Pathway Targeted Metabolomic Analysis in Oral Cancer Cells Using High Performance Ion Chromatography Coupling to a New HR/AM Orbitrap Mass Spectrometry

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Overview

Purpose: Demonstrate ion chromatography (IC) coupling with high resolution and accurate mass measurement (HR/AM) MS for targeted metabolomics analysis and the possibility of HR/AM full scan to perform targeted quantitattion.

Methods: A high pressure Thermo Scientific™ Dionex™ ICS-5000$^+$ HPIC™ IC system coupled with Thermo Scientific™ Q Exactive™ HF mass spectrometer for nontargeted profiling and targeted TCA cycle quantitative analysis in oral cell lysates using stable isotope labeled standards as external calibration reference.

Results: Higher flow IC achieved short run time (18 min) and reproducible separation with RT variation less than 2 sec during 150 real samples analysis. LOQ (CV<15%) at 0.1 pg/μL for six stable isotope labeled TCA metabolites at five orders of magnitude linear range from 0.2 pg to 20000 pg with $R^2 > 0.995$. The absolute quantitative analysis results agree well with untargeted differential analysis results, demonstrating the capability of using HR/AM Orbitrap full mass scan for targeted quantitative analysis.

Introduction

Mass spectrometry based metabolomics approach has obtained increasing attention in oral cancer study. Recently, a highly analytically sensitive platform coupling capillary ion chromatography (CapIC) with Q Exactive mass spectrometer has been successfully developed for nontargeted metabolic profiling of head and neck cancer cells [1]. The outstanding resolution of IC has led to the differentiation of many isobaric and isomeric polar metabolites, and it has shown a broad coverage to glycolysis and TCA intermediates. Interesting changes of TCA cycle metabolites in cancer stem cells versus non-stem cancer cells were observed.

In this work, we utilized a higher flow IC system for higher throughput targeted analysis to validate our previous observation. Isotopically labeled standards are ideal external calibration references to spike into the metabolomic sample for targeted quantitative analysis in LC/MS experiment because of their similar ionization effect and chromatographic retention. Six stable isotope labeled standards available for TCA cycle were used for the targeted quantitative analysis. The corresponding endogenous metabolites in a large scale of samples were quantified easily against the standards by using the software tool Thermo Scientific™ Tracefinder .

Methods

Sample Preparation

UMS1, UMS2, UMSCC5 and UMSCC6 head and neck cancer cells were cultured in Dulbecco's modified eagle medium (DMEM) plus 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL). The cells were maintained at 37° C in a humidified 5% CO_2 incubator and passaged when they reached 90-95% confluence. Cell number were counted and the representative numbers per plate were: UM1:3.46 millions, UM2: 2.88 millions, UMSCC5: 2.88 millions, MSCC6: 3.06 millions. The cells on the remaining plates for IC/MS analysis were not counted but similar to these numbers because they were cultured under the same conditions. Cellular metabolites were extracted using liquid nitrogen snap-freezing method with methanol/water according the literature [2]. Six isotope supplied by Cambridge Isotope Laboratories: Sodium pyruvate (13C3, 99%), Malic acid (13C4, 99%), Fumaric acid (13C4, 99%), Succinic acid (13C4, 99%), *alpha*-ketoglutaric acid (13C5, 99%) and Citric acid (2,2,4,4-D4, 98%) were made into water at concentrations from 10000, 5000, 1000, 500, 100, 50, 10, 5, 1, 0.5, 0.1 pg/μL (11 levels). 200 uL of such water with the standards was used for cell lysate sample (dried) reconfiguration. The 11 calibration level standards were randomly spiked into the samples in 3 replicates.

Ion Chromatography

A Dionex ICS-5000$^+$ HPIC ion chromatography was coupled to a new Q Exactive HF mass spectrometers for the analysis. The IC was equipped with an anion electrolytic suppressor (Thermo Scientific™ Dionex™ AERS 500) to convert the potassium hydroxide gradient to pure water before the sample enters the MS. A 2 μL partial loop injection on a 5 μL size loop of cellular metabolites were separated using a Thermo Scientific™ Dionex™ IonPac™ AS11HC-4μm, 2 × 250 mm column. IC flow rate was 0.38 mL/min supplemented post-column with 0.060 mL/min make-up flow of MeOH/HOAc (2 mM).

Data Processing

Differential analysis of profiling data was performed using Thermo Scientific™ SIEVE 2.2. Targeted analysis of TCA compounds was performed using Thermo Scientific™ Tracefinder 3.2.

Mass Spectrometry

A new high field Thermo Scientific™ Q Exactive HF™ quadrupole-Orbitrap mass spectrometer was operated under ESI negative mode for all detections. The resolution setting is from 15 000-240 000. Full mass scan (m/z 67-1000) used resolution 120 000 with automatic gain control (AGC) target of 1x106 ions and a maximum ion injection time (IT) of 100 ms. Source ionization parameters were optimized with the spray voltage 3.5kV; transfer temperature, 320 °C; S-Lens level, 50; heater temperature 325 °C; Sheath gas 36; Aux gas 5.

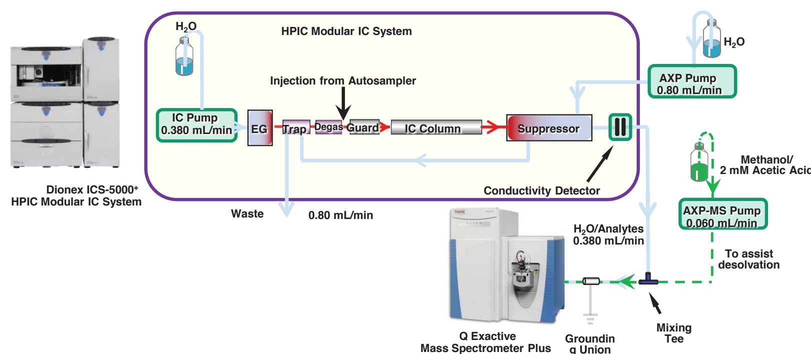


FIGURE 1. Schematic diagram of a High Performance IC-Q Exactive HF platform for targeted and nontargeted metabolomic analysis.

Results

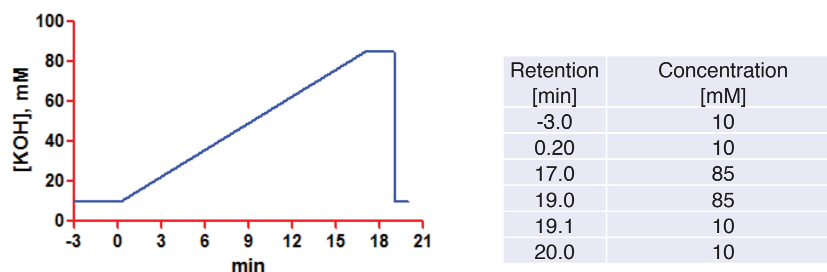
IC/MS Optimization for Higher Throughput Analysis

We applied a high pressure IC system 5000+ in this work to take several advantages:

•Higher speed separation. This system can operate at flow rate up to 380 μL/min with the pressure limit 5000 psi, while on ICS 4000 capillary system we ran 25 μl/min.

•Higher reproducibility thanks to the greater column capacity (2.0 mm vs. 0.4 mm on the capillary system).

The transfer line from autosampler to injection valve was red PEEK tubing (0.13 mm i.d.) with a 14 μL volume, which was found to substantially improve the sensitivity and peak shape as compared to a black (0.25 mm i.d.) or blue tubing (0.5 mm i.d.) with volumes greater than 40 μL. A black PEEK tubing was used from the waste valve, which was found to reduce the carryover to negligible levels (<0.01%). PushPartial sample injection mode was used on a 5 μL loop (0.13 mm i.d., red) with 2 μL injection and 5 μL cut volume. This achieved a total sample consumption volume of ~12 μL, which allows better suitability for the precise biological sample injection. Compared to CapIC running at a capillary flow rates which used a very long gradient (45 min), the Dionex ICS-5000+ system allows a short gradient of only 20 min shown in Figure 2.



Retention [min]	Concentration [mM]
-3.0	10
0.20	10
17.0	85
19.0	85
19.1	10
20.0	10

FIGURE 2. Best separation of Glucose 6P and Fructose 6P isomer was achieved using a linear gradient (among ~20 methods we tried) for metabolite separation in 20 minutes on Dionex IonPac™ AS11HC-4μm, 2 × 250 mm column.

TABLE 1. Six TCA Cycle Stable Isotope Standards

#	Metabolite Name	Formula	*Obs. m/z*	Ion	RT (min)
1	Sodium pyruvate (13C3, 99%)	[13]$C_3H_4O_3$	90.0188	[M-H]-	3.27
2	Succinic acid (13C4, 99%))	[13]$C_4H_6O_4$	121.0328	[M-H]-	6.71
3	Malic acid (13C4, 99%)	[13]$C_4H_6O_5$	137.0277	[M-H]-	6.73
4	*alpha*-ketoglutaric acid (13C5, 99%)	[13]$C_5H_6O_5$	150.0310	[M-H]-	8.08
5	Fumaric acid (13C4, 99%)	[13]$C_4H_4O_4$	119.0172	[M-H]-	8.63
6	Citric acid (2,2,4,4-D4, 98%)	C_6H_4[2]H_4O_7	195.0449	[M-H]-	11.90

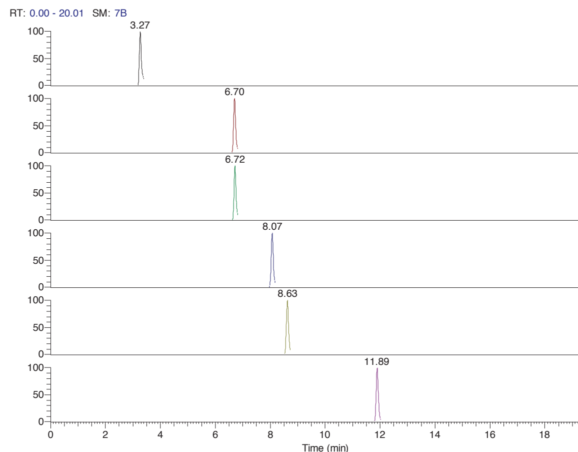
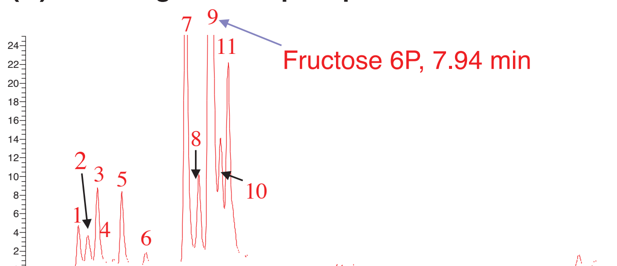
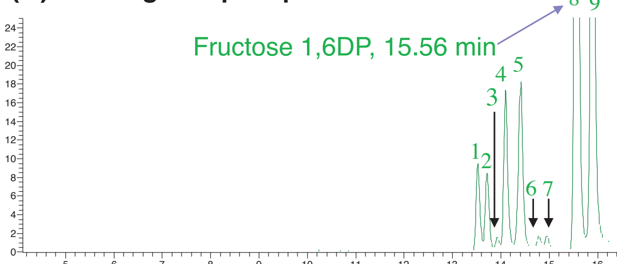


FIGURE 3. Detection of six TCA isotope label standards at 1 μg/mL concentration by IC-Q Exactive HF platform.

(A) 11 sugar mono-phosphate isomers



(B) 9 sugar di-phosphate isomers



(C) RTs of Peak 9 (7.93±0.02 min, *p*-value=0.02, n=150)

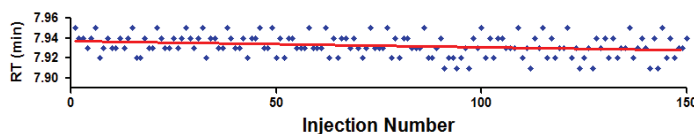


FIGURE 4. HPIC-Q Exactive HF detected (A) 11 Sugar Monophosphates and (B)9 Sugar Diphosphates from cancer cell lysates. (C) RT reproducibility of Peak #9 for 150 injection in (A).

Study Design

UMSCC1 and UMSCC2 cells were initially established from the same tumor of a tongue cancer patient. However, UM1 cells are highly invasive whereas UM2 cells are less invasive. Similarly UMSCC5 cells are significantly more invasive than UMSCC6 cells. Also, we found that the cancer stem like cells (CSC) were present in the highly invasive UM1 cells, the metabolic feature of CSC compared to nonstem cancer cells (NSCC) is also very interesting. In previous nontargeted analysis, we found metabolites in the TCA cycle show significant changes between CSCs and NSCCs. It is very interesting that the first half cycle (pyruvate/citrate/cisaconitate/ isocitrate/2-oxoglutarate) had an increasing upregulation in CSC cells, although the second half cycle (succinate/fumarate/malate) showed progressive down-regulation in CSCs versus NSCC. The study is to investigate the metabolic difference between highly invasive cancer cells and less invasive cancers and to confirm the previous observation.

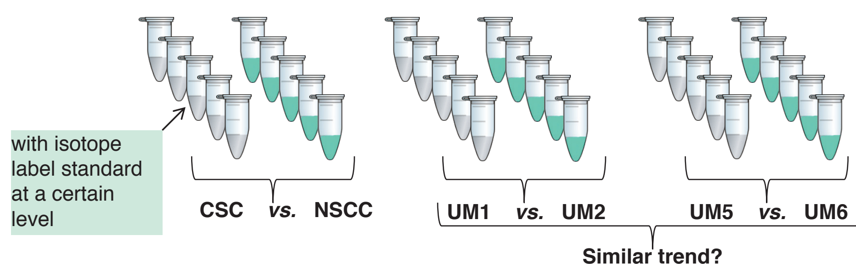


FIGURE 5. UM1 and UMSCC5, highly invasive. UM2 and UMSCC6, less invasive. CSC was present in highly invasive UM1. Isotope label compounds were made in water at 11 cal levels. The water with isotopes was used for reconstituting the lyophilized cell sample.

Calibration Curve for Stable Isotope Label Standards

Cell lysates containing the stable isotopes from low to high concentration were injected onto HPIC/Q Exactive HF for analysis. The data was analyzed using Thermo Scientific™ Tracefinder, the linear curve is shown in Figure 6.

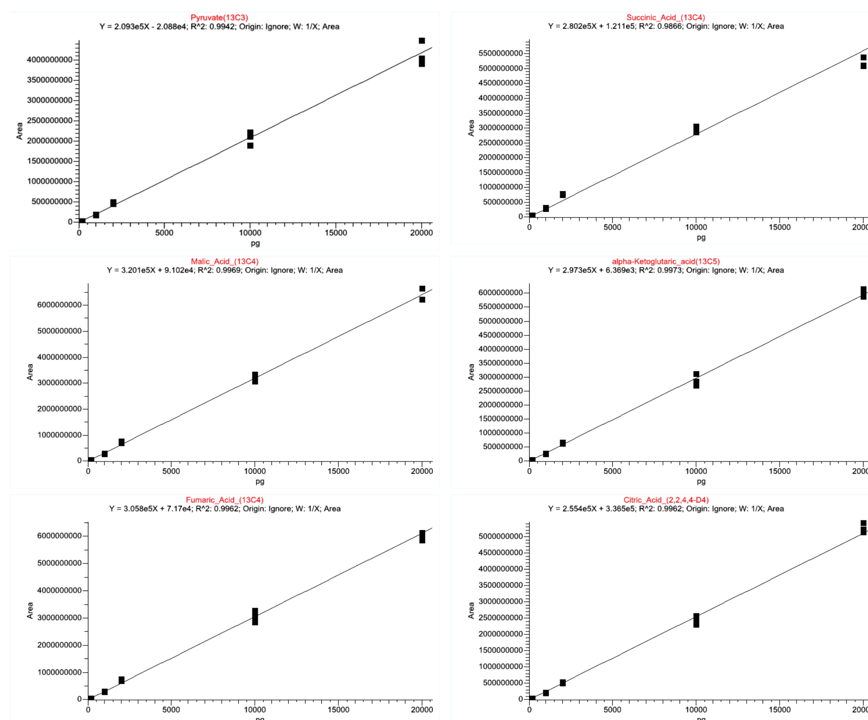


FIGURE 6. The HPIC/Q Exactive HF achieved five orders of magnitude linear range from 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000, 5000,to 10000 pg/μL (2 μL injection) with R^2 = 0.995 and above (except for succinic acid-13C4, R^2 = 0.987).

Quantify Targeted Endogenous Metabolites

Tracefinder software offers a method for quantitative analysis by linking the target compound to an external standard reference and uses its calibration curve.

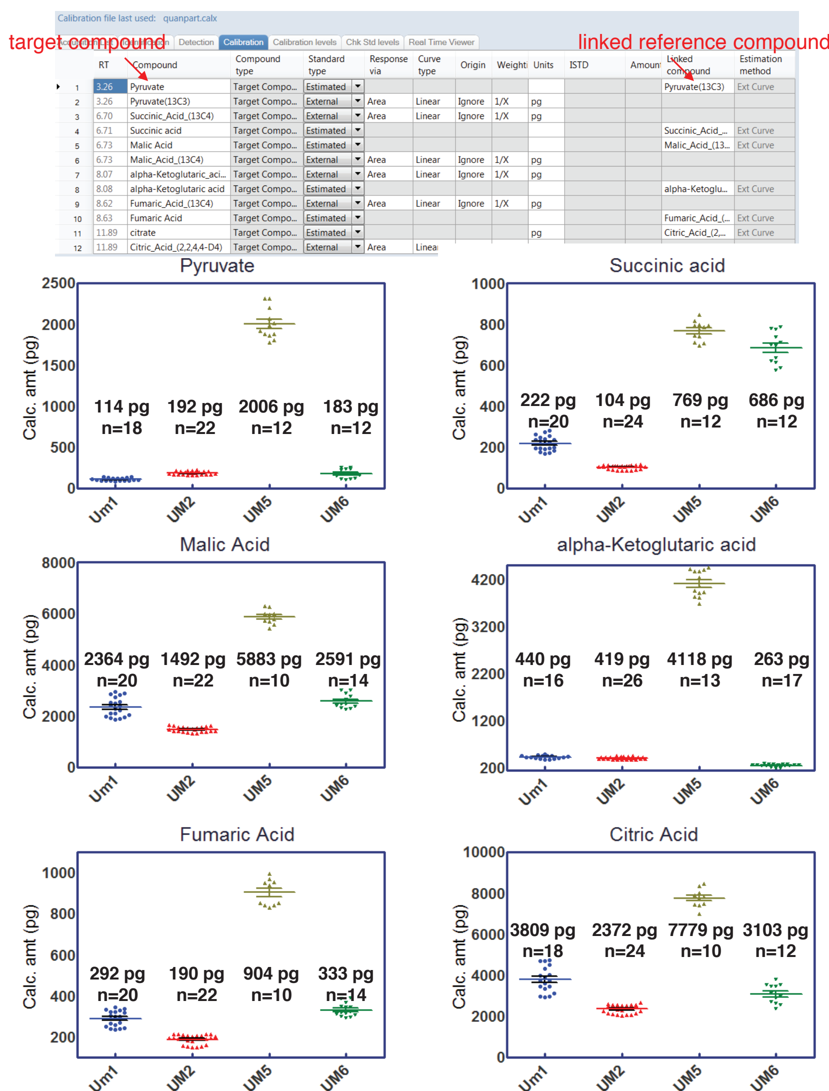


FIGURE 7. Calculated amounts of TCA metabolites per plate cells (avg. ~ 3M) and replicate numbers in UM1, 2, 5, and 6. It is known that UM5 cells consume a very large amount of glucose and secret a lot of lactate in cell culture. Therefore, it is not surprising that pyruvate and other metabolites level are significantly higher in UM5.

Conclusion

➢Based on our quantitative analysis, UM1 cells display significantly higher levels of TCA metabolites, including malate, fumarate, citrate, succinate and *alpha*-ketoglutarate. Similarly UMSCC5 cells express dramatically higher levels of these metabolites than UMSCC6 cells. These results suggests that highly invasive HNSCC cells possess a more active TCA cycle than less invasive HNSCC cells.

➢The targeted quantitation results for the 6 TCA metabolites in CSC versus NSCC match exactly with previous differential analysis (not shown here).

References

1. Wang, J. et al *Anal. Chem.*, 2014, 86, 5116–5124.
2. Lorenz, M.A.et al *Anal. Chem.*, 2011, 83, 3406–3414.

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