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Formation of Iron Complexes from Trifluoroacetic Acid Based Liquid Chromatography Mobile Phases as Interference Ions in LC-ESI-MS Analysis

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Abstract

Two unexpected singly charged ions at m/z 1103 and 944 have been observed in mass spectra obtained from electrospray ionization-mass spectrometric analysis of liquid chromatography effluents with mobile phases containing trifluoroacetic acid that severely interfered with sample analysis. Accurate mass measurement and tandem mass spectrometry studies revealed that these two ions are composed of three components; clusters of trifluoroacetic acid, clusters of mass 159 and iron. Formation of these ions is inhibited by removing trifluoroacetic acid from the mobile phases and using formic acid in its place, replacing the stainless steel union with a titanium union or by adding a small blank fused silica capillary column between the chromatography column and the electrospray tip via a stainless steel union without any adverse effects to chromatographic separation, peak broadening or peptide identifications.

Keywords

Electrospray ionization; Liquid Chromatography-Mass Spectrometry; Peptides; Iron complex

Introduction

Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) is a widely used platform for analyzing complex biological samples¹. An important, but sometimes undesirable aspect of ESI is the formation of ions from contaminants present in the solution or in the gas phase in the laboratory atmosphere (e.g., siloxanes², common contaminant in laboratory air and plasticizers^{3, 4}). Lee et al.⁵ have shown that volatile organic compounds present near the ESI tip are easily ionized into protonated molecules either by their absorption into droplet and subsequent ion-molecule reactions or by ion-molecule reactions when volatile organic molecules collide with protonated molecules generated by electrospray of solvents. Solvents used for LC mobile phases also form ionized clusters/mixed clusters when effuse from the ESI tip and enter the mass spectrometer. Keller et al.⁶ have compiled long list of contaminants and interferences that have been experimentally observed in this and various other mass spectrometry laboratories.

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Recently, we observed abundant singly charged ions at m/z 1103 and 944 (nominal) from ESI-MS of LC effluent from peptide separations when trifluoroacetic acid (TFA), an ion pairing agent commonly used to improve chromatographic resolution, was present in the mobile phase. These ions, which in many cases dominated the mass spectra to the extent that they hindered sample analysis, could not be assigned to any known peptides or to any other molecules as possible contaminants in the solvents, stationary phase or laboratory atmosphere. All possible efforts to eliminate these ions failed until a short, ~5-cm long, 50 μm i.d. blank column (i.e., a fused silica column of 50 micron id without packing material) was incorporated between the packed LC column and the ESI tip and high voltage was applied to the union holding the tip. These two ions were also not observed when a titanium union was used in place of the stainless steel (SS) union. Herein, we present evidence that these two ions are formed during ESI by clustering and reactions of TFA with iron from the SS union used for connecting the LC column with the ESI tip.

Experimental

The m/z 1103 and 944 ions were observed in varying degrees in spectra collected on all of our mass spectrometers used for high throughput proteomics analysis; the most severely affected data were obtained using Fourier-transform ion cyclotron resonance (FTICR) mass spectrometers. We therefore used a modified 9.4 Tesla FTICR mass spectrometer from Bruker Daltonics (Billerica, MA) that was coupled with a home-built dual column LC system to study these ions. This instrument with its modified source and ion transfer optics has been described in detail in an earlier publication,⁷ so only essential features are given here. Ions formed by ESI pass through a heated metal capillary (500 μm i.d.) at 150°C and the resulting desolvated ions are delivered into an electrodynamic ion funnel⁸ at ~1 Torr for efficient transfer into the collisional quadrupole. In the collisional quadrupole, which is maintained at ~20 mTorr, ions collisionally relax and are axially focused and then transferred to the RF-only quadrupole for further transmission to the next vacuum stage. In this stage, ions are accumulated in a quadrupole for 300 ms prior to transfer into the ICR cell via electrostatic ion optics. Ions are excited and detected in the ICR cell, and the resulting mass spectrum is recorded. We also used a quadrupole time-of-flight (Q-TOF) mass spectrometer from Waters Corporation (Milford, MA) with a similar electrospray source and ion funnel for tandem mass spectrometry (MS/MS) studies of these ions. MS/MS spectra were obtained at 30 eV collision energy. All experiments were done using positive ions.

LC was performed using 150- μm -i.d. fused silica columns of 70 cm length that were packed in-house⁹ with 3 μm C₁₈ Jupiter particles (Phenomenex, Torrance, CA). These columns were connected to a chemically etched ESI tip¹⁰ via a SS union that was floated at ~2 kV for ESI of the LC effluent. LC columns were operated at 5000 psi. All chemicals and solvents were HPLC grade. Mobile phase A consisted of nanopure water with 0.2% acetic acid and 0.05% TFA (v/v) and mobile phase B, acetonitrile : nanopure water : TFA (90:10:0.1; v/v). A 100 minute linear gradient was used from zero to 100 % mobile phase B. All solvents were tested for impurities by direct infusion ESI, and no ions at m/z 1103 and 944 were observed. The sample used in this study consisted of a known trypsin digested mixture of peptides and proteins¹¹ (QC mixture), and blank analyses were performed by injecting 7 μL of mobile phase A.

Results and Discussion

Figure 1 provides a total ion chromatogram (TIC) and two mass spectra from LC-ESI-FTICR MS of the QC mixture that illustrate the interference from m/z 1103 and 944 ions. Note that the background in the TIC slowly increases and overtakes the LC peaks at longer elution times, which results in mass spectra dominated by m/z 1103 and 944 ions and

renders them useless for analytical purposes as ions of low abundance are completely suppressed. These two ions were continuously observed throughout the 100 minute gradient. The calibrant signal in the TIC is from direct infusion of a peptide mixture used for recalibration of the mass spectrometer for each sample run. These two ion peaks were not observed when LC mobile phases did not contain TFA and the TIC looked normal, as shown in Fig. 2. Interestingly, m/z 1103 and 944 peaks were not observed in the spectra obtained from direct infusion ESI of the QC mixture, mobile phases, and solvent extract of column packing material that contained a mixture of mobile phases. A LC run using a longer column and without the packing material to maintain higher pressure did not produce these two ions. We also accumulated solvents eluted from LC runs of blank and QC sample through the packed column and used that for direct infusion experiments but again, these ions were not observed. The presence of these ions was not dependent upon the electrospray voltage either. However, these ions disappeared when TFA was replaced with formic acid in the mobile phases. Together, these findings suggested that TFA in the LC effluent was responsible for the formation of these two ions. Because TFA results in better chromatographic separation and peak shapes, removing it from the mobile phases was not considered a viable option for our high throughput LC-ESI-MS proteomics approach. As a result, we deemed it necessary to find the origin of these ions and ways to eliminate them.

To characterize these two ions, we studied their dissociation patterns by accumulating them for longer periods in the accumulation quadrupole and by monitoring their unimolecular decay, as well as their dissociations by collisions with the background gas in the accumulation quadrupoles in the 9T FTMS instrument since we did not have proper setup (a collision cell) for doing MS/MS experiments in the ICR cell on these instruments. We observed that m/z 1103 slowly diminished and that m/z 944 increased in abundance over time, which suggested that m/z 944 is a fragment ion of the m/z 1103 ion via loss of 159 mass units. Further studies of their dissociations were performed on the Q-TOF instrument. Scheme 1 depicts the two main fragmentation pathways via loss of 159 and loss of 114 mass units that account for most of the fragmentation processes observed for these two ions. High-resolution analysis of the primary ions and their fragment ions gives the mass of these neutral losses as 159.0502 and 113.9930. The mass of TFA is 113.9929, which matches the measured mass of 113.9930, however, mass 159 does not correspond to any of the individual components in the solvent system and column material or to any known contaminants/interferences commonly observed in ESI. Since this is a neutral fragment, we cannot perform any further direct investigation on its identity by mass spectrometry.

In search of further clues as to the origin and identity of the m/z 1103 and 944 ions, we observed that the isotopic patterns for both ions were similar and somewhat unusual for the molecules (composed of C, H, O, N and F) present in the solvent system. For example, the smaller contribution from the ^{13}C isotope suggested fewer carbon atoms than expected from the solvents/chemicals and more mass deficient elements (e.g., oxygen, fluorine) and possibly some metal atoms, as the actual masses were very close to m/z 1103.0 and 944.0. Also, there is a lower intensity peak at two mass units before m/z 1103 and 944 peaks, as shown in Figure 3 for m/z 944. A search for the isotopic patterns of elements in the periodic table revealed that Fe and Cr have similar isotopic patterns. However, Cr has two additional isotopes of significant intensity at higher masses that are in much higher abundance than ^{13}C and ^{57}Fe and hence it is ruled out as a constituent for these two ions. Thus, Fe is the only element that seems to be responsible for the observed isotopic distribution. The experimental isotopic pattern suggests that both 1103 and 944 ions contain a single Fe atom. As shown in Scheme 1, m/z 1103 and 944 lose 114 and 159 neutrals in a series of dissociation processes and we can obtain the correct molecular mass of these ions by including one Fe and a combination of masses 159 and 114. We can therefore tentatively assign m/z 1103 and 944

ions to $(159)_3(114)_5\text{Fe}^+$ and $(159)_2(114)_5\text{Fe}^+$, respectively, thus further confirming that Fe instead of Cr is the metal constituent in these ions.

In our experiments, we used TFA (mass 114), acetic acid (mass 60), acetonitrile (mass 41), silica capillary with proprietary coating (that manufacturer will not disclose) and C_{18} based packing material. As mentioned earlier, on direct infusion ESI of solvent extracts from these materials and solvents themselves, we did not observe ions at m/z 159 or a combination of it with others. However, it is well established that ESI often results in clustering of solutes and solvents and the presence of TFA clusters along with 159 clusters is clearly evidenced in the present study. We therefore explored the possibility of neutral 159 being a cluster of several solvent components present in the LC effluent at the time of ESI. There are several possibilities: a mixed cluster of acetonitrile and water, $(\text{CH}_3\text{CN})_3(\text{H}_2\text{O})_2$ corresponding to mass 159.1008; $(\text{H}_2\text{O})_6(\text{OH})_3$ corresponding to mass 159.0716; $\text{CH}_3\text{COOH}(\text{CH}_3\text{CN})_2\text{OH}$ corresponding to mass 159.0770; propenenitrile cluster $(\text{C}_3\text{H}_3\text{N})_3$ corresponding to mass 159.0529 and $(\text{CH}_3\text{COO})_2(\text{CH}_3\text{CN})$ corresponding to mass 159.0531. We can rule out the first three clusters as the mass error from the measured mass of 159.0502 is quite large, even though neutral and charged iron hydroxides, such as FeO , FeOH , $\text{Fe}(\text{H}_2\text{O})$, $\text{Fe}(\text{OH})\text{H}_2\text{O}$ and FeO_mH_n are involved in the redox chemistry of iron in the aqueous media¹² and clusters of acetonitrile and water have also been observed¹³. Clusters of CH_3COO with Fe have been observed in ESI when a SS tip is used. For example, mass 537.88 corresponding to $(\text{CH}_3\text{COO})_6\text{Fe}_3\text{O}^+$ has been commonly observed as an interference ion in direct infusion and LC experiments when acetic acid based mobile phases are used in conjunction with SS spray tip or union⁶. However, it does not balance the total charge to give a singly charged ion. Also, none of these possible components for mass 159 give the correct masses for m/z 1103 and 944 that are close to the observed masses. The full compositions of these two ions therefore remain uncertain at this time.

The presence of Fe as a constituent of these complexes is not surprising considering that the SS union contains Fe that is likely leached by TFA into the spray solution. For example, Ijames et al.¹⁴ observed some Fe containing-ions from peptide solutions containing TFA when a SS capillary was used for electrospray and a positive voltage applied to the capillary. An alternative possibility for the formation of these ions may be that Fe ions are released electrochemically during ESI. It has been shown by Blades et al.¹⁵, Van Berkel et al.^{16–18}, Konermann et al.¹⁹ and Liu et al.²⁰ that electrospray ion source is in effect a controlled-current electrolytic cell where ions of the same polarity as the voltage applied to the electrospray tip migrate from the bulk solution and are emitted from the tip. Blades et al.¹⁵ observed Zn- and Fe-containing ions in the ESI when a Zn or SS capillary tip was used. These authors suggested that iron and zinc dissolution from the ESI capillaries is due to electrospray-induced oxidation reaction occurring at the metal-ligand interface. A similar process may be occurring in our experiments, especially in the presence of TFA.

Using direct infusion experiments, we excluded all possibilities of contamination from solvents, chemicals, and column packing material. Therefore, formation of the two ions during the ESI process was most likely due to a chemical/electrochemical process involving TFA with the SS union when LC effluent is present. To understand these processes and to stop the formation of these two ions, we explored several options of applying high voltage differently but it did not succeed as we lost ions of interest as well. We observed that addition of a ~5 cm long unpacked fused silica column (50 μm i.d) after the 70 cm LC column, which did not lead to any noticeable deterioration in chromatographic separation. Interestingly, the two ions disappeared when the ESI voltage was applied to the union holding the ESI tip, but were observed when the ESI voltage was applied to the SS union connecting the blank capillary to the packed LC column. These variations in the application of high voltage are shown in Fig. . When the blank column was removed, these two peaks

reappeared in the spectrum. In another experiment, we replaced the SS union with a titanium union (without the blank column) and the two ions disappeared while no titanium containing ions were observed, thus confirming our hypothesis that Fe from the SS union interacts with TFA to form clusters observed in the present study. However, the mechanism for the removal of these cluster ions with the addition of a blank column is not clear at the present time.

In summary, we determined that these unexpected ions were formed by the interaction of TFA with Fe from the SS union in the electrospray process resulting in m/z 1103 and fragment ion m/z 944. These ions are eliminated when a small fused silica blank column (no packing material) is added between the packed column and the ESI tip using a metal union where high voltage is applied for electrospray. This arrangement does not adversely affect the separation process or the identification of peptides present in the sample.

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Fig. 1. TIC of the LC-FTICR MS analysis of the QC mixture. Two mass spectra are shown for (a) when a peptide elutes showing normal mass spectrum, and (b) when two background noise peaks at m/z 1103 and m/z 944 dominate the mass spectrum.

TIC OF QC Mixture

Fig. 2.
TIC of a normal LC-FTICR MS run with the QC mixture when there is no interference by the m/z 1103 and m/z 944 ion peaks.

Experimental Isotopic Distribution of m/z 944

Fig. 3.
Experimentally observed isotopic distribution for the m/z 944 ion.

**Unexpected
1103/ 944 Ions** **Fig. 4.**

Schematics of the LC-MS setup showing the addition of a short blank column between the packed LC column and the electrospray tip and the location where high voltage is applied to remove m/z 1103 and m/z 944 ions.

Fragmentation Sequence

Scheme 1.