Hypercarb, 3.0 x 50 x 2.1 x 50 mm

LC-MS/MS Method Development of Endogenous Small Molecule Biomarker: Case Studies

Jingguo Hou, Xiaodong Zhu, Sarah Garcia, Steven Hoehne, Alicia Bian, and Perry Fan

Worldwide Clinical Trials, Austin TX

Overview

Purpose:

- Provide several cases to discuss challenges and solutions during method development of endogenous small molecule biomarker. .

Methods:

Solid- Phase Extraction (SPE), Liquid-Liquid Extraction (LLE), or Protein Precipitation (PPT) were used to sample preparation of different analytes. LC-MS/MS methods were performed using RP-HPLC, HILIC or hypercarb columns with a Shimadzu SIL-30AC HPLC system coupled to a Sciex API 4000/5000/6500 mass spectrometer.

Introduction

Endogenous molecules that serve as indicators of drug safety, mechanism of action, efficacy, and disease state progression. These indicators, referred to as biomarkers, are very important to improve the efficiency of drug discovery and development. Small molecule biomarkers, including peptide, are typically quantified using LC-MS/MS. During method development, bioanalytical scientists must overcome some special or unexpected challenges to develop a ruggedness bioanalytical method to meet criteria of regulated bioanalysis. Here, we provide several cases to highlight some of the practical solutions. Based on these cases, important roles of matrix choice without interference, background subtraction approach, chromatographic optimization, sample handling and sample preparation will be discussed for achieving a fit-for-purpose LC-MS/MS method of biomarker analysis.

1. Biological Matrix Choice

The surrogate matrix works well for certain biological matrices like urine and CSF, but poses challenges in plasma, serum, or whole blood due to recovery differences and matrix effects. The background subtraction approach encounters difficulties when commercially available stripped biological matrices are lacking for some endogenous compounds.

Our lab adopts alternative approaches, focusing on minimal changes to authentic biological matrices and effective removal of endogenous compounds. Examples include ion-exchange with resin (ornithine/arginine in plasma), photodegradation with UV light (vitamin K in plasma), and dialysis in PBS buffer (uridine).

2. Mass Conditions Optimization

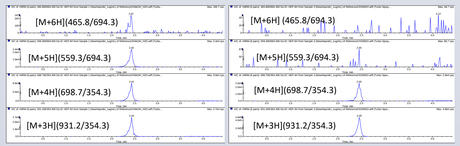
Hepcidin-25, a 25-amino acid hormone, exhibits sensitivity impacted by mobile phase composition. Adding 0.01% 2-nitrobenzyl alcohol (NBA) results in the strongest charged state being $[M+6H]^+$ in Q1 scan. Conversely, 1% DMSO yields the strongest charged state as $[M+3H]^+$ in Q1 scan, with the highest chromatographic response on $[M+3H]^+$ (931.2/354.3) using DMSO. A comprehensive strategy is crucial for optimizing mass parameters for multiple charged peptides. For 4β-Hydroxycholesterol, selecting $[M-H2O+H]^+$ as the precursor ion eliminates tedious derivatization steps.

Hepcidin/LEAP-1 (Human)

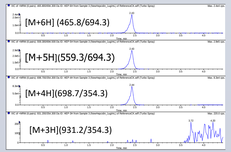
Liver-Expressed Antimicrobial Peptide 1 (Human)

Asp-Thr-His-Phe-Pro-Ile-Cys-Ile-Phe-Cys-Cys-Gly-Cys-Cys-His-Arg-Ser-Lys-Cys-Gly-Met-Cys-Cys-Lys-Thr (Reported disulfide bonds between Cys^7-Cys^{23}, Cys^{10}-Cys^{13}, Cys^{11}-Cys^{19}, and Cys^{14}-Cys^{25})

(M.W. 2789.4) $C_{111}H_{170}N_{34}O_{31}S_8$

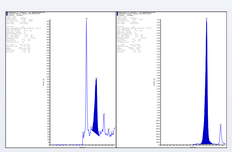


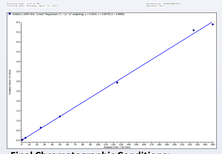
Without additive in mobile phase | **Add 0.02% NBA in mobile phase**



Add 1% DMSO in mobile phase

Chromatographic Conditions:
Column: UPLC Peptide CSH C18, 130A, 1.7um, 2.1x50mm
Mobile Phase:
(A) Formic acid/Water/Methanol (2:900:100)
(B) Formic acid/Methanol/Water (2:900:100)
Flow rate: 0.300mL/min.
Sample: Hepcidin-25 (1.00 ug/mL)



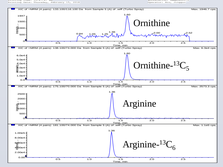


Expected Conc. (ng/mL)	Sample Name	Number Of Values Used	Mean	Standard Deviation	%CV	Accuracy
0.500	LLOQ	8 of 8	0.517	0.058923	10.62646	103.4
1.50	QC L	3 of 3	1.32	0.067702	5.159742	87.5
20.0	QC M	6 of 6	19.5	0.429652	2.205976	97.4
200	QC H	6 of 6	198	6.018187	3.045875	98.8

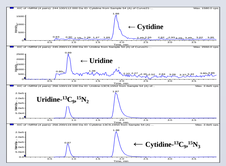
Final Chromatographic Conditions:
Column: Kinetex XB-C18, 2.6μm, 2.1×100 mm,
Mobile Phase:
(A) Formic Acid/Water/Methanol/DMSO (2:900:100:10)
(B) Formic Acid/Methanol/Water/DMSO (2:900:100:10)

3. Chromatography Optimization

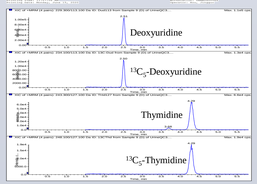
The choice of an appropriate chromatographic mode (RP-HPLC, HILIC) and column can overcome challenges in biomarker bioanalysis. HILIC mode enhances sensitivity and retention of ornithine and arginine. Using a graphite column with a fully organic mobile phase achieves excellent selectivity and sensitivity for cytidine/uridine and thymidine/deoxy uridine.



Poroshell 120 HILIC-Z, 2.1x50 mm, 2.7 μm



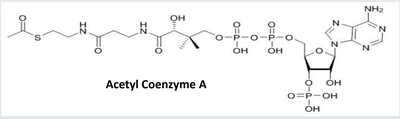
Hypercarb, 2.1 x 50 mm, 3μm
MPA: MeOH/HCOOH/Acid/NH_4HCO_2
MPB: ACN/IPA/HCOOH/NH_4HCO_2



Hypercarb, 3.0 x 100 mm, 3.0 μm
MP: 1.0% HCOOH/2 mM NH_4TFA/5 mM NH_4HCO_2 in MeOH/H_2O; 95:5

4. Stabilization of Biomarker

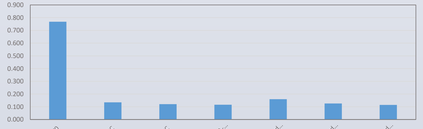
Acetyl-coenzyme A, a polar and zwitterionic small molecule, serves as a biomarker in metabolic disease. However, it is extremely unstable and rapidly decomposes in whole blood after freezing/thawing. Attempts to find an inhibitor or stabilizer were unsuccessful. A practical solution involves on-site crashing of whole blood samples with organic solvent/buffer, proving successful in stabilization.

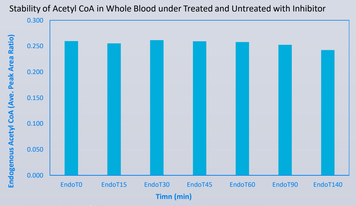


Acetyl Coenzyme A

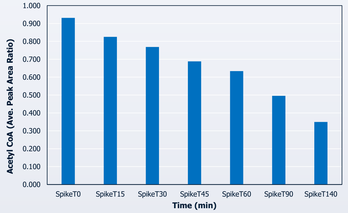
Challenges:

- **Very Polar and Ziwtteronic** -----Chromatographic challenge
- **Extremely unstable in plasma and whole blood** ------Sample preparation challenge
- **Acetyl CoA is decomposed after frozen/thaw challenge** ------Sample collection and storage

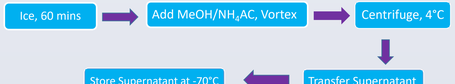




Stability of Endogenous Acetyl CoA in House Draw Whole Blood (Fresh)



Stability of Endogenous+Spiked Acetyl CoA in House Draw Whole Blood



Expected Concentration (ng/mL)	Sample Name	Number Of Values Used	Mean	Standard Deviation	%CV	Accuracy
1.00	LLOQ	6 of 6	1.05	0.037914	3.606693	105
3.00	QC L	6 of 6	2.79	0.151699	5.444287	92.9
3.00	QC L_BTS_Ice24h	6 of 6	2.68	0.1536	5.741262	89.2
3.00	QC L_BTS_RT24h	6 of 6	2.67	0.231214	8.665803	88.9
3.00	QC L_FTS_-20C4Cyc	6 of 6	2.68	0.087998	3.284708	89.3
30.0	QC M	6 of 6	27.1	0.638058	2.354757	90.3
30.0	QC M_BTS_Ice24h	6 of 6	27.3	0.788286	2.886333	91.0
30.0	QC M_BTS_RT24h	6 of 6	25.9	0.465125	1.798139	86.2
30.0	QC M_FTS_-20C4cyc	6 of 6	27.6	0.393002	1.423561	92.0
320	QC H	6 of 6	296	5.649489	1.906363	92.6
320	QC H_BTS_Ice24h	6 of 6	294	6.231833	2.122319	91.8
320	QC H_BTS_RT24h	6 of 6	274	5.872696	2.142608	85.7
320	QC H_FTS_-20C_4Cyc	6 of 6	290.4485	5.902036	2.032042	90.8

Worldwide Clinical Trials