Quantitative LC-MS/MS analysis of polyamines and their metabolic precursors in lung tissue

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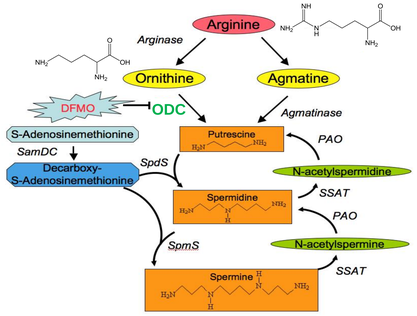
Overview

- Development of an efficient and robust sample preparation strategy for analysis of healthy & diseased lung tissue
- LC-MS/MS analysis of two types of polar analytes: polyamines and amino acids, in a single method

Introduction

Polyamines play a critical role in various processes involved in cell growth and differentiation, such as DNA synthesis and stability, regulation of transcription, and ion channel regulation. Polyamine biosynthesis is upregulated in actively growing cells, including cancer cells, thus levels of polyamines are expected to be higher in cancerous tissue than in surrounding normal tissue. The enzyme ornithine decarboxylase (ODC) catalyzes the transformation of amino acids into polyamines, linking our two classes of analytes. To better understand metabolic processes in tumor cells, it is essential to accurately monitor polyamine levels as well as those of their metabolic precursors, arginine and ornithine. This study focuses on the development of an LC-MS/MS assay to address this analytical need.

FIGURE 1. Polyamine biosynthesis pathway. Ornithine decarboxylase (ODC) is the rate-limiting enzyme in polyamine biosynthesis, and decarboxylates ornithine to form putrescine. ODC has become a promising target for anti-cancer investigation in recent years.



Materials

Standards: Commercially available standards for arginine, ornithine, putrescine, spermine and spermidine, and their corresponding stable isotope labeled internal standards (Sigma-Aldrich, IsoSciences, C/D/N Isotopes Inc., Cambridge Isotope Laboratories, Inc.)

Methods

Instrumentation
LC/MS: Accela 1250 LC, TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific)
Ionization mode: positive ESI
Scanning mode: Selected Reaction Monitoring (SRM)
Data analysis: Xcalibur software (Thermo Fisher Scientific)

Calibration curve range: 2 nM – 100 µM, equivalent to 10 fmol – 1 nmol on column

Lower Limits of Quantitation (LLOQ) (on column):
ARG – 50 fmol; ORN – 100 fmol; PUT – 750 fmol;
SPD – 5 fmol; SPM – 2 fmol.

Biological samples: Lung tissue homogenates from five patients were pooled for method development. The optimized protocol was then applied to patient-matched pairs of healthy and tumor lung tissue samples from 25 adenocarcinoma patients.

Sample preparation:
- Enzymatic homogenization: collagenase from *Clostridium histolyticum* (130 CDU/mg), 5 mg/mL solution in PBS
- Mechanical homogenization: Lysis Matrix D 1.4 mm, 4 x 30 sec. 5500 rpm, Precellys24 Homogenizer
- Extraction:
  - Method A – 70% methanol/1% acetic acid
  - Method B – 6% trichloroacetic acid (TCA) in methanol

TABLE 1. Extraction comparison. Two extraction protocols were evaluated for analyte recovery by spiking the tissue homogenate with internal standard. **A:** 6% trichloroacetic acid (TCA) in methanol, **B:** 70% methanol/ 1% acetic acid. Extraction with TCA was more efficient (A/B ratio > 1) for a majority of the analytes and generated cleaner extracts.

Extraction comparison	ARG	ORN	PUT	SPD	SPM
Method A/B ratio	1.18	2.05	0.81	1.42	1.14

TABLE 2. Analytes. Five major metabolites of the polyamine biosynthesis pathway were monitored. Two to three SRM transitions were monitored for each analyte and for its corresponding stable isotope labeled internal standard (IS).

Analyte	Abbreviation	MW (g/mol)	SRM Transitions
Arginine	ARG	174.1	175.1 > 60.2, 70.1, 116.1
$^{13}C_6$-Arginine	ARG IS	180.0	181.0 > 61.2, 74.1, 121.1
Ornithine	ORN	132.1	133.1 > 70.1, 116.1
d7-Ornithine	ORN IS	139.1	140.1 > 76.0, 123.2
Putrescine	PUT	88.11	89.1 > 30.3, 55.1, 72.2
d8-Putrescine	PUT IS	96.15	97.1 > 32.1, 56.2, 80.2
Spermine	SPM	202.2	203.2 > 84.1, 112.1, 129.1
d20-Spermine	SPM IS	222.3	223.3 > 94.2, 126.2, 143.2
Spermidine	SPD	145.1	146.1 > 72.2, 84.1, 112.1
d8-Spermidine	SPD IS	153.2	154.2 > 80.2, 92.2, 120.2

Results

FIGURE 2. Comparison of LC conditions. Three chromatographic systems were optimized and evaluated for peak shape and retention capabilities. A: multi-mode ODS/IEX, B: silicon hydride in aqueous normal phase mode, C: porous graphitic carbon. All three columns are widely used to separate polar analytes.

	A	B	C
Column	**Scherzo SM-C18** (Imtakt) 100 mm x 2 mm, 3 µm	**Diamond Hydride** (Cogent) 100 mm x 2 mm, 4 µm	**Hypercarb** (Thermo Fisher Scientific) 100 mm x 2.1 mm, 3 µm
Mobile Phase A	10 mM AmAc	0.1%FA/50%MeOH/H_2O	10 mM AmF
Mobile Phase B	100 mM AcOH in 50% ACN	0.1% FA in ACN	methanol
Flow rate	300 µL/min	300 µL/min	300 µL/min
Gradient	0 to 50 %B	95 to 0 %B	0 to 70 %B

AmAc – ammonium acetate; FA – formic acid; MeOH – methanol, ACN – acetonitrile, AmF – ammonium formate; AcOH – acetic acid

SRM chromatograms

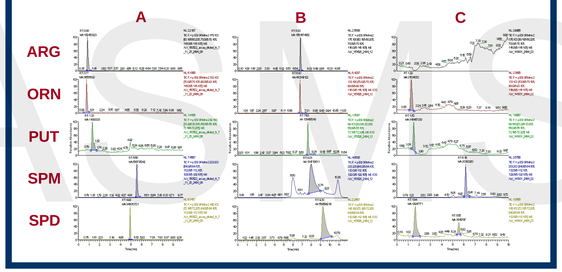


FIGURE 3. Sample preparation of human lung tissue samples. Homogenization: Lung tissue is composed of rich fibroblast-like cells which are considered "tough" for homogenization, thus both enzymatic and mechanical homogenization were applied to generate a fine homogenate[1].

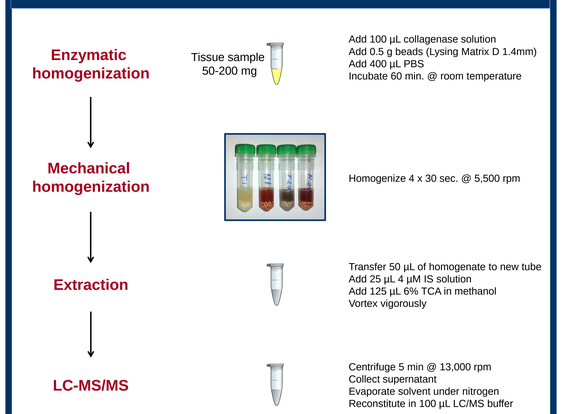
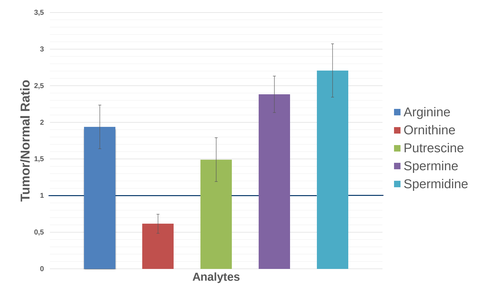


FIGURE 4. Analysis of human lung tissue. Two patient-matched lung tissue samples from each adenocarcinoma patient (n=25) were compared. A tumor-to-normal ratio (T/N) greater than 1 indicates upregulation in tumor tissue sample. Levels of spermidine, spermine and arginine are significantly higher in tumor tissue (T/N > 1) while levels of ornithine are higher in normal tissue (T/N < 1).



Conclusions

- In this study we developed a sensitive LC-MS/MS method enabling efficient analysis of polyamines and their amino acid precursors in lung tissue samples.
- The Scherzo SM-C18 LC column gave the best overall performance. Analytes were better retained on the Diamond Hydride column, but the Scherzo produced superior peak shape and thus the best sensitivity.
- Due to extremely high endogenous levels of the analytes (especially ARG and ORN) in lung tissue and the unavailability of a true blank matrix, highly diluted homogenate (1:50,000) served as calibration curve matrix.
- Benefits of trichloroacetic acid: The highest overall extraction yield was achieved with 6% TCA. Residual TCA in the prepared LC/MS sample acted as an ion pairing agent and enhanced retention of the polar analytes on the Scherzo SM-C18 column.
- Patient-matched tumor and normal lung tissue from 25 patients were analyzed. By comparing samples from the same patient, the effect of inter-individual variation was minimized, sharpening the focus on important differences between normal and cancerous tissue.
- Spermine and spermidine were clearly upregulated in tumor tissue in comparison to normal tissue collected from the same patient.

References

1) Liang, H et al. *Future Science* **2011**, 1923-1933

Acknowledgements

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