LC-MS/MS analysis of nucleotides

Targeted profiling of nucleotide metabolites was conducted at Duke University. 300 µl of tissue homogenates prepared at 50 mg/ml in 70% methanol were spiked with nine internal standards: $^{13}C^{10}$,$^{15}N^{5}$-adenosine monophosphate, $^{13}C^{10}$,$^{15}N^{5}$-guanosine monophosphate, $^{13}C^{10}$,$^{15}N^{2}$-uridine monophosphate, $^{13}C^{9}$,$^{15}N^{3}$-cytidine monophosphate, $^{13}C^{10}$-guanosine triphosphate, $^{13}C^{10}$-uridine triphosphate, $^{13}C^{9}$-cytidine triphosphate, $^{13}C^{10}$-adenosine triphosphate, and nicotinamide-1, N^{6}-ethenoadenine dinucleotide (eNAD) (Sigma-Aldrich). Nucleotides were extracted using an equal volume of hexane as described by Cordell et al. and Gooding et al.[51,52]. The samples were vortexed and centrifuged at 14,000 x g for 5 minutes. The bottom layer was collected and centrifuged again. Chromatographic separations and MS analysis of the supernatants were performed using an Acquity UPLC system (Waters) coupled to a Xevo TQ-XS quadrupole mass spectrometer and (Waters). The analytical column (Chromolith FastGradient RP-18e 50-2mm column, EMD Millipore, Billerica, MA, USA) was maintained at 40°C. The injection volume was 2 µL. Nucleotides were separated using a mobile phase A consisting of 95% water, 5% methanol, and 5 mM dimethylhexylamine adjusted to pH 7.5 with acetic acid and a mobile phase B consisting of 20% water, 80% methanol, and 10 mM dimethylhexylamine. Flow rate was set to 0.3 ml/min. The 22-minute gradient (t=0, %B=0; t=1.2, %B=0; t=22, %B=40) was followed by a 3-minute wash and 7-minute equilibration. Nucleotides were detected in the negative ion MRM mode based on characteristic fragmentation reactions. The endogenous nucleotides were quantified using calibrators prepared by spiking tissue homogenates with authentic nucleotides (Sigma-Aldrich).