BioPharma

Sensitive detection of post transcriptional modifications in tRNA by microflow UHPLC-HRAM-MS/MS

Shweta Chhajed, Robert L. Ross, Thermo Fisher Scientific, Lexington, MA, USA

Abstract

Transfer Ribonucleic Acid (tRNA) contain the greatest density of post transcriptional modifications than any other RNA in the cell. These modifications range from simple methylations to more intricate chemistry resulting from extensive enzymatic pathways. Historically, RNase digestion followed by LC-MS/MS analysis has been applied for analysis of tRNA modifications. A challenge for nucleoside analysis has been the need to inject microgram quantities (>10µg) of the enzymatic digest to detect and characterize these modifications. Here we show the utility of microbore chromatography for the UHPLC-HRAM-MS/MS characterization of *E. coli MRE 600* tRNA nucleosides using less than 1 ug of sample

Introduction

tRNA modifications play a crucial role in various biological processes, including translation where Wobble position modifications assist in codon-anticodon pairing. Modification of the tRNA occurs post transcriptionally and can affect tRNA stability, folding, and interaction with other molecules. Liquid chromatography coupled to tandem mass spectrometry is the gold standard for characterization of post transcriptional modifications. One challenge for performing this assay is the large amounts of cells or tissue needed to produce enough tRNA for analysis. Here we show the capability of the Thermo Scientific™ Vanquish™ Neo UHPLC system coupled to the state-of-the art Orbitrap™ Ascend BioPharma Tribrid™ mass spectrometer for the analysis of modified nucleosides utilizing a microflow LC separation. By utilizing microflow chromatography, we decrease sample consumption while simultaneously increasing sensitivity due to a more facile desolvation due to the lower flow rate used. The Thermo Scientific™ ProFlow™ XR pump technology enables robust operation up to 1500 bar from 1 nL/min to 100 µL/min with active flow control for the entire flow range, enabling excellent retention time precision for long gradients as well as ultra-short run times. The Orbitrap™ Ascend BioPharma Tribrid™ MS has an improved high-sensitivity atmospheric pressure interface, combining a high-capacity transfer tube and a modified electrodynamic ion funnel, which provides increased ion flux resulting in lower limits of detection. Enhanced Vacuum Technology improves the Thermo Scientific™ Orbitrap™ mass analyzer performance by decreasing residual gas molecules which can interfere with ion transmission. Together the Vanquish Neo UHPLC coupled to the Orbitrap Ascend BioPharma Tribrid MS results in a state-of-the art analytical platform for low sample consumption analysis of post transcriptional modifications in tRNA.

Materials and methods

Sample Preparation

Total tRNA from *E.coli* MRE 600 (Sigma Aldrich) were aliquoted at four different amount (20 µg,10 µg,5 µg and 2.5 µg). Samples were digested enzymatically to monomers as previously reported.[1] The digested nucleosides were then dried in speed vac and resuspended in 10ul of mobile phase A for analysis.

LC Method

1 ul of each sample was injected onto an Evosphere® AQUA C_{18} UHPLC column, 0.5 x 100 mm, 1.7 um, with the Thermo Fisher Scientific™ Vanquish™ Neo UHPLC system at a flow rate of 30 µL min^{-1} using an ammonium acetate/acetonitrile buffer system. Mobile Phase A was 5 mM Ammonium acetate pH 5, Mobile Phase B was 60% MPA and 40% ACN. Gradient starts at 0% B held for 0.1 min then increasing to 1.5% at 7.2 min, 3% at 9.7 min, 5% at 15 min, 25% at 18 min, 50% at 20 min, 75% at 23 min, and held for 1.5 min before increasing to 90% at 30 min, 99% at 35 min then returning to 0% for 10 column volume re-equilibration.

MS Method

Acquisition was performed on a Thermo Scientific™ Orbitrap™ Ascend Tribrid™ mass spectrometer running Xcalibur™ 4.6. For all experiments, data was collected in small molecule application mode. Full scan at a 60000 orbitrap resolution with a scan range of 240 to 600 m/z was used. AGC target was set to standard, microscans was 1, the RF lens was 35% and maximum injection time was auto. MS2 was performed with decision tree method involving CID (collision induced dissociation) and HCD (higher energy collisional dissociation) in orbitrap with a Resolution of 30000. For CID collision energy of 40%, activation time of 10 ms and activation q of 0.25 was used. For HCD, 60V of energy, activation time of 100 ms and microscans of 1 was used.[2]

Data Analysis

Data was processed using Thermo Scientific™ Freestyle™ 1.8 and Compound Discoverer 3.3 as previously reported.[2]

Nucleoside digestion workflow

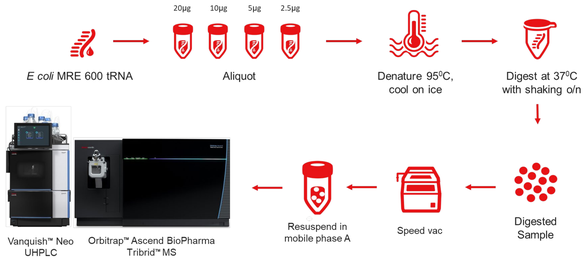

Figure 1. Workflow used for total nucleoside analysis by microbore LC-MS/MS

Results

Total tRNA from *E coli* MRE 600 was the sample used in this assay. The intact tRNA was aliquoted in 4 different concentrations 20 µg, 1 µg, 5 µg, and 2.5 µg. These samples were taken to the nucleoside level by enzymatic digestion, dried in speed vac and resuspended in 10 µL of mobile phase A. One µL of each sample was injected onto the microbore column and acquired with the Orbitrap™ Ascend BioPharma Tribrid™ mass spectrometer for detection of the individual post-transcriptional modifications in the sample. **Figure 1** shows the workflow for the assay.

From the known modifications in tRNA, Dihydrouridine is the most polar. This chemical property results in the modification being the first to elute from the column, and thus can be used as a marker for determination of digestion and chromatographic performance. Here we used dihydrouridine to monitor the efficiency of the digestion. Given that the modification is highly polar its ionization efficiency is poor, resulting in a lower response in the chromatogram. Should the digestion fail to reach completion, the response of dihydrouridine in the mass spectrometer would vary. **Figure 3A** shows extracted ion chromatograms of four digests, ranging from 2 ug to 250 ng. An exponential fit (93%) was found by plotting the peak areas of the four digests. We found that the response of the 2 ug injection was slightly under the fit where the 1 ug injection was slightly over. This could suggest the optimal on column amount of digest is between 0.5 to 1 ug. More work would be needed to verify the observation.

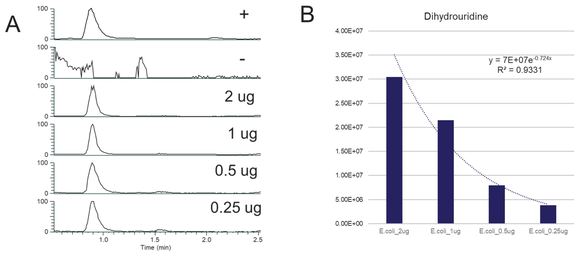

Figure 2. A) Extracted ion chromatograms of dihydrouridine at four different injection amounts. Top two panes show a positive (50 ng) and negative control response. B) Peak areas of the four injected amounts plotted as bar graphs. A 93% exponential fit suggests that the optimal amount of sample to inject lies between 1 and 0.5 ugs.

Table 1. Post transcriptional modifications of *E. coli* MRE600 total tRNA detected in a 2 ug injection

Name	Formula	Delta Mass [ppm]	Calc. MW	m/z	RT [min]
D	C9 H14 N2 O6	-0.34	246.0851	247.09236	0.907
Y	C9 H12 N2 O6	-0.53	244.06941	245.07668	1.006
chm5U	C11 H14 N2 O9	-0.44	318.06979	319.07704	1.957
ho5U	C9 H12 N2 O7	0.53	260.06459	261.07189	2.524
s2C	C9 H13 N3 O4 S	-0.47	259.06255	260.06982	3.002
m1A	C11 H15 N5 O4	-0.33	281.11231	282.11959	3.329
mnm5s2U	C11 H17 N3 O5 S	-0.85	303.08863	304.0959	4.02
cmnm5s2U	C12 H17 N3 O7 S	-0.64	347.0785	348.08576	4.132
Cm	C10 H15 N3 O5	-1	257.10091	258.10821	5.095
m7G	C11 H15 N5 O5	-0.32	297.10722	298.1145	5.616
cmnm5Um	C13 H19 N3 O8	-0.91	345.1169	346.1242	5.816
I	C10 H12 N4 O5	-0.88	268.08053	269.08779	6.11
T	C10 H14 N2 O6	-0.77	258.08499	281.07418	6.382
s4U	C9 H12 N2 O5 S	-0.89	260.04646	283.03568	7.933
cm5s2U	C11 H14 N2 O7 S	-0.9	318.05188	319.05915	7.946
oQtRNA	C17 H23 N5 O8	-1.19	425.15416	426.16143	10.496
k2C	C15 H25 N5 O6	-1.38	371.17997	372.18725	11.188
Gm	C11 H15 N5 O5	-0.81	297.10708	298.11436	11.618
m1G	C11 H15 N5 O5	-0.87	297.10706	298.11434	12.214
ac4C	C11 H15 N3 O6	-1.23	285.09574	286.10306	13.143
mchm5U	C12 H16 N2 O9	-0.8	332.08532	333.09259	16.524
Am	C11 H15 N5 O4	-0.72	281.1122	282.11948	17.484
L-t6A	C15 H20 N6 O8	-0.66	412.13399	413.14125	17.808
m2A	C11 H15 N5 O4	0.09	281.11243	282.11971	18.058
m6A	C11 H15 N5 O4	-0.15	281.11236	282.11964	18.303
m6t6A	C16 H22 N6 O8	-0.84	426.14955	427.15682	18.581
m6,6A	C12 H17 N5 O4	-0.29	295.12797	296.13525	19.681
i6A	C15 H21 N5 O4	-0.87	335.15906	336.16634	21.908
ms2i6A	C16 H23 N5 O4 S	-0.23	381.14699	382.15427	26.265

The published number of known, mature *E. coli* modifications is thirty-two (32).[3] Thirty (30) of the 32 known *E. coli* tRNA modifications were detected across the 4 amounts sampled. Two exceptions were the hyper-modification Queuosine (Q) and its glutamyl containing variant. Queuosine is present in *E. coli* during mid log phase and presents as a hydroxylated variant Hydroxyqueuosine (oQ) when in stationary phase. In this assay we detected oQ in place of Q using the commercial MRE 600 sample and is tabulated in **Table 1**. **Figure 3** shows the comparison of extracted ion chromatograms for the 30 modifications listed in **Table 1**. **Figure 2A** shows the results from the 2 ug injection where **Figure 2B** that of the smallest injection amount (0.25 ug). We found excellent reproducibility in the elution profiles of the four samples with ΔRTs < 0.1 min.

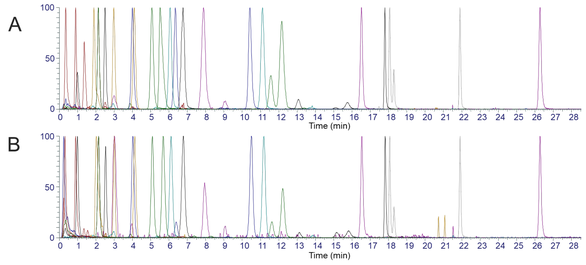

Figure 3. A) Overlayed Extracted Ion Chromatograms (EIC) of the known PTMs in a 2 ug injection of *E. coli* MRE600. B) Overlayed Extracted Ion Chromatograms (EIC) of the known PTMs in a 0.25 ug injection of *E. coli* MRE600. Every predicted nucleoside at 2 ug was detected within the 0.25 ng injection and verified through MS/MS (not shown).

The hyper-modification 2-Lysidine (k2C), is a cytidine derived modification found only at position 34 of the *E. coli* isoleucine decoding tRNA. Where dihydrouridine can be used for digestion and sampling efficiency, dihydrouridine (D) is present in multiple tRNAs, and in some tRNAs at multiple positions. K2C's sole presence makes its abundance in the sample an excellent marker for verification of digestion and LC-MS/MS efficiency as its polarity is such that it elutes later in the gradient. We can use the elution time of D vs k2C to show reproducibility injection to injection and could possibly be used to standardize nucleoside analysis of other sample types.

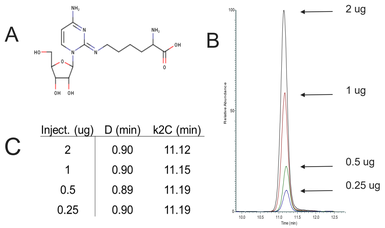

Figure 4. A) Structure of 2-Lysidine (k2C) B) Overlayed extracted ion chromatograms of k2C at the four acquired sample amounts. is detected even at a low concentration of 0.25 µg in the sample. C) elution times of k2C vs D.

Conclusions

For nucleoside analysis we have shown the application of microbore column separations for routine analysis of RNA modifications. Microbore separation on columns with internal diameters less than 1 mm is an attractive method as it has the advantages of the robustness of analytical separation, with the low sample consumption and enhanced sensitivity of nanoflow separations. The analytical platform we present here allows for the low flow (< 50 ul min^{-1}) sampling of RNA modifications from nanogram amounts of starting material. The solvent delivery of the **Vanquish™ Neo UHPLC** yields precise elution profiles from complex samples as well as reproducibility in sample injection amounts shown by comparing the peak areas of dihydrouridine and 2-lysidine. Increased sensitivity is obtained through a more facile desolvation in the NG Max Source as well as improvements in the **Orbitrap™ Ascend BioPharma Tribrid™** mass spectrometer's architecture. Confidence in sample identification is improved through tandem MS. By simultaneously fragmenting in the linear ion trap as well as the ion routing multipole (CID and HCD) the modified nucleoside is identified, and modification position is obtained.[2,4] Data is processed using **Compound Discoverer 3.3**, a powerful software solution that quickly identifies and annotates features in the acquisition through multiple points of identity (spectral matching, HRAM, elution time etc.).

In conclusion, the presented work results in a robust analytical platform for routine RNA modification monitoring where initial sample amounts are in consideration. Here we show a 40-fold decrease in sample consumption with a greater than 3-fold decrease in mobile phase use.

References

1. Ross, R. L., Cao, X., & Limbach, P. A. (2017). Mapping post-transcriptional modifications onto transfer ribonucleic acid sequences by liquid chromatography tandem mass spectrometry. *Biomolecules*, 7(1), 21.
2. Ross, R. L., Yu, N., Zhao, R., Wood, A., & Limbach, P. A. (2023). Automated Identification of Modified Nucleosides during HRAM-LC-MS/MS using a Metabolomics ID Workflow with Neutral Loss Detection. *J Am Soc Mass Spectrom*, *34*(12), 2785-2792.
3. El Yacoubi B, Bailly M, de Crécy-Lagard V. Biosynthesis and function of posttranscriptional modifications of transfer RNAs. *Annu Rev Genet*. 2012;46:69-95
4. Jora M, Burns AP, Ross RL, Lobue PA, Zhao R, Palumbo CM, Beal PA, Addepalli B, Limbach PA. Differentiating Positional Isomers of Nucleoside Modifications by Higher-Energy Collisional Dissociation Mass Spectrometry (HCD MS). *J Am Soc Mass Spectrom*. 2018 Aug;29(8):1745-1756.

Trademarks/licensing