LC-MS Analysis of Reserpine Using High pH Buffer

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ABSTRACT

Reserpine is being used as one of the gold standards for calibration and sensitivity testing in liquid chromatography coupled to mass spectrometry. In most cases, chromatography is performed using methanol and acetic acid (or formic acid) to improve the ionization of reserpine. A high pH buffer can be used for the LC-MS analysis of reserpine. Detection of trace level reserpine by LC-MS is now possible using high pH mobile phase.

OVERVIEW

- Purpose: analysis of reserpine using a high pH buffer and comparison of the peak response (area, height and peak shape) with conventional acid containing mobile phase was performed.
- Methods: peak responses were monitored by selected ion monitoring (SIM) using a single quadrupole mass spectrometer; a parallel reaction monitoring method was established using high resolution Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer.
- Results: superior peak shape and higher peak intensity for reserpine was obtained with high pH buffer under the analysis conditions.

INTRODUCTION

- Reserpine is used as a calibration standard for evaluating the performance of mass spectrometers (MS) such as Thermo Scientific ion trap and triple quadrupole MS. Addition of formic acid or acetic acid to the mobile phase is necessary for sufficient ionization of reserpine. We report the use of a high pH buffer to monitor the signal response of reserpine by SIM in single quadrupole MS and compared it to the response obtained using acidic mobile phase.
- UHPLC system coupled with MS enables the generation of narrow analyte peaks in chromatographic elution. High resolution MS operated in SRM or multiple reaction monitoring (MRM) enables an acquisition frequency that is well suited for these narrow peaks generated by UHPLC.
- Reserpine was analyzed with high resolution mass spectrometry. Peak response was monitored using three different columns; both acidic and basic mobile phases were used to compare the peak response by parallel reaction monitoring. A superior peak response was obtained using high pH buffer.

METHODS

- Instrumentation:
  - Thermo Scientific high resolution Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer with Vanquish™ UHPLC system
  - Agilent 1260 system and 6150 MS (single quadrupole) with jet stream ion focusing technology, diode array detector (DAD) and auto-sampler
  - ESI-MS: Agilent 1100 with MSD SL (single quadrupole)
- Columns:
  - Agilent Poroshell 120 EC-C18 column (50 x 4.6 mm, 2.7 micron)
  - Agilent XDB extend C18 column (50 x 2.1 mm, 3.5 micron)
  - Thermo Scientific Acclaim™ 120 column (50 x 3.0 mm, 3 micron)
- Mobile phases and reagents:
  - Water (W8), methanol (A458), formic acid (A117), acetonitrile (A956), ammonium bicarbonate, and ammonium hydroxide solution are obtained from Fisher Scientific (Pittsburgh, PA)
  - Reserpine was purchased from Sigma Chemical Company (St. Louis, MO)
- LC-MS testing was performed using three different mobile phases:
  - 10 mM ammonium bicarbonate, pH 10 with 5% methanol as mobile phase A and methanol as mobile phase B
  - 0.1% formic acid in water and acetonitrile
  - 0.1% acetic acid in water and methanol
- LC-MS run conditions:
  - Flow rate 0.5 mL/min and 0.3 mL/min; injection volume 5 µL
  - Gradient runs were performed as follows:

LC with single quadrupole MS		
Time (min)	Mobile phase A%	Mobile phase B%
0	60	40
2	60	40
10	0	100
20	0	100

LC with high resolution MS		
Time (min)	Mobile phase A%	Mobile phase B%
0	60	40
2	60	40
5	0	100
7	0	100

- Mass spectrometer (single quadrupole) data acquisition condition:
  - Fragmentor: 70 V
  - Dry gas temperature: 250º C
  - Dry gas flow: 12 mL/min
  - Capillary voltage: 3000 V
  - Gain: 1
  - Threshold: 150
  - Dwell msec: 290
  - Peak width: 0.1 min
  - Cycle time: 0.84 sec/cycle
- High resolution hybrid mass spectrometer data acquisition condition:
  - Full MS - SIM
  - Polarity: Positive
  - Resolution: 70,000
  - Microscans: 1
  - AGC target: 1e6
  - Scan ranges: 50 - 750 m/z
  - Maximum IT: 200 msec
  - CE: 40

Figure 1. Linear Response of Reserpine in High pH Buffer Using ESI-MS

Conc. ng/mL	Area Counts	Height Counts
1	1.24E+04	1787.93
2.5	1.76E+04	2531.08
5	3.34E+04	4771.08
10	6.09E+04	8.76E+03
25	1.43E+05	2.05E+04
50	3.26E+05	4.65E+04
100	5.38E+05	7.61E+04

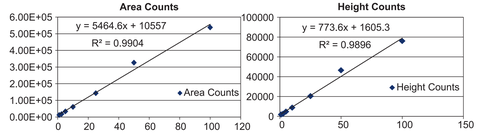


Figure 2. Linear Response of Reserpine in High pH Buffer Using Jet Stream Ion Focusing Technology

PPB Conc.	Area Counts	Height Counts	Peak Symmetry	Width	Plates	S/N
1	1.33E+05	2.26E+04	0.8	0.1009	48197	1073.9
2.5	2.15E+05	3.63E+04	0.8	0.1007	48017	1952.6
10	7.39E+05	1.24E+05	0.78	0.0996	49154	6530.3
25	1.68E+06	2.80E+05	0.79	0.1005	47927	1.20E+04
50	3.03E+06	4.90E+05	0.78	0.1201	46711	1.80E+04
100	5.61E+06	8.88E+05	0.77	0.1037	45018	2.80E+04

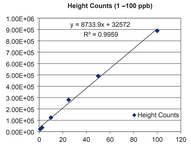


Figure 3. LC-UV Response of Reserpine

Serial dilution of 100 ppb to 1000 ppb reserpine; chromatograph run in ammonium bicarbonate buffer. Corresponding UV absorption profile is shown for the 1000 ppb reserpine peak.

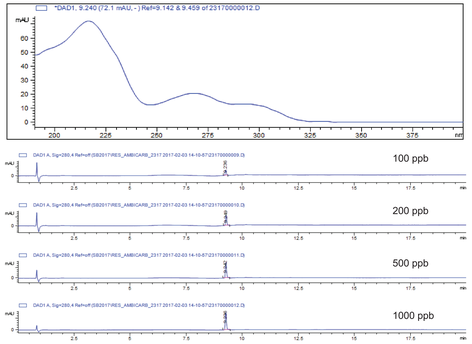


Figure 4. Sample Preparation in Basic and Acidic Media

- Reserpine serial dilution was made in ammonium bicarbonate buffer solution
- Serial dilution of reserpine was made using methanol/0.1% acetic acid
- Not much variation observed in peak area or peak height counts

	Sample Dilution in High pH Buffer		Sample Dilution in Acid	
Conc (ppb)	Res_Area_SIM-Am	Res_Height_SIM-Am	Res_Area_SIM	Res_Height_SIM
1	6.04E+04	1.07E+04	1.33E+05	2.26E+04
2.5	1.10E+05	1.97E+04	2.13E+05	3.64E+04
25	1.23E+06	2.18E+05	1.68E+06	2.82E+05
50	3.56E+06	5.99E+05	3.02E+06	4.94E+05
100	4.14E+06	6.89E+05	5.55E+06	8.92E+05
200	7.23E+06	1.19E+06	9.15E+06	1.46E+06

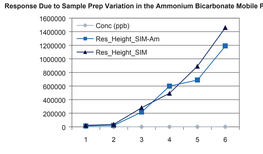


Figure 5. Mobile Phase Variation and Peak Shape

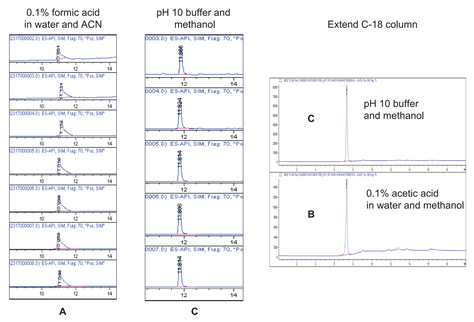


Figure 6. Poroshell Column Peak Comparison

Data from Q Exactive Plus MS

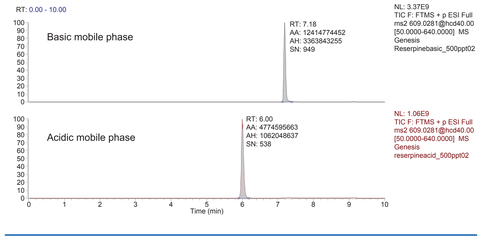


Figure 7. Poroshell Column Response Comparison

	0.5 ppb_Basic_MP Retention time	Area Counts_ Basic_MP_0.5 ppb	Height Counts_ Basic_MP_1 ppb	0.5 ppb_Acidic_MP Retention Time	Area Counts_ Acidic_MP_0.5 ppb	Height Counts_ Acidic_MP_1 ppb
	7.19	3359359736	963496136	6.03	1372539294	322313955.4
	7.19	3458213157	947163179.2	6.00	1297126030	285680324.9
	7.19	3509687724	957612800	5.98	1280872585	286943748.6
	7.19	3490808795	972500319.8	5.97	1283056204	278062146.2
	7.19	3427998405	906834438.7	5.96	1287331905	282471499.1
% RSD	0	1.714	2.693	0.46	2.968	6.11

0.3125 in

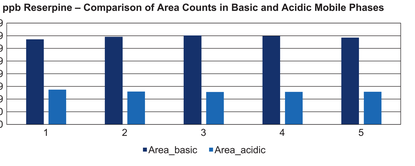
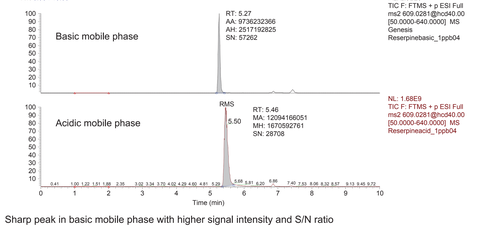


Figure 8. Extend C-18 Column Peak Shape Comparison



Sharp peak in basic mobile phase with higher signal intensity and S/N ratio

Figure 9. Extend C-18 Column Response Comparison

	Basic MP_ Retention Time	Area Basic_0.5 ppb	Height Basic_ 0.5 ppb	Basic MP_ Retention Time	Area Basic_1 ppb	Height Basic_1 ppb
	5.26	1615604706	419573669.3	5.25	2794326074	664057389.1
	5.25	1648019482	418147739.6	5.26	2842878662	713823433.1
	5.25	1642678341	422784792.8	5.25	2802918557	710656244.8
	5.25	1633546449	392988036.8	5.26	2773112496	710865418.8
	5.26	1639729081	415457010.4	5.25	2818301106	743470533.6
% RSD	0.104	0.764	2.882	0.104	0.932	4.014
	Acidic MP_ Retention Time	Area_Acid_ 0.5 ppb	Height_Acid_ 0.5 ppb	Acidic MP_ Retention Time	Area_Acid_1 ppb	Height_Acid_1 ppb
	5.65	2181292046	270738713.8	5.55	3482397367	452480601
	5.77	2785998641	343216947.2	5.51	3480584052	434671807.9
	5.71	2447589109	234182824.9	5.49	3457168861	463023031.8
	5.68	2219159223	243945729.5	5.46	3571131365	472346367.7
	5.61	2087157573	238543999.9	5.42	3555020086	488992294.2
% RSD	1.067	12.001	5.814	0.898	1.437	4.424

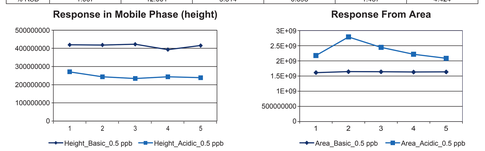
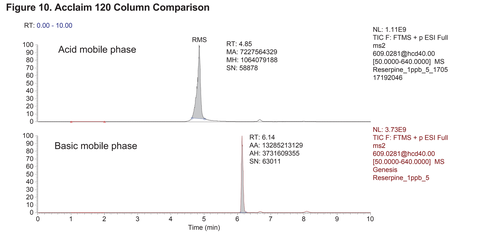


Figure 10. Acclaim 120 Column Comparison



- Better response and superior peak shape is observed for reserpine in basic mobile phase compared to acidic mobile phase

Figure 11. Acclaim Column Response

	RT (min)	Area_basic_1 ppb	Height_basic_1 ppb	RT (min)	Area_acid_1 ppb	Height_acid_1 ppb
	6.14	13285213129	3731609355	4.91	9201382918	1194122828
	6.15	14169181911	3953875426	4.9	9425014687	1214235671
	6.14	14140038237	3879012751	4.87	9197329817	1177185024
	6.13	13850837177	3806993349	4.87	9306667581	1078361488
	6.13	14121002086	3985710584	4.85	9220494445	1110898369
% RSD	0.136	2.685	2.697	0.502	1.049	5.002
	RT (min)	Area_Basic_500 ppt	Height_Basic_500 ppt	RT (min)	Area_acid_500 ppt	Height_acid_500 ppt
	6.13	8550258728	2585552626	5.17	4644990293	998154447.4
	6.13	8617236724	2669115296	5.09	4722876199	975989256.2
	6.15	8773344095	2608197053	5.03	4952938723	808171869.1
	6.15	8695834172	2551960348	4.98	5119724179	783648592.6
	6.13	8454522392	2539560071	4.95	5122550809	767897484.9
% RSD	0.178	1.433	1.987	1.749	4.508	12.809

Figure 12. Fragmentation Pattern in Acidic and Basic Mobile Phase

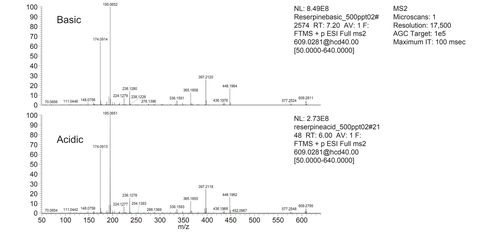
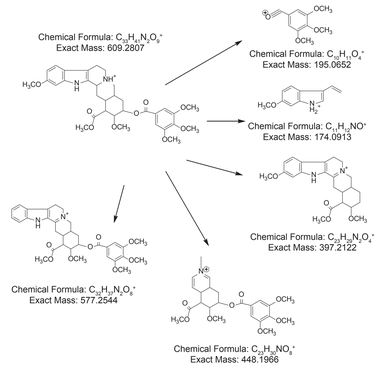


Figure 13. Proposed Fragmentation Pathway



RESULTS AND DISCUSSION

- **Figs. 1** and **2** show a linear response of reserpine from 1 ppb to 100 ppb concentration range with a good fit linear regression ($R^2 = 0.99$) when high pH ammonium bicarbonate buffer was used as the mobile phase.
- Data were acquired both in regular ESI-MS and the jet stream ion focusing ESI-MS system. An order of magnitude higher response was observed when jet stream ion focusing was used.
- **Fig. 3** shows the LC-UV chromatogram of 100 to 1000 ppb reserpine and the UV absorption profile of the eluted peak; chromatogram run in high pH buffer.
- **Fig. 4** depicts that the reserpine standard prepared in acidic or basic solution does not affect the response at the lower range (1 ppb to 200 ppb) concentration in the high pH buffer – both sample preparations showed similar response.
- A comparison of peak shape in various mobile phases is shown in **Fig. 5**. We observed broad peak shape and peak tailing in water-acetonitrile mobile phase with 0.1% formic acid for 1 ppb to 100 ppb concentration of reserpine (**Fig. 5A**). Peak shape was observed much sharper in water/methanol with 0.1% acetic acid (**Fig. 5B**) and even better peak shape was obtained from basic mobile phase (**Fig. 5C**).
- Next we ran reserpine in high resolution MS and checked parallel reaction monitoring under the same condition of analysis, but with changes to the mobile phase. **Figs. 6** and **7** represent the peak shape as well as SIM response comparison using Poroshell column from 0.5 ppb and 1 ppb reserpine when both acidic and basic mobile phases were used. Under the same operating condition, the reserpine peak with basic mobile phase was sharp and its intensity three times higher than reserpine peak with acidic mobile phase.
- Extend C18 column performance is depicted in **Figs. 8** and **9**. Interestingly, a broader peak shape was noticed in this column when reserpine was eluted from acidic mobile phase. Area counts of the peak were relatively higher in comparison to basic mobile phase, however, peak height counts were higher in basic mobile phase.
- Peak elution in basic mobile phase from Acclaim 120 column was also observed superior compared to acidic mobile phase (**Figs. 10** and **11**).
- Fragmentation pattern of reserpine is identical in basic and acid mobile phases as revealed in **Fig. 12** under the same mass spectrometric condition; fragmentation pattern is proposed in **Fig. 13**.

CONCLUSIONS

- All three columns used in the reserpine testing revealed that peak shape is better in ammonium bicarbonate pH 10 buffer solution
- High resolution mass spectrometer data for 0.5 ppb and 1 ppb concentration of reserpine is significantly higher in S/N ratio using the high pH buffer
- A linear response of peak area and height was obtained in high pH buffer from 1 ppb to 100 ppb concentration range
- Mass spectrometer response of reserpine is superior in high pH buffer compared to acid containing mobile phase per the conditions of our analysis
- High pH buffer is an alternative mobile phase for detection of reserpine-like compounds in mass spectrometry