Case 1: Dimethylarginines

Nitric oxide (NO) is an important biological mediator produced from arginine by the enzymes nitric oxide synthase (NOS). Aggregative dimethylarginine (ADMA) is a competitive inhibitor of all isoforms of NOS. ADMA is implicated in the pathogenesis of pulmonary hypertension, lung and coronary artery disease, and thrombosis. We were asked to develop an assay for ADMA in lung homogenate extract by newly purchased high performance liquid chromatography (HPLC) and mass spectrometry (LC-MS/MS) for a study on the role of cytokines in regulating lung function. We chose protein micro precipitate HILIC (10 mM) rather than other mobile phases containing 100 mM formic acid in 95:5 MeCN:water (A) and 0.1% formic acid (aq) (B). Note: B is water with acetic acid, but it does not 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 sample for digestion with trypsin and then analysis with HPLC-MS/MS chromatography. Mixing samples with methanol first, then acetonitrile, resulted in a final precipitate and -fold improvement in analyte over use of acetonitrile.

HILIC gave good separation of analytes, especially arginine (m/z 133) and citrulline (m/z 172). However, sensitivity was marginal for the di-methylarginines, the analytes of most interest.

Final Fixes

No change of mobile phase or gradient was able to give better separation of the 15 XIC shown. The minimum needed for acceptable quantitation.

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 progression.

Established LC-UV and LC-fluorescence methods for quantifying GSH, GSSG, and other thiols in plasma, sera, and tissue homogenates require time-consuming sample preparation and lengthy chromatographies. Identification is based on retention time because detection is based on a single absorbance wavelength or fluorescence excitation/emission pair. This means that chromatographic scans for precursors of GSH and GSSG may not be readily available single-quadrupole mass spectrometry. In addition, there has been no work to date included the cysteine-cystine redox pair (Cys and CysS-S, respectively) during development, in the hope of achieving a more generally applicable method.

Why neutralize?

To make the chromatography work:

PC-performant plates with high pH and high ion strength.

To make the derivatization work:

Standards in water.

Ammonium bicarbonate added to standards

Product ions of

Product ions of

Product ions of

What could cause poor peak shape? Protein precipitation, while simple, does yield a "dirty" extract. A chromatographic scan for precursors of 134 re-veals base peak 184 (GSH), 210 (GSSG), and 203 (ADMA) phenylboronic acid (PhB) and tfa-q, known to cause ion suppression.

However, our investigations have a large number of samples prepared using standard PC-MS analysis. i.e., the sample contains 10% acetic acid, acetonitrile, 100 mM formic acid, and 0.1% formic acid (aq).

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I thought this could be simple...

NEM and ammonium bicarbonate should be the preferred combination:

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

Ammonium bicarbonate in aqueous solution has a pH of 8. But the additional 10 uM GSSG

What did the triple quad say?

Cys-NEM in the ammonium bicarbonate solution changed in structure, yielding a peak with a slightly lower retention time (~ t0.75). With 100 uM GSSG and a different product ion spectrum, the peak was entirely different to GSH-NEM.

Cys-NEM in NH4CO3 (pH ~8)

GSH+HCl ~48

GSH+HCl ~75

HCl ~48

GSH+HCl ~108

HCl ~108

Resting Remaining Problems

Sulfur compounds, GSSG, cysteine, Cys-NEM, CysS-S, with GSH and NEM. The more important indicator of the redox status of cells, tissues and organisms in various states of health and disease progression.

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