity [4], and elution in a mobile phase having a higher organic content, which is beneficial for LC/MS detection.

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One possible drawback to using high pH could be a severe decrease in sensitivity of mass-spectrometric detection under conditions that suppress analyte ionization in solution. As the ionization state of analyte molecules depends on the pH of the mobile phase, it is expected that the ionization efficiency in LC/MS with electrospray (ESI) in positive ion mode (PIM) will be drastically lowered in high pH mobile phases. It is common practice to employ volatile weak acids for enhancing the ionization of basic compounds in ESI^+. Thus, the LC/MS analysis of basic drug compounds in mobile phases of high pH (>pK_a+2) could be compromised if gas-phase ionization yields in ESI^+ were closely linked to acid-base equilibria in solution. Nevertheless, several workers have reported the successful detection of particular basic compounds in ESI^+ when using high pH buffers in the mobile phase [5-11].

In our previous work [5], we reported the successful detection of several important classes of basic drugs – antihistamines, beta-blockers, opiates, tricyclic anti-depressants – under mobile phase conditions which suppress their ionization in solution. We compared the ESI^+ responses of various groups of basic drugs within a wide range of polarity (log P 0.09 ~7.6) and pK_a values (8 ~10), in low and high pH mobile phases. Analyte signal intensities observed in 0.1 % formic acid with acetonitrile were compared to intensities observed in 10 mM ammonium bicarbonate buffers at different pH (7.8-11) with acetonitrile. Contrary to common expectations, high pH mobile phases do NOT suppress the ionization of basic compounds in ESI^+; positive ions are formed abundantly, and analyte responses are often better in high pH compared to acidic mobile phases. The results show that the use of high pH mobile phases for the analysis of basic compounds offers a good alternative to the current practice of using low pH mobile phases in ESI^+ LC/MS. This finding is significant as it extends the applicability of generic elution methods to the analysis of polar basic compounds previously difficult to retain by reversed phase chromatography without compromising the ability to detect them by mass spectrometry.

We investigated the effectiveness of using high pH mobile phases for the quantitation of basic compounds in ESI^+ LC/MS/MS by comparing limits of detection (LOD), limits of quantitation (LOQ), linearity ranges, precision and accuracy observed in high and low pH mobile phases.

Table 1: Analyte Characteristics

Compound	Transition	pK_a, pK_{a2}	logP	Compound	Transition	pK_a, pK_{a2}	logP
Diltiazem	415→178	8.91	2.70	Terfenadine	472→436	9.57	7.62
Lidocaine	235→86	8.01	2.44	Carbamazepine	237→194	----	2.45
Atropine	290→124	9.43	1.83	Tetracaine	265→176	8.2	3.51
Diphen-hydramine	256→167	8.98	3.27	Procainamide	236→163	9.32	0.88
Haloperidol	376→165	8.66	4.3	Amiloride	230→171	8.7, 9.30	0.09
Acebutolol	337→116	9.20	1.71	Verapamil	456→165	8.92	3.79
Cimetidine	253→159	6.8	0.4	Trimethoprim	291→230	7.12	0.91

Experimental Conditions

Instrumentation

HPLC System: HP 1100 series (www.agilent.com)
Pump: G1312A (Binary Pump)
Autosampler: G1329A ALS
MS Detector: API 3000 LC/MS/MS (www.appliedbiosystems.com), with ESI (TurboIonSpray®) operated in PIM

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 Ammonium Bicarbonate pH=7.8, 9.0, 10.0, or 11.0; B: Acetonitrile

Gradient Program:
10 % B to 90 % B in 10 min, hold for 2 min; re-equilibrate for 4 min

Flow Rate: 0.6 mL/min
Column: Gemini 5 µm C18, 150 x 3.0 mm
Part No.: 00F-4435-Y0
Concentration Levels: 0.05-200 ng/mL
Injection Volume: 5 µL

Figure 1. LC/MS/MS Responses for Basic Compounds in pH=2.7 and pH=10.0 Mobile Phase

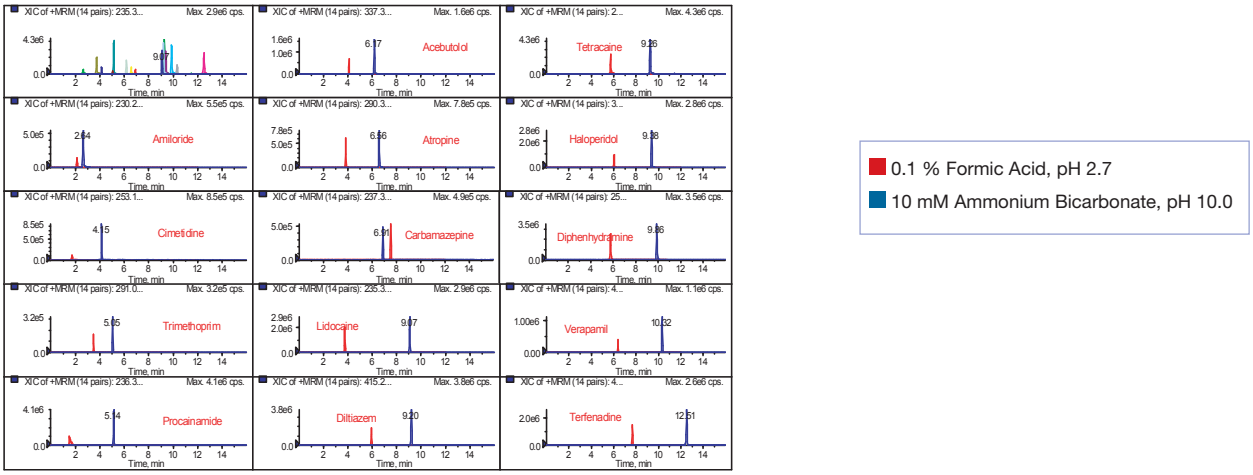


Figure 2. Comparison of LC/MS/MS Responses in Acidic and Basic Mobile Phases

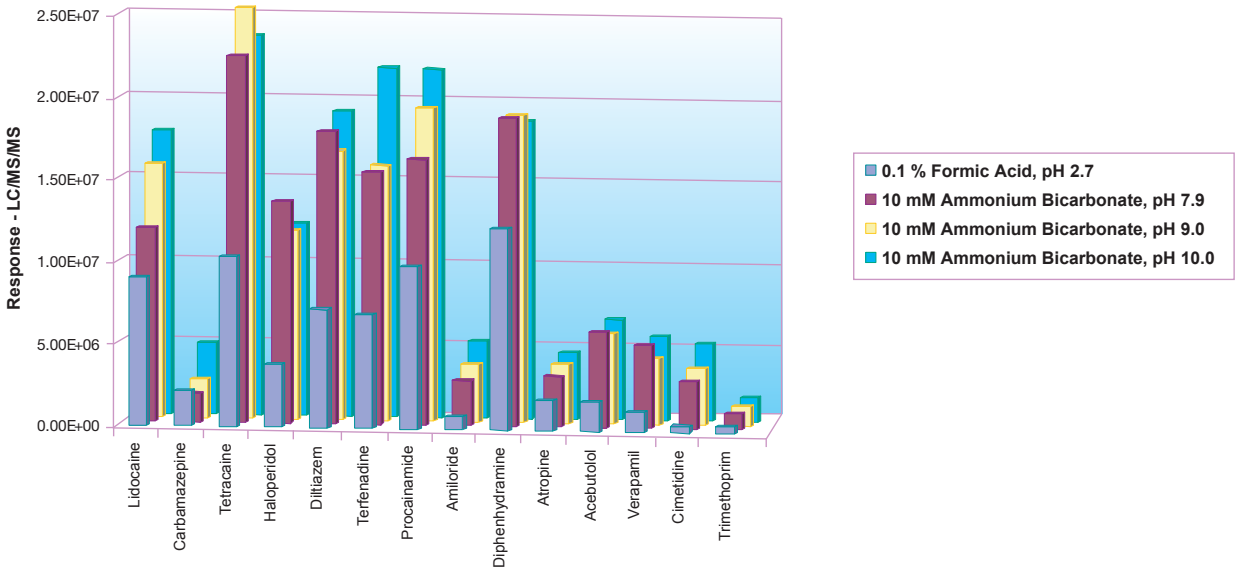


Figure 3. Peak Asymmetry of Basic Compounds in Acidic and Basic Mobile Phases

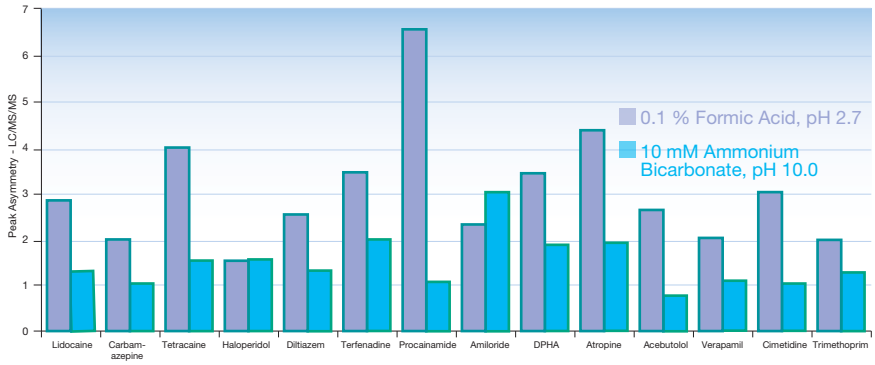
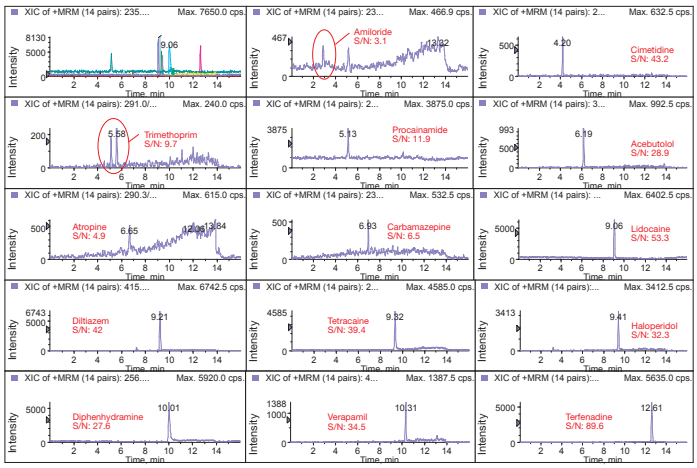


Figure 4. LC/MS/MS Responses of Basic Compounds at the 50 pg/mL level (pH 10)



Results and Discussions

LC/MS Method and Test Probes

We monitored signal intensities for a wide variety of basic drugs in SIM with electrospray as a function of mobile phase pH. Routinely, ion source optimization and mass spectrometer tuning is performed in an acidified mobile phase, rich in organic modifier, which enhances the abundant formation of positive ions (protonated bases). Thus, we optimized all detector settings under acidic conditions in acetonitrile and 0.1 % of formic acid solution, 1:1. To study the effect of mobile phase pH on the MS response of basic compounds, we included in this study a variety of polar and non-polar bases, covering a wide range of pK_a values **(Table 1)**. Standard mixtures were prepared at the 200 ng/mL level in a weak solvent to avoid peak distortion for the early eluting polar basic compounds analyzed on a narrow-bore reversed phase (Gemini C18) column.

Figure 5. S/N in LC/MS/MS in Acidic and Basic Mobile Phases at the 50 pg/mL Level

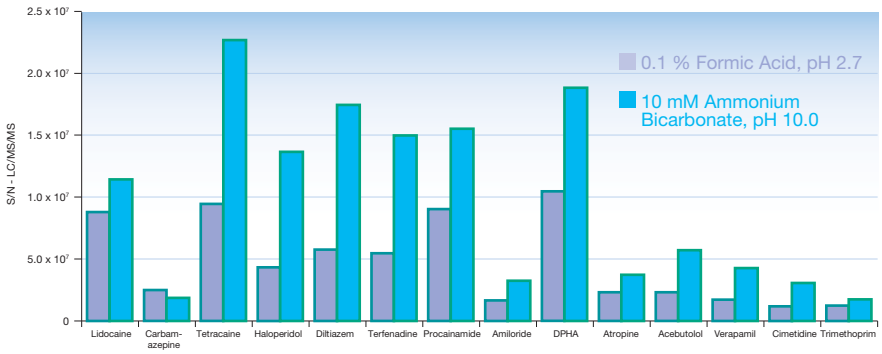
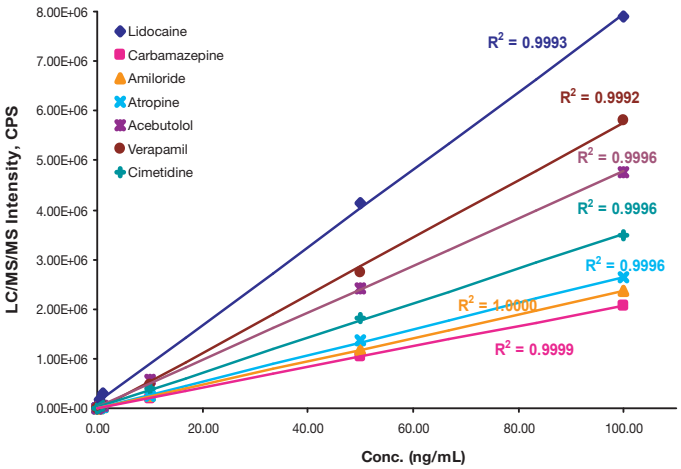


Figure 6. Response Linearity of Basic Compounds in pH 10 Mobile Phase



Influence of Mobile Phases and pH Buffer Selection

The reversed phase elution of basic compounds in an uncharged state results in significantly longer retention times **(Figure 1)**, sharper peak shapes **(Figure 3)** and, often, a change in elution order (indicating alternative column selectivity). Such changes result in a significant improvement in column resolving power. Ammonium bicarbonate-ammonia hydroxide (NH_4HCO_3-NH_4OH) buffer has a wide pH range of 7.8-11 with high buffering capacity. Its thermally labile property makes it compatible with MS detectors. Although this buffer suppresses the ionization of basic drugs in solution at high pH, signal intensities for basic species in ESI^+ LC/MS are higher in ammonium bicarbonate buffers as compared to 0.1 % formic acid **(Figure 1, 2)**.

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Limit of Quantitation and Response Linearity

The limit of quantitation (LOQ) is considered to be the minimum analyte quantity on-column giving a S/N of 10. Most basic compounds included in this study were successfully quantified at the level of 1.25 pg on-column or better, some at levels as low as 50 fg. A comparison of S/N ratios at very low concentration levels (50 pg/mL) reveals that most basic compounds included in this study can be detected with better sensitivity in high pH mobile phases **(Figure 5)** compared to acidic conditions. Only Carbamazepine (LOQ>1250 fg), and Amiloride (LOQ>750 fg), were detected with lower S/N in high pH mobile phase. Response linearity was studied in the concentration range 0.05-100 ng/mL for all basic compounds. The results **(**some are shown in **Figure 6)** demonstrate good linearity with R^2 values above 0.997.

Precision and Accuracy

Method precision was determined by replicate analyses (n=6) at four concentration levels: 0.05 ng/mL, 0.25 ng/mL, 1.00 ng/mL and 100 ng/mL. **Table 2** shows RSD < 15 % at the 0.05 ng/mL level, and <12 % at the 100 ng/mL level. Method accuracy was evaluated by comparing the mean value of six replicate analysis with the expected concentration value at different levels. The analyzed concentrations were determined by linear regression of results obtained on standard calibrators. Accuracy was better than 80 % for most analytes when quantified at their respective LOQ level, and 100 % at the 100 ng/mL level **(Table 2)**.

Table 2. Quantitation of Basic Compounds - Precision and Accuracy

Compound Name	Precision, RSD % (pH = 10) *1				Accuracy (%) (pH = 10) *1			
	0.05 ng/mL	0.25 ng/mL	1.00 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	1.00 ng/mL	100 ng/mL
Lidocaine	12.21	11.90	4.70	3.84	107	100	96	100
Carbamazepine	9.92 *2	7.23	8.19	2.72	72	122	100	100
Tetracaine	12.99	3.93	4.70	4.03	115	89	99	100
Haloperidol	9.49	3.07	5.62	4.37	135	90	103	100
Diltiazem	14.67	6.70	6.35	5.60	94	84	108	100
Terfenadine	6.09	5.75	1.47	7.26	161	80	114	100
Procainamide	11.52	8.03	6.63	5.19	86	81	100	100
Amiloride	-----	2.93	11.10	4.92	----	101	93	100
Diphenhydramine	11.41	7.12	4.34	8.38	102	82	111	100
Atropine	13.03 *2	6.07	7.38	5.94	76	69	100	100
Acebutolol	11.47	6.43	10.18	5.06	94	84	93	100
Verapamil	13.21	8.62	11.41	11.58	138	85	104	100
Cimetidine	11.70	9.78	7.22	5.72	93	111	97	100
Trimethoprim	11.03 *2	5.79	6.98	6.87	90	102	96	100

*1.Inter-assay (n=6); *2. S/N > 10

Conclusions

- The effectiveness of using high pH mobile phases for the quantitation of basic drugs covering a wide range of polarity and pK_a values in ESI^+ LC/MS/MS was evaluated by comparing limits of detection and quantitation, linearity ranges, and accuracy observed in low and high pH mobile phases.
- The successful quantitation of basic compounds by ESI^+ LC/MS/MS in a mobile phase containing a high pH buffer was demonstated.
- Most basic compounds included in this study can be detected with better sensitivity in high pH mobile phase compared to low pH mobile phase.
- Results demonstrate the feasibility of performing basic compound analysis in high pH mobile phases by ESI^+ LC/MS/MS with higher sensitivity, good linearity, precision, and accuracy when using the high performance Gemini® 5 µm C18 HPLC column.

References

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