Matching Old Samples to New Assays: Two Case Studies

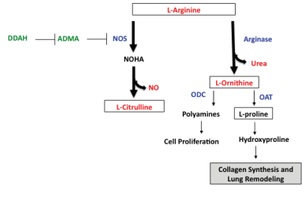
Alan W. Taylor
Oregon State University, Corvallis, OR

Case 1: Dimethylarginines

Nitric oxide (NO) is an important biological mediator produced from arginine (Arg) by the enzyme nitric oxide synthase (NOS). Asymmetric dimethylarginine (ADMA) is a competitive inhibitor of all isoforms of NOS. ADMA is implicated in the pathogenesis of pulmonary hypertension, burn and smoke inhalation injury, sepsis, and endothelial dysfunction.

We were asked to develop an assay for ADMA in lung tissue and plasma to support the work of colleagues studying burn and smoke injuries. Also needed were measurements of SDMA (symmetric DMA), citrulline, and ornithine in lung and plasma.

Arginine Metabolism

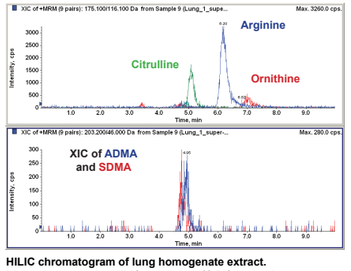


Published assays have used high aqueous, high acid mobile phases on silica for LC separation, which did not perform reproducibly in our lab. ADMA and SDMA did not separate sufficiently for MS detection, but do produce unique product ions. We therefore began work on a new LC-MS/MS assay using materials, columns and instruments readily available in our lab.

Starting Method

To keep the assay simple, we chose protein precipitation with organic solvent to prepare samples for direct injection on HILIC (hydrophilic interaction liquid chromatography). Mixing plasma with methanol first, then acetonitrile, resulted in a finer precipitate and ~4-fold improved extraction of analytes over use of acetonitrile alone.

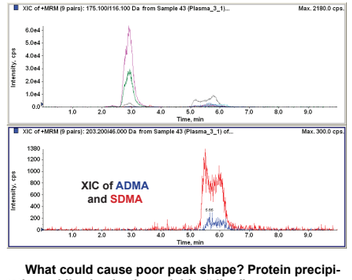
HILIC gave good separation of analytes, especially of arginine (*m/z* 175) and citrulline (*m/z* 176). However, sensitivity was marginal for the di-methylarginines, the analytes of most interest.



HILIC chromatogram of lung homogenate extract. Phenomenex 15 cm HILIC column, 10% B for 0.5 min, ramp to 40% B in 2.5 min, and hold. Mobile phases are 10 mM ammonium formate, 100 mM formic acid in 95:5 MeCN:water (A) and water (B).

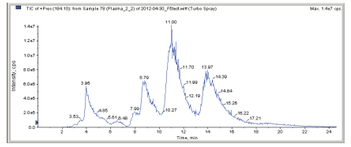
Final Fixes

No change of mobile phase or gradient was able to give good peak shape with 5 μL sample injections, the minimum needed for acceptable quantitation.



What could cause poor peak shape? Protein precipitation, while simple, does yield a "dirty" extract.

A chromatographic scan for precursors of *m/z* 184 revealed large amounts of phosphotidyl choline (PC) and lyso-PC, known to cause ion suppression.



Case 2: Glutathione

Glutathione is the most abundant non-protein thiol in the body. Reduced glutathione (GSH) is important for protection from oxidative damage. Accurate determination of GSH, and its oxidized form (GSSG), provides an important indicator of the redox status of cells, tissues and organisms in various states of health and disease.

Established LC-UV and LC-fluorescence methods for quantifying GSH, GSSG, and other thiols in plasma, cells, and tissue homogenates require time-consuming sample preparation and lengthy chromatography. Identification is based on retention time because detection is based on a single absorbance wavelength or fluorescence excitation/emission pair. The more specific identification provided by mass spectrometry enables faster chromatography that need not resolve every analyte in time. While free thiols must be protected for accurate measurement of the redox state, no chromophore or fluorophore is needed for MS detection, compared to UV or fluorescence.

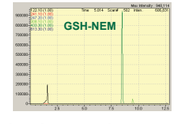
However, our investigators have a large number of samples prepared using standard PCA sample preservation, i. e., the samples contain 5% w/v perchloric acid. Thus, I have the challenge of adapting these samples to a new analytical method: derivatization with NEM to protect free thiols, separation by hydrophilic interaction chromatography (HILIC), and detection by selective ion monitoring (SIM) on a readily available single quadrupole mass spectrometer. As some investigators are interested in other thiols, I included the cysteine-cystine redox pair (Cys and CysSSCys, respectively) during development, in the hope of achieving a more generally applicable method.

Why neutralize?

To make the chromatography work:

PCA-preserved plasma with GSH and NEM

Same, but neutralized with KOH to pH 7-8



To make the derivatization work:

Standards in water

15 min

75 min

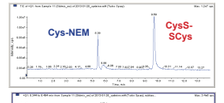
48 hours

Ammonium bicarbonate added to standards

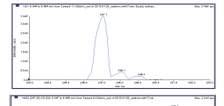
Change in Cys-NEM

Cys and NEM in water (pH ~5)

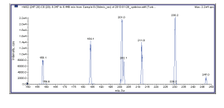
TIC of Q1 scan, *m/z* 240 - 280



Q1 spectrum, RT 5.39 min

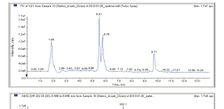


Product ions of *m/z* 247.2, RT 5.39 min

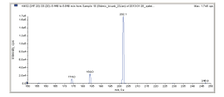


Cys and NEM in NH_4HCO_3 (pH ~8)

TIC of Q1 scan, *m/z* 240 - 280

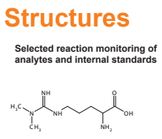


Product ions of *m/z* 247.2, RT 5.51 min

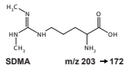


Structures

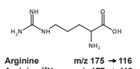
Selected reaction monitoring of analytes and internal standards



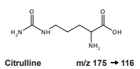
ADMA m/z 203 → 46
ADMA-D_3 m/z 210 → 46



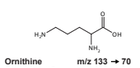
SDMA m/z 203 → 172



Arginine m/z 175 → 116
Arginine-$^{15}N_2$ m/z 177 → 116



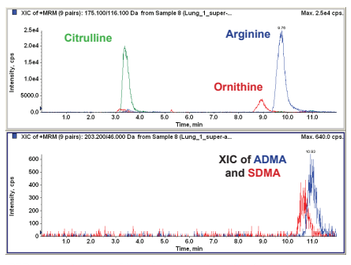
Citrulline m/z 175 → 116
Citrulline-D_2 m/z 177 → 116



Ornithine m/z 133 → 70
Ornithine-$^{13}C_5$ m/z 138 → 74

Switch to New Column

A new PFP (perfluorophenyl) column was tested with the same samples, giving improved sensitivity, perhaps because of the absence of salts in the mobile phase.

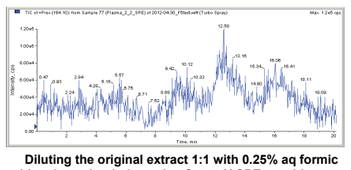


PFP chromatogram of lung homogenate extract. Supelco Ascentis Express 15 cm F5 column, 40% B for 1 min, ramp to 50% in 2 min, and hold. Mobile phases are MeOH (A) and 0.1% formic acid (aq) (B). Note: B is water, as in HILIC.

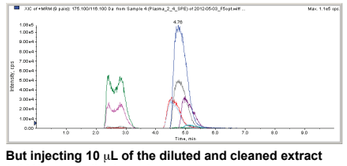
However, the same chromatography did not work well with plasma extracts, something discovered after a large number of samples had already been prepared.

Several adjustments to the chromatography were tried in an effort to salvage extracts that could not be re-prepared from original samples.

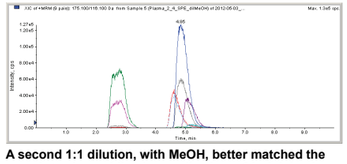
During this testing, retention times shortened. Use of mobile phases containing 1 mM ammonium formate yielded stable RTs, but at a cost of lesser sensitivity, only 3X that in the original HILIC (10 mM), rather than the 10X seen at first on PFP without salt.



Diluting the original extract 1:1 with 0.25% aq formic acid and passing it through a Strata X SPE cartridge removed the PC contamination...

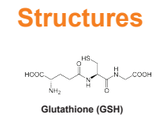


But injecting 10 μL of the diluted and cleaned extract (~60% water) didn't work...

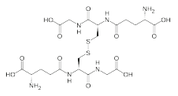


A second 1:1 dilution, with MeOH, better matched the sample to the mobile phase, allowing a 20 μL injection, equivalent to 5 μL of the original, uncleaned extract.

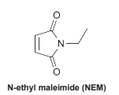
Structures



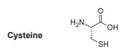
Glutathione (GSH)



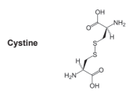
Glutathione disulfide (GSSG)



N-ethyl maleimide (NEM)



Cysteine



Cystine

I thought this would be simple...

NEM and ammonium bicarbonate should be the perfect combination:

NEM reacts with thiols at pH 6.5 - 7.5, making it ideal for working with blood, for example. At higher pHs, it may react with amines, as well. But that shouldn't be a problem, because:

Ammonium bicarbonate in aqueous solution has a pH of 7.6. It has the additional virtue that it is volatile in an ion source. If used to neutralize perchloric acid, the resulting ammonium perchlorate may decompose to N_2, O_2, Cl_2, and water with heating, e. g., in a heated ion source. And a little excess should keep the pH just right, so no need to titrate.

At first, everything appears to be working:

Both Cys and GSH derivatize quickly with NEM when buffered with ammonium bicarbonate, while it takes hours for the reaction to complete in water. With no buffer, the apparently more vulnerable Cys progressively oxidizes to CysSSCys, demonstrating the need to quickly protect thiols for accurate redox determination.

So what is going wrong?

Cys-NEM in the ammonium bicarbonate solution quickly begins to change, showing a double peak in the *m/z* 247 chromatogram within a short time. This continues, until the later peak is the only one remaining.

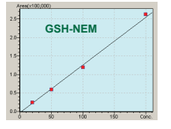
While the objective is to have a method based on the single quad, more information is needed: time to run some tests on the triple quad!

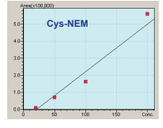
What did the triple quad say?

Cys-NEM in the ammonium bicarbonate solution changes in structure, yielding a peak with a slightly later retention time in the *m/z* 247 chromatogram and a different product ion spectrum. In fact, this is the spectrum reported by Shuford et al. (Anal Bioanal Chem (2012) 402: 357-366), and their method uses 50 mM ammonium bicarbonate. They quench the reaction with acetic acid, but it's too late! I would prefer to use the initial reaction product, prior to the rearrangement Shuford proposed. I'm working on the right balance to achieve this.

Remaining Problems

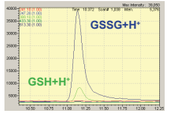
Standard curves for GS-NEM are linear, but not for Cys-NEM: probably side reactions: discover and stop





At higher concentrations, more GSSG cleaves to produce *m/z* 308 ion ($GSH+H^+$): reoptimize ion source and entrance optics settings

10 uM GSSG



100 uM GSSG