Non-Target Analysis of Urine by Electrospray Ionization – High Field Asymmetric Waveform Ion Mobility – Tandem Mass Spectrometry (ESI-FAIMS-MS/MS)

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S-1: Additional Experimental Details

Ammonium acetate and HPLC grade water and methanol were purchased from Fisher Scientific (Nepean, Ontario). The standard solution used for intensity optimization consisted of ~500 nM of 1-methylguanine, 9-ethylguanine, 2’-deoxyguanosine, 2’-deoxyinosine, N^2- methylguanosine (Sigma-Aldrich, Oakville, ON) and O^2’-methylcytidine (R.I. Chemicals, Inc., Orange, CA) in 9:1 methanol:water with 0.1 mM ammonium acetate. An overnight urine sample was collected from a healthy volunteer into a 50 mL PTFE tube and centrifuged at 19 000 rpm for 120 min. The supernatant was decanted and frozen in 4 mL aliquots at –20 °C until further purification. Thawed aliquots of supernatant were loaded onto an activated 200 mg Oasis HLB glass solid phase extraction cartridge (Waters, UK), washed with 4 mL of water and eluted with 4 mL of 70% methanol in water. A 10 µL subsample of the eluent was diluted to 1 mL with the buffer described above.

Dilute urine samples were infused using the auto-sampler and pump from a nano-Acquity nano-flow capillary UPLC system (Waters, UK). Injections were made using a 50 µL sample loop into a 400 nL/min flow of the same composition as the dilution buffer. Ionization was carried out in positive mode using a nano-electrospray source with a spray voltage of 5000 V (4000 V relative to FAIMS curtain plate). Detection was carried out using a Q-TOF micro mass spectrometer (Waters, UK) in either full scan (MS) mode over a range of CV values or in tandem (MS/MS) mode at a particular CV value. In MS mode a cone voltage of 14 V and a collision energy of 4 V were used to minimize ion dissociation in the MS source after FAIMS separation, as well as a scan time of 15 sec. In MS/MS mode, a cone voltage of 25 V and a scan time of 5 sec were used and collision energy (CE) was optimized and is presented in figure captions. External mass calibration was carried out using Glu-Fibrinopeptide and internal mass calibration...
was carried out using the well characterized ions of known composition shown in Fig. 1. Mass accuracies in MS mode were < 10 ppm and those in MS/MS mode ranged from 1 – 120 ppm with a typical value around +/-20 ppm. These represent realistic mass accuracies for the instrument employed and were sufficient to determine the elemental composition of the product ions presented.

The LC-ESI-MS/MS method used for comparison of the quality of spectral data included a nano-flow capillary reversed phase separation with online pre-concentration using the nano-Acquity LC system and MS detection using the same nano-ESI source and Q-TOF-MS as the FAIMS experiments. The urine sample was the same as the sample used for FAIMS analysis but was 400 times more concentrated. Injections of 3.5 µL were made onto a 10 mm x 180 µm, 5 µm C18 trapping column where it was washed with aqueous mobile phase before being eluted onto a 150 mm x 75 µm, 3 µm analytical column with a 45 min linear gradient to 100% organic mobile phase. This method used data dependant acquisition (DDA) to acquire MS data for all eluting compounds and MS/MS spectra for a few hundred of the most abundant species. Conditions in MS mode were the same as those described above but with a capillary (nanospray) voltage of 3700 V. A 1 sec MS scan was used to determine the three most abundant ions at a particular retention time. These were then automatically selected for MS/MS detection and sampled alternately for nine 1 sec scans each at a CE alternating between 15 and 30 V. These energies represent conditions optimized to show abundant protonated bases as product ions of modified nucleosides (15V) as well as product ions of base fragmentation useful for establishing the site of base modification (30V). After MS/MS acquisition, an ion was excluded from selection for 30 seconds to avoid continuous selection of the same precursors.
When the Ionalytics Selectra was coupled to the Q-TOF using the supplied source interface and manufacturer recommended specifications practically no ion current was detected from a mixture of modified nucleoside and NA base standards. Trace 1 in Fig. S-1 shows the extremely low intensity of total ion current for the standards that was detected when the compensation voltage (CV) was scanned. This trace, referred to as a total ion compensation voltage spectrum, was collected by continuously scanning CV from 0 to 35 V at a rate of 0.4 V/min with MS acquisition at 0.1 V steps. Even at this low ion current, it was evident that a CV separation of analytes was occurring but analytical sensitivity of the method was too low to carry out any practical analysis. Using the same mixture of standard compounds, we set out to improve and optimize the performance of ESI-FAIMS-MS with respect to ion transmission and reproducibility of CV separation.

The purity of the carrier gas in FAIMS has a profound effect on the instrument’s performance. Even at small amounts, water vapor has a significant impact on the reproducibility of CV separation because of interactions between ions and water molecules that change their ion mobility at either high or low field strength. Three measures were taken to minimize the impact of residual water vapor on the FAIMS operation in our relatively humid climate. First, to minimize the water vapor contribution from the carrier gas we installed two hydrocarbon/moisture traps in series (1:1 by volume molecular sieves: activated carbon), which was critical for stable operation of FAIMS and achieving reproducible CV separations. Second, we changed the configuration of the nano-electrospray source which originally had the spray tip located only a few mm from and in direct alignment with the orifice in the FAIMS curtain plate. This direct mode of spraying resulted in fast contamination of the FAIMS electrodes and the
introduction of droplets and solvent vapors into the carrier gas. By installing the sprayer off-axis by 20°, solvent droplets and neutrals strike the curtain plate to one side of the inlet and are not introduced between the FAIMS electrodes. This modified off-axis configuration resulted in the modest signal increase seen in trace 2 in Fig. S-1 but effectively eliminated contamination problems. This allowed the total carrier gas flow (used in main part for ion desolvation and in small part for ion transport in FAIMS) to be decreased from 2 to 1 L/min producing another increase in the intensity of detected ions (trace 2 in Fig. S-1). Finally, including a relatively large proportion of CO$_2$ (20%) as a component of the carrier gas gave an increase in signal intensity (trace 4 in Fig. S-1) but also significantly improved reproducibility of CV separations.

Fig. S-1: Additive effects of sequential optimization of signal intensity from the manufacturer recommended configuration (1) to an off-axis ESI (2), a carrier gas flow of 1 L/min (3), addition of 20% CO$_2$ to the carrier gas (4), and installation of an additional o-ring around the MS-sampling cone (5).
This can be attributed to the fact that an excess of polar CO$_2$ will out-compete trace amounts of water vapor for polar interactions with analyte ions, increasing the threshold where water impacts the separation. Together, these modifications have increased the time for which the CV value of an analyte remains constant from just minutes with the default configuration to approximately 5 days, even when using much less expensive liquid nitrogen boil-off.

The most significant gain in detection sensitivity (trace 5 in Fig. S-1) was obtained by improving ion transmission at the interface between the FAIMS device and the inlet of the mass spectrometer. Figure 1 illustrates the configuration of the commercial interface which consists of the interface lens (a) with a concave orifice on both the side of the FAIMS electrodes (b) held in place by a PEEK spacer (c) and the inner MS sampling cone (d). The lens (a) and the inner cone (d) are both held at the same voltage controlled by the MS software. This interface has been designed with the purpose of analyzing ions of large biomolecules so even at the optimal experimental conditions the ion transmission of small species is very low (trace 4 in Fig. S-1). We have investigated different modifications of the interface and developed a simple solution. Plugging the 0.3 mm gap between the interface lens and the inner cone with a 1.5 mm ID x 1 mm Viton O-ring (e) (Able Seal & Design, Concord, ON) has greatly improved transmission of small ions (trace 4 to trace 5 in Fig. S-1). Poor ion transmission in the commercial source is due to a disruption of the ion beam from the FAIMS outlet (f) to the entrance of the sampling cone (d).

Gas flow in the gap between the interface lens and the sampling cone causes turbulence at the entrance of the sampling cone and significant loss of ions. This is supported by the fact that ion transmission deteriorates significantly even with small increases in gas flow, especially during sampling of smaller ions which exhibit a small momentum during ion transport. In fact, one of the only successful couplings the commercial Selectra to MS has previously been
accomplished using a highly sophisticated custom RF-ion funnel\textsuperscript{10,11} instead of the commercial interface. Based on our experience, the poor ion transmission from the FAIMS device into the mass spectrometer, especially for small ions, is the main reason for the lack of success of this technique. Our simple modification of the commercial interface and the optimization of experimental conditions provide an increase in signal intensity of over two orders of magnitude as illustrated in Fig. S-1. Most importantly, with our modifications an investigation of standard compounds and real urine samples was possible.

Fig. S-2 shows the first FAIMS separation of electrospray-generated, protonated ions of standard nucleosides and NA bases at optimized conditions. Such ions exhibit larger ion mobility at a high electric field and are transmitted at positive values of CV. Modified bases with a more rigid and compact structure are separated at higher positive CV than modified nucleosides. The narrow range of CV where modified nucleosides are transmitted suggests that the dangling sugar moiety represents the dominant feature in their mobility separation. Separation properties of nucleosides and bases in FAIMS are important because they can be used for selecting the most appropriate CV conditions at which modified nucleosides, modified bases and other similar metabolites are transmitted during ESI-FAIMS-MS analysis of a real urine sample.
**Fig. S-2:** Extracted ion CV spectra showing the separation of the standard mixture of nucleosides and modified bases at signal optimized conditions.
S-3: Gas Phase Reactivity of 3-Methylcytosine During Collision Induced Dissociation

The MS/MS spectrum in Fig. 3D is consistent with dissociation of N3-methylcytosine but many observed dissociation pathways have not previously been explained. The gas phase ion chemistry of cytidine has previously been investigated using several isotopically labeled and structurally modified analogues. Gas phase reactions of protonated N3-methylcytosine are quite complex because during collisional activation tautomerization, proton transfer, and charge redistribution reactions can occur, all of which are directed by the positive charge. For brevity, only tautomeric forms of precursor ions leading to specific dissociation products will be considered in our discussion. Scheme S-1 illustrates proposed reactions describing the formation of all product ions detected in Fig. 3D using similar charge directed mechanism to that of water loss from unmodified cytosine in Scheme 1A.

Scheme S-1A illustrates formation of the major CID products corresponding to loss of ammonia (m/z 109) and methylamine (m/z 95) detected in Fig. 3D. Ring opening at the C2-N3 bond, charge redistribution from N^4, and proton transfer from N^4 to N3 lead to elimination of methylamine (-31 Da) and formation of the highly resonance stabilized ion detected at m/z 95. The analogous reaction (not shown) involving the ring opening, charge redistribution from N3 and proton transfer from N3 to N^4 leads to elimination of ammonia (-17 Da) and formation of a methyl analogue of the m/z 95 detected at m/z 119. Formation of the m/z 82 fragment (Scheme S-1B) is associated with a proton transfer to exocyclic N^4 without ring opening which results in concerted loss of ammonia (-17 Da) and hydrogen cyanide (-27 Da). The charge on the product ion of this reaction could be stabilized by both resonance from N and O lone pairs but also by homoconjugation through space from π electron density in the C-C triple bond. The N3-imino tautomer shown in Scheme S-1C generates an intermediate ion in which a proton transfer from
C6 to N⁴ through a 5-membered ring arrangement assists in elimination of CO (-28 Da) and spontaneous loss of ammonia to produce the highly resonance stabilized product detected at m/z 81. An analogous reaction (not shown) to Scheme S-1C proceeding from the N3 protonated N⁴-imino tautomer leads to ring opening at the C2-N3 bond, proton transfer from C6 to N3 and CO elimination followed by spontaneous loss of methylamine to form the product ion detected at m/z 67. These two reaction are fully supported by a previous isotope labeling study of another pyrimidine base, uracil, were the analogous [M+H-CO-NH₃]⁺ ion is produced by loss of a proton from C6, as proposed here. Scheme S-1D shows reactions of the N⁴ protonated N⁴-imino tautomer leading to formation of the fragment ion detected at m/z 69. The intra-molecular nucleophilic attack by N1 at C4 forms a reactive bicyclic intermediate ion which eliminates methyl isocyanic acid (-CH₃NCO, -57 Da) to produce the highly resonance stabilized product. Each of the reactions presented in Scheme S-1 help confirm the structure of the investigated ion. The fact that all product ions detected originate from 3-methylcytosine nicely demonstrates the strength of FAIMS to separate analytes of interest from unresolved electrospray background ions. Even in ESI-MS/MS or LC-ESI-MS/MS experiments involving pure standards, product ions are detected that are not associated with the targeted precursor. This problem becomes much more severe in complex mixtures and when low abundance precursors are selected.
S-4: Additional References
