MICROFLOW LC–MS/MS WITH TIP-BASED REVERSE PHASE ON-LINE CLEANUP FOR CHALLENGING ANALYTES

W0930-01-01

Amritangshu Chakravarty, Ph.D, Andrew Bergdorf, Ryan Darling and Rathna J. Veeramachaneni, Ph.D

KCAS Bio

CONTACT INFORMATION: amrit.chakravarty@kcasbio.com, https://kcasbio.com/contact-us/

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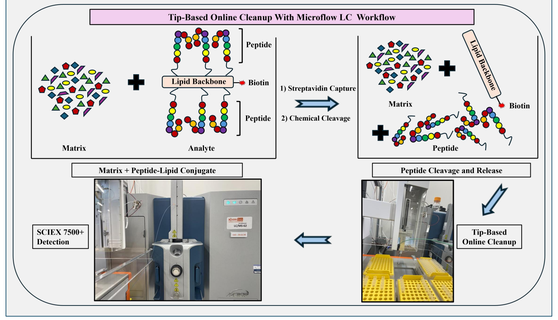
PURPOSE

Some of the pharmacokinetic and many of the low-abundant biomarker quantitation assays require highly sensitive detection of analytes, often at low nanogram-to-picogram/mL levels. Conventional extraction methods, such as protein precipitation, liquid-liquid extraction, direct digestion, as well as selective capture methods such as immunoprecipitation, can fall short of the assay's targeted sensitivity due to inadequate sample cleanup. As a proof of concept, we have utilized a novel single-use tip-based reverse-phase cleanup integrated with microflow LC-MS/MS to effectively detect analytes at low nanogram/mL concentrations across diverse biological matrices.

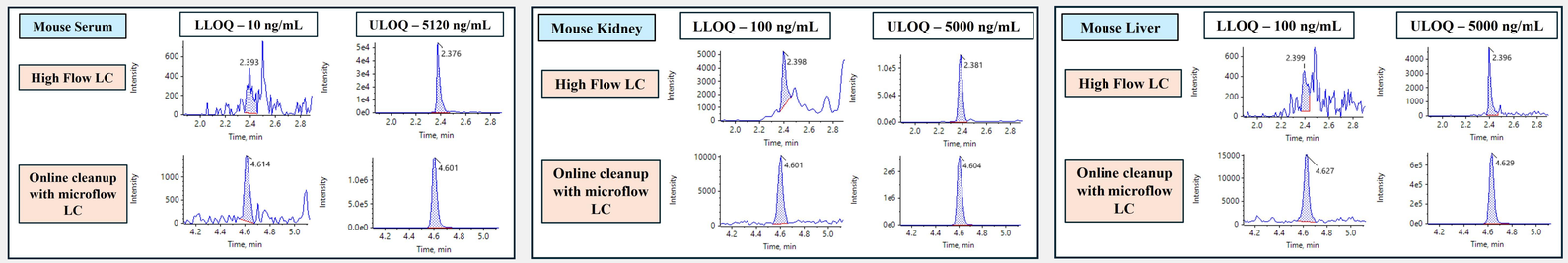
METHOD(S)

We used this novel approach to develop an assay for a small polymeric protein composed of peptide chains conjugated to a lipid backbone. The biotinylated peptide-lipid conjugate was resistant to trypsin digestion, preventing traditional surrogate peptide quantitation following specific capture with streptavidin beads from mouse serum. Thus, we adopted a chemical cleavage strategy to cleave the target peptide off the lipid backbone. However, unwanted adduct formation during chemical cleavage reduced sensitivity, increased carryover, and compromised injection linearity, as well as a concentration-dependent increase in response, necessitating additional sample cleanup. After initial extraction, samples were loaded onto C18 resin tips using the Evosep Eno platform, where they were captured, desalted, selectively cleaned up, and concentrated, thereby removing interfering adducts. The target analytes were then eluted, leaving hydrophobic contaminants on the tip, which were discarded after each use. The single-use tips reduce autosampler carryover, minimize troubleshooting time on the mass spectrometer, and extend the column's lifespan. Post-cleanup, samples were injected into a Sciex 7500+ LC-MS/MS system to quantify the peptide-of-interest from mouse serum. The online cleanup, combined with microflow LC using an 8 cm × 150µm, 1.5µm column, improved sensitivity, chromatographic performance, and data robustness.

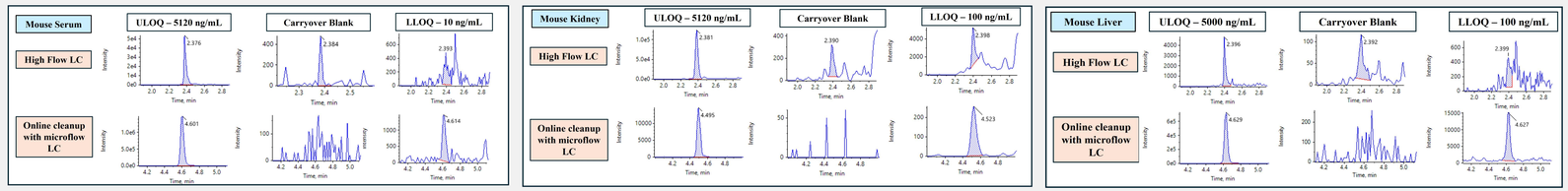


RESULT(S)

For mouse serum, this innovative method met the acceptance criteria of ±20% of the nominal value (±25% at LLOQ), enabling an LLOQ of 10 ng/ml. It improved sensitivity by 3-fold at the LLOQ and 40-fold at the ULOQ, improved peak shape, and enhanced data robustness. We also applied this tip-based cleanup technique to the mouse kidney and liver, reducing the chromatographic background and improving the signal-to-noise ratio at the LLOQ. In general, this approach resolved autosampler carryover, particularly in mouse serum, which initially showed ~100% of LLOQ with the conventional high-flow method.



Improved peak shape and sensitivity with online tip-based reverse phase cleanup followed by microflow LC-MS/MS

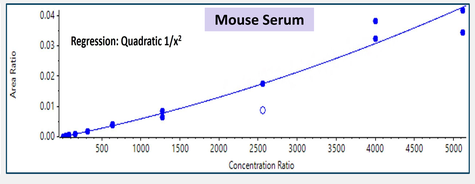


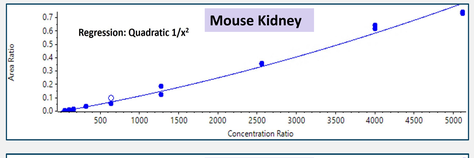
Resolution of autosampler carryover with online tip-based reverse phase cleanup followed by microflow LC-MS/MS

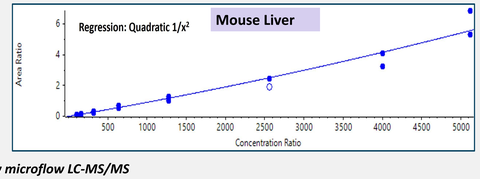
Conc (ng/mL)	10.0	20.0	40.0	80.0	160.0	320.0	640.0	1280.0	2560.0	5120.0
Mean (ng/mL)	10.4	16.6	44.0	76.2	150.0	319.7	632.0	1324.9	2512.6	4367.9
CV (%)	10.0	4.9	6.8	3.7	4.4	8.3	3.0	16.3	7.4	5.9
Accuracy (%)	104.4	83.2	110.1	95.3	93.7	99.9	98.8	103.5	98.2	109.2
n	4	4	4	4	4	4	4	4	4	4

Conc (ng/mL)	50.0	100.0	160.0	320.0	640.0	2560.0	4000.0	5120.0
Mean (ng/mL)	52.4	89.5	150.9	348.6	573.1	2863.3	4279.4	4671.5
CV (%)	8.8	13.7	16.4	11.3	19.7	3.9	9.7	7.6
Accuracy (%)	104.9	89.5	94.3	108.9	89.6	111.8	107.0	91.2
n	4	4	4	4	4	4	4	4

Conc (ng/mL)	50.0	100.0	160.0	320.0	640.0	1280.0	2560.0	4000.0	5120.0
Mean (ng/mL)	49.4	107.8	151.0	292.7	631.8	1339.2	2576.7	4097.2	4971.7
CV (%)	9.0	12.3	6.9	11.4	5.8	2.8	11.7	14.6	5.0
Accuracy (%)	98.7	107.8	94.4	91.5	98.7	104.6	100.7	102.4	97.1
n	4	4	4	4	4	4	4	4	4







QC level (Mouse Serum)	60 ng/mL	2000 ng/mL	3840 ng/mL
Mean conc (ng/mL)	57.9	2023.4	3631.5
Inter-run %CV	17.9	9.8	9.7
Inter-run %Accuracy	96.7	101.2	94.6
n	6	6	6

QC level (Mouse Kidney)	150 ng/mL	2000 ng/mL	3840 ng/mL
Mean conc (ng/mL)	149.0	2062.5	4196.8
Inter-run %CV	4.1	9.9	3.0
Inter-run %Accuracy	99.3	103.1	109.3
n	6	6	6

QC level (Mouse Liver)	150 ng/mL	2000 ng/mL	3840 ng/mL
Mean conc (ng/mL)	157.1	2274.9	3876.0
Inter-run %CV	9.2	3.4	15.1
Inter-run %Accuracy	104.7	113.7	100.9
n	6	6	6

Analytical range and precision-accuracy with online tip-based reverse phase cleanup followed by microflow LC-MS/MS

CONCLUSION(S)

- We have utilized and optimized a novel single-use tip-based online cleanup method using the Evosep Eno platform that, when combined with a microflow setup on a Sciex MS 7500+ system, enables detection of analytes at low nanogram/mL levels.
- This technology provides a reliable, transferable approach adaptable to various matrices and front-end extraction techniques, allowing the quantitation of low-abundance biomarkers at the picogram/mL level.
- This method reduces autosampler carryover and the time required to optimize chromatography, enabling high-throughput sample analysis to determine pharmacokinetic parameters.

Matrix	LLOQ sensitivity (x) increase	ULOQ sensitivity (x) increase	LLOQ S/N (High Flow)	LLOQ S/N (online cleanup and microflow)	Fold (x) increase in LLOQ S/N w microflow
Mouse Serum	3.0	40.0	3.0	8.0	2.6
Mouse Kidney	2.0	20.0	3.0	18.0	6.0
Mouse Liver	37.0	150.0	2.0	7.0	3.5

Matrix	Carryover (%) (High Flow)	Carryover (%) (online cleanup and microflow)	Carryover improvement (x) w online cleanup and microflow
Mouse Serum	100.0	N/A	100.0
Mouse Kidney	8.0	N/A	8.0
Mouse Liver	31.0	N/A	31.0

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