Assessment of Ammonium Fluoride as a Mobile Phase Additive for Sensitivity Gains in Electrospray Ionization

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Abstract

Rationale

Ammonium fluoride has been shown to improve sensitivity in electrospray ionization in mass spectral detection when used as a mobile phase additive. Internal investigation showed sensitivity improvement for steroid compounds with LCMS. Our work presented here investigates ammonium fluoride as a mobile phase additive in improving mass spectral ionization compared with other mobile phases under fully optimized instrument conditions.

Methods

For our research, we investigated ammonium fluoride in comparison with formic acid and ammonium hydroxide as mobile phase additives to measure sensitivity effects in electrospray ionization sensitivity. Full source optimization was performed for nine commercially available compounds at three different organic concentrations (30, 60, or 90%) with formic acid, ammonium fluoride, and ammonium hydroxide adjusted mobile phase sets. Optimization results were compiled to generate individual methods by compound, polarity, mobile phase additive, and organic concentration, respectively. These methods were used for final flow injection analysis to objectively compare compounds analyzed with the different solvent systems under optimal conditions for each mobile phase composition.

Results

Negative ESI data showed 2 – 22-fold sensitivity improvements for all seven compounds with ammonium fluoride. Positive ESI data showed > 1 - 11-fold improvement in sensitivity for four of seven compounds and equivalent (or slight drop in) sensitivity for three of seven compounds with ammonium fluoride adjustment. Acetaminophen showed the greatest sensitivity improvement in both polarities, with 7-22-fold (ESI-) and 4-11-fold (ESI+) improvements in sensitivity, respectively.

Conclusions

With fully optimized source conditions there is good likelihood of improving MS sensitivity (ESI-) in using ammonium fluoride adjusted mobile phase. Investigation with ESI+ analyses showed mixed results, with four of seven compounds showing improvement and others showing equivalency or slight loss in sensitivity. Our investigation focused solely on sensitivity gains achievable in ESI-MS by source optimization and mobile phase additive selection by flow injection without chromatography.

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Introduction

High performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) is well represented in many quantitative applications with demonstrated analytical strengths in separation efficiency, detection, and specificity. Applications ranging from trace-level clinical monitoring, academic, manufacturing, private and governmental research, and more, have showcased HPLC/MS as a must-have lab instrument in today’s laboratory.

Electrospray ionization (ESI) has become the accepted standard ionization technique in the MS instrumental community, having demonstrated robust efficiency in liquid effluent introduction from the chromatography system to a variety of modern mass spectrometers. ESI works well with compounds across a range of polarities, charge, and size. There are many factors that may influence ESI ionization efficiency (mobile phase composition, co-elution of components, source conditions, etc.). The source heat, gas, and voltage parameters are adjusted to optimize the desolvation of liquid effluent from the chromatography system. Although ESI is regarded as a standard and rugged MS introduction technique, it may still require additional optimization beyond routine instrumental tuning, especially for trace level analytical measurements in complex matrices.

Improving ESI ionization efficiency is one option to improve sensitivity for LC/MS methods. Ionization can be optimized by selecting appropriate mobile phases with pH adjustment to promote the formation of protonated/deprotonated species in solution. When selecting mobile phase options chromatographic separatory needs should be balanced with optimal ionization efficiency as both are important to the sensitivity and robustness of the methodology. Mobile phase selection includes assessing chromatographic separatory needs balanced with respective desolvation characteristics and ionization efficiency to drive overall assay sensitivity and robustness of the methodology. There are only a few MS-friendly mobile phase modifier options without lasting instrumental effects. For low pH adjustment, formic, acetic, and trifluoroacetic acids are all MS-friendly mobile phase additives that aid in generation of protonated species (notably for compounds with primary or secondary amines) and provide good chromatographic separatory characteristics. These acids promote protonation of chromatographic stationary phase free silanols that can cause undesired secondary ion exchange interactions and lead to band broadening or peak splitting. For high pH adjustment, ammonium hydroxide is a volatile and common mobile phase modifier that aids in deprotonation for acidic or hydroxy functional groups. Other volatile mobile phase additives rely on ion-pairing interactions which causes ion-suppression or remain in the instrument after use.
Ammonium fluoride has been reported in the literature across applications as an effective volatile mobile phase additive in improving ESI sensitivity and is hypothesized to work as a sequestration agent. Sensitivity improvements with ammonium fluoride are not fully characterized but have been associated with binding competing ions in ESI gas phase formation, helping improve formation of more desirable protonated or deprotonated species. Multiple application notes have been published, including analysis of fat-soluble vitamins, veterinary drugs, pesticides, and hormones, all using ammonium fluoride adjusted mobile phase systems. Specifically, several separate application notes identify success in optimizing sensitivity for hormone measurement with improvements in switching to an ammonium fluoride based mobile phase system. Internal efforts in developing a trace level analytical method for steroids showed an approximate 5-10 fold improvement in sensitivity by replacing ammonium hydroxide with ammonium fluoride and making no other changes. Additionally, recent publications describe sensitivity improvements with substituting ammonium fluoride as a mobile phase modifier (vs formic acid or ammonium acetate) in metabolism screening work. Our experience, along with review of multiple literary references, lead us to question how ammonium fluoride might impact ionization efficiency across analogs when compared against other mobile phases in a controlled design.

In this study, we compare ammonium fluoride with formic acid and ammonium hydroxide mobile phases in measuring changes in ESI signal intensity for nine compounds across three experiments. In Experiment 1, nine compounds were injected with MS Scanning in positive and negative ion modes, with formic acid, ammonium fluoride or ammonium hydroxide adjusted mobile phases, with three different organic compositions (30, 60, and 90%) to confirm (de)protonated species across mobile phase conditions. In Experiment 2, each compound was analyzed to identify optimal source conditions for each respective mobile phase conditions. These conditions were recorded and compiled to generate optimized methods for each compound, according to mobile phase composition. In Experiment 3, a final analysis was performed (in triplicate) for all compounds with optimized source conditions from the prior experiment. Results allowed for objective comparison of MS sensitivity attributable to mobile phase conditions.

This is the first work to our knowledge investigating source optimization to compare sensitivity of ammonium fluoride as a mobile phase additive with ESI MS. All analyses were performed by flow injection analysis (no chromatographic separation) to focus attention on source optimization and mobile phase composition as key variables in ESI sensitivity enhancement or suppression.

**Materials and Methods**

**Materials**

Three different sets of mobile phases were prepared for use throughout experimentation. Mobile Phase A was prepared in MS Grade water and Mobile Phase B was prepared in 1:1 Acetonitrile: Methanol (MS grade). Mobile Phase A and Mobile Phase B were adjusted accordingly for each set as described below:

*Mobile Phase Set 1: 0.1% Formic Acid (Fisher, MS Grade)*

*Mobile Phase Set 2: 0.2 mM Ammonium Fluoride (Sigma, 99%)*

*Mobile Phase Set 3: 0.1% Ammonium Hydroxide (Fisher, MS Grade)*

We chose to use 0.1% concentrations of formic acid and ammonium hydroxide respectively as these are common adjustment concentrations for LC/MS separatory conditions. Ammonium fluoride mobile phases were adjusted to 0.2 mM concentrations based on an application note we had followed in our lab in steroid assay development.

Reference standards for antipyrine, tetrabromobisphenol A, leucine enkephalin, thyroxine, verapamil, labetalol, estrone, and acetaminophen were purchased as powders from Sigma Aldrich, with each having purity [?]. Reference standard for aminopyralid was secured internally (Corteva Agriscience) as a certified reference standard with purity of 99%. Compound structures are shown in Figure 1.

*Figure 1. Structure and MS Polarity for Nine Compounds Tested*
Stock solutions were prepared by individual weighing and dissolution of each material to yield final 1 mg/mL preparations. Individual stock solutions were diluted 1000-fold to yield 1 ppm individual stock solutions (for use in Experiment 1 and 3) and as a single collective stock containing all analogs at 1 ppm concentration (for use in Experiment 2). All stocks were prepared with dimethyl formamide (Fisher Scientific, Reagent Grade) and stored at 4 °C when not in use.

Chromatographic Instrumentation
Analyses were performed with an Agilent 1290 Infinity II UPLC system comprised of binary pump (G7120A), multisampler (G7167B) and column compartment (G7116B).

Isocratic flow rate of 0.5 mL/min and injection volume of 1 μL were maintained throughout all experiments. All injections were performed with a union (no HPLC column or guard cartridge) to optimize the source for each mobile phase condition, without having to consider chromatography.

MS Instrumentation
Analyses were performed with an Agilent G6495A triple quadruple mass spectrometer. Instrument control was facilitated with Agilent Masshunter Workstation Acquisition software (Version 10.0.127). The mass spectrometer was equipped with an Agilent Jet Spray source and operated in positive/ negative polarities with either Q3 scanning for initial parent mass confirmation (Experiment 1), or in Multiple Reaction Monitoring (MRM) mode for source optimization and confirmatory analyses (Experiments 2 and 3).

MRM optimization and testing was performed with Agilent Masshunter Workstation Optimizer software. The program does not allow for optimization in Single Ion Monitoring (SIM) mode. As such, all MRM analyses were performed using the respective protonated/ deprotonated species assigned for both Q1 and Q3 masses. Collision energy was set very low (5 V) to promote full ion transmission through the collision cell to Q3. This was the simplest approach we could identify that would ensure full ion transmission and measurement for comparison, without incorporating bias from product ion formation and allow use of the source optimization software.

In Experiment 2, results from source optimization were reviewed for each parameter for each MRM trace using Agilent Masshunter Workstation Quantitative Analysis software (B.09.00). The chromatograms for each injection in each MRM trace were integrated, allowing for sorting by area counts in the table view to easily rank parameters from lowest to highest. The sorting capability allowed for quick review of results and selection of optimal condition for each trace. This process was repeated for all optimization conditions in all mobile phase sets and organic mobile phase conditions for each respective trace. Once completed, the results were compiled and used to generate optimized methods for each compound.

Results
Experiment 1 - Ion Confirmation by MS Scan
Flow injections were performed with Mobile Phase Sets 1, 2, and 3 with Organic (%B) concentration set to 30, 60, and 90%, respectively, resulting in 9 different isocratic mobile phase conditions in all. Individual 1 ppm stock solutions were analyzed to generate spectra for Experiment 1. Initial MS Scans were performed with conditions shown in Table 1. Spectral data were reviewed against anticipated [M-H]⁻ and [M+H]⁺ ion formation in respective negative/ positive ion mode results with Agilent Masshunter Workstation Qualitative Analysis software (Version B.08.00). If [M-H]⁻ or [M+H]⁺ ions were observed across mobile phase conditions, the ion was included for source optimization in Experiment 2. A summary of results for confirmation of protonated/ deprotonated species by analyte is presented in Table 2.
Table 1. LC/MS Method Starting Conditions for Experiment 1

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Table 2. Results Summary from Experiment 1 in Confirming Observed \([M+H]^+\) and \([M-H]^+\) Ions

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Experiment 2 - Source Optimization

Experiment 2 was conducted to identify optimal source conditions for all compounds with each mobile phase with three different organic mobile phase compositions (30, 60 and 90%). A stock solution containing all 9 compounds at 1 ppm concentration was used for all analyses in Experiment 2. Source optimization was performed with Agilent MassHunter Workstation Optimizer software (Version 10.0.127). Respective parameters are shown in Table 3. Optimization was performed with a single mobile phase system (formic acid, ammonium fluoride, or ammonium hydroxide adjusted) at each organic mobile phase concentration before changing solvent systems, equilibrating, and proceeding with the next round of source optimization injections.

Table 3. Source Optimization Parameters and Ranges for Experiment 2

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Example data in selection of optimal gas flow conditions for acetaminophen in negative ion mode with Mobile Phase Set 2 at 30% organic composition are presented in Table 4.

Table 4. Example Optimization Results for Acetaminophen \((M-H)^-\) (Sorted by Area Response) for Mobile Phase Set 2 – 30% Organic Mobile Phase

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Example compiled optimization source condition data for all optimized MRM traces with Mobile Phase Set 2 at 30% organic composition is presented in Table 5. An example flow injection chromatogram for acetaminophen with optimized gas flow setting with Mobile Phase Set 2 at 30% organic composition is presented in Figure 2.

Figure 2. Example Flow Injection Chromatogram for Acetaminophen \((M-H)^-\) with Mobile Phase Set 2 - 30% Organic Composition with Optimal Gas Flow Setting (12 L/min)
Table 5. Compiled Source Optimization Results - Mobile Phase Set 2 - 30% Organic Mobile Phase

For Experiment 3, final optimized methods were constructed for each respective MRM trace, for each mobile phase set/organic condition set using the results generated in Experiment 2. To generate final data with optimized methods, individual 1 ppm stock solutions were analyzed in triplicate and processed in Agilent Masshunter Workstation Quantitative Analysis software (B.09.00). Review of data included integration of the flow injection MRM peaks and export of peak areas to Microsoft Excel for final processing and comparison.

Example results for Estrone [M-H]− analyzed with 30, 60, and 90% B mobile phase composition in Mobile Phase Sets 2 and 3 are presented in Table 6. Example results for Acetaminophen [M-H]+ as analyzed with 30, 60, and 90% B mobile phase composition in Mobile Phase Sets 1 and 3 are presented in Table 7.

Table 6. Mobile Phase Sensitivity Comparison for three replicate injections – Mobile Phase Set 2 vs 3 - Acetaminophen [M-H]⁺

Table 7. Mobile Phase Sensitivity Comparison – Mobile Phase Set 1 vs 3 - Verapamil [M+H]⁺
Results comparing ammonium fluoride with ammonium hydroxide optimized analyses for [M-H]− species is presented in Figure 3. All seven compounds showed 2 to 22-fold improvement in sensitivity. Acetaminophen showed the greatest improvement in sensitivity with ammonium fluoride adjustment, with 7 to 22-fold sensitivity improvement across all organic mobile phase compositions. The greatest sensitivity gains were observed at 30% organic composition for five of the seven analogs. Two of seven analogs (thyroxine and leucine enkephalin) showed the greatest sensitivity improvements at 60% organic composition.

Figure 3. Sensitivity Fold Improvement - Ammonium Fluoride vs Ammonium Hydroxide – [M-H]−

Results comparing ammonium fluoride in with formic acid optimized analyses for [M+H]+ species is presented in Figure 4. Four of seven compounds (acetaminophen, antipyrine, labetalol, and verapamil) showed improved sensitivity with ammonium fluoride conditions. Three of seven compounds (aminopyralid, leucine enkephalin, and thyroxine) showed equivalent or slightly lower sensitivity when analyzed with ammonium fluoride. Acetaminophen showed the greatest improvement in sensitivity overall, similar with results from negative ion mode data, with 4 to 11-fold sensitivity improvement across all organic compositions. Four of seven compounds (acetaminophen, antipyrine, labetalol, and thyroxine) showed outperformance in ammonium fluoride with 90% organic composition. Verapamil results showed approximately equivalent improvement across 30, 60, and 90% organic compositions. Data show that using ammonium fluoride vs formic acid improved sensitivity for half of the analogs, with greater sensitivity gains observed at higher organic mobile phase composition. Data were not included for aminopyralid with 90% organic mobile phase as there was low response with formic acid modifier compared with ammonium fluoride resulting in a dramatic 29-fold improvement in sensitivity with ammonium fluoride. These results are an outlier compared with performance from other compounds and not included here.

Figure 4. Sensitivity Fold Improvement - Ammonium Fluoride vs Formic Acid – [M+H]+

Conclusions

Results show unanimous improvement in sensitivity for [M-H]− species, with greatest gains observed at low (30%) organic mobile phase composition (four compounds) and 60% organic mobile phase composition (three compounds). Results suggest that analytical detection limits may be improved substantially with substitution of 0.2 mM ammonium fluoride in place 0.1% ammonium hydroxide, with elution profiles that are low to moderate organic content.
[M+H]+ results showed improvement in overall sensitivity for four of the seven analogs and equivalent or slight loss in sensitivity for three analogs. Mobile phase compositions with higher organic composition (90% B) showed the greatest sensitivity improvements for three of four analogs – contrary to [M-H]- results.

Observed improvement for [M-H]- sensitivity with ammonium fluoride adjustment makes sense as it is hypothesized that fluoride in the mobile phase may scavenge for any additional ion species competing with deprotonation and bind or sequester them, improving overall deprotonation. There is not a clear explanation at this time for improved sensitivity of [M+H]+ with ammonium fluoride.

Ammonium fluoride (0.2 mM adjusted) substitution in mobile phase is shown to improve MS sensitivity for [M-H]-, particularly at low to moderate organic composition, and potential to improve sensitivity for [M+H]+ species, more readily at higher organic mobile phase compositions.

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