Case Study

A Showcase of Ultra-Sensitive Peptide Hormone Quantitation: Measuring Human ACTH [1-39] at Low pg/mL Levels Using Three-Column Chromatography and HRMS

Introduction

Peptide hormones are short amino-acid chains (typically less than 100), that play critical roles in human physiology, making accurate and robust quantification essential for disease diagnosis and drug development. Immunoassays have been traditionally used for peptide hormone quantitation. Immunoassays depend heavily on critical reagents and often cannot distinguish hormones from their pro-hormone or degradation products, limiting assay specificity.

LC-MS can offer higher selectivity and specificity for quantifying biological molecules, and coupling nano-flow chromatography with High-Resolution Mass Spectrometry (HRMS) significantly enhances sensitivity — sometimes surpassing immunoassays. The employment of nano flow chromatography (Nano LC) interfaced with HRMS greatly improves the sensitivity. Here we present a showcase study demonstrating sensitive quantitation of ACTH [1-39] in human plasma at low pg/mL levels using this nano-LC/HRMS approach.

The challenge

To achieve high chromatographic efficiency and sensitivity at nL/min flow rates, NanoLC systems employ small internal-diameter tubing and columns, which necessitates careful control of sample cleanliness and system configuration in LC-MS applications. During extended analytical sequences, elevated backpressure can occasionally be observed, particularly when analyzing samples extracted from complex biological matrices such as plasma, serum, or tissue homogenates.

Notably, this phenomenon is rarely observed when analyzing enzyme-digested samples, suggesting that specific matrix components contribute to the backpressure behavior. Based on these observations, we hypothesized that high-molecular-weight and surface-active proteins present in extracted samples may accumulate within narrow-bore flow paths, leading to increased system pressure.

To proactively address this analytical consideration, we implemented a three-column chromatography strategy incorporating orthogonal separation mechanisms. A Size-Exclusion Chromatography (SEC) column is first used to selectively divert high-molecular-weight proteins, followed by a C18 trap column to concentrate analytes and their corresponding internal standards, and finally a NanoLC analytical column interfaced with HRMS for detection. The quantification of human ACTH [1–39] serves as a representative application of this workflow. This robust platform has been successfully extended to the quantification of additional low-abundance peptide hormones, including human Atrial Natriuretic Peptide (ANP), glucagon, angiotensin II, angiotensin 1-5, and angiotensin 1-7, demonstrating the broad applicability and reproducibility of the approach.

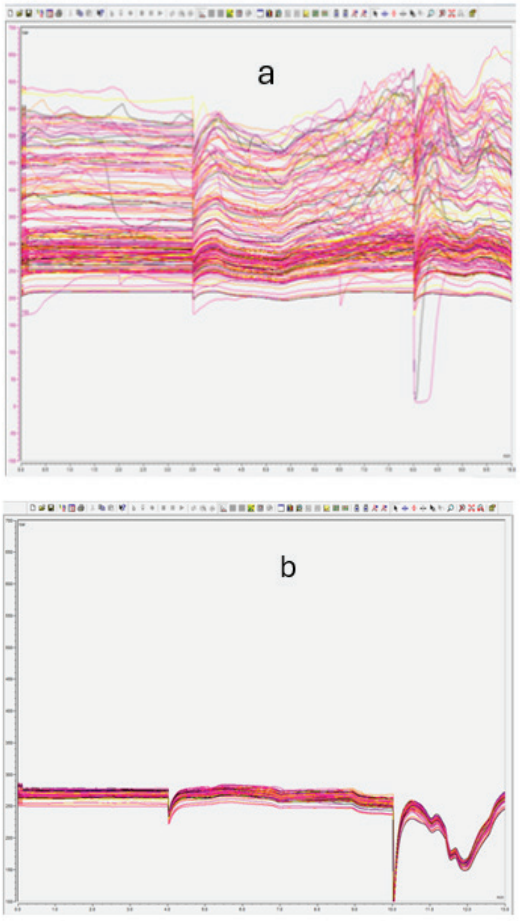
Our approach

Because of their small dimensions and high separation efficiency, single-column NanoLC systems inherently have limited sample loading capacity and are therefore less frequently employed for routine LC-MS

quantification. As a result, two-column configurations — consisting of a trap column coupled to an analytical NanoLC column — have become the established standard, enabling larger injection volumes, matrix tolerance, and improved sensitivity.

In routine bioanalytical workflows involving extracted plasma samples, long-term robustness can depend on effective management of matrix-derived macromolecules. Without appropriate upstream separation, large or adhesive proteins may progressively accumulate in the trap column, analytical column, or narrow-bore tubing. By integrating a size-exclusion step prior to analyte trapping, our workflow systematically mitigates this risk, thereby enhancing pressure stability, extending column lifetime, and improving overall system robustness during repeated bioanalytical runs.

Figure 1: Nano LC pump pressure traces from 96 sequential injections of extracted plasma are overlaid to show the effect of backpressure buildup when a two-column system is employed (panel a) compared to a three-column LC approach utilizing online size exclusion column as first dimension of separation (panel b).



Experimental details

The quantitation of human ACTH [1-39] adopts a surrogate approach. Calibration standards were prepared in a surrogate matrix, 5% BSA in 10 mM PBS, pH 7.4 in the following concentrations: 5.0, 10, 20, 50, 100, 200, 500, 900, and 1000 pg/mL. Two types of QCs were employed to monitor the assay performance: surrogate QCs at 5.0 and 15 pg/mL which were prepared by spiking human ACTH [1-39] into surrogate matrix, and endogenous QCs at 50, and 700 pg/mL which were prepared by spiking human ACTH [1-39] into human plasma K_2EDTA. The human plasma K_2EDTA used to prepare the endogenous QCs was purchased from BioIVT and a very weak signal of human ACTH [1-39] in this plasma was detected, which was well below LLOQ at 5 pg/mL, making the matrix appropriate for use in the method. The internal standard was a mouse ACTH [1-39]. Samples were extracted with a SPE method utilizing an Oasis™ HLB 96-Well Plate 30µm (30 mg) plate from Waters in the following steps: first, 300 µL of sample was spiked into a 2 mL Eppendorf™ (Enfield, CT, US) Protein LoBind® 96 well plate. Then 20 µL of 10 ng/mL internal standard working solution and 1,000 µL of 5% formic acid in water were added to the samples. The sample plate was centrifuged and shaken to mix. The SPE plate was conditioned with methanol and equilibrated with 5% formic acid in water. Samples were then transferred from the preparation plate to the SPE plate and loaded to the stationary phase under negative pressure. After the wells of the SPE plate were washed three times with 300 µL of 5% formic acid, the analyte and internal standard were eluted with 100 µL of 50/50/5 methanol/water/formic acid into a 1 mL Protein LoBind® 96 well plate, which was subsequently diluted with 400 µL 0.1% formic acid in water.

The LC separation was performed with a Thermo Scientific™ (Waltham, MA, USA) Ultimate 3000 Nano system consisting of the following modules: WPS-3000TPL Autosampler with a 250 µL sample loop, DGP-3600RS conventional flow pumps, NCS-3500RS Nano LC pump, and two 10-port switching valves. Three columns were used in the LC separation. The first column was a SEC (Waters™ Acquity APCTM AQ 125, 2.5 µM, 4.6 x 30 mm,

P/N 186006977) , the second column was a trap column (Thermo Fisher™ Acclaim PepMap100 C18, 5 µm, 100Å, 300 µm x 5 mm, P/N CPLC0095) , and the third column was a Nano LC column (Thermo Fisher™ EASY-Spray PepMap C18, 3 µm, 75 µm x 15 cm, P/N ES900). 200 µL of the extracted samples were first injected into the LC system flowing at 300 µL/min and directed to the SEC column to remove large molecular weight molecules. Subsequently, the analyte and internal standard were loaded to the trap column via a 10-port switching valve, also flowing at 300µL/min. Finally, the trap column was placed in-line with the analytical Nano LC C18 column via a second 10-port switching valve for gradient separation. The flow rate on the 75µm analytical column was 0.6 µL/min. Mobile phase A was 0.1% formic acid in 980/20 water/acetonitrile, and mobile Phase B was 0.1% formic acid in 100/900 water/acetonitrile. The total LC cycle time was 12.5 min.

Mass spectrometry detection was performed using a Thermo Scientific™ Q-Exactive Plus HRMS instrument using positive ionization with an Easy Spray nano source. A 6+ charge state precursor ion was selected for both human ACTH [1-39] (set at m/z 757.7) and murine ACTH [1-39] (set at m/z 764.4). The quadrupole isolation width was set to 1 Dalton. Normalized Collision Energy (NCE) of 10 eV for human ACTH [1-39] and 13 eV for murine ACTH [1-39] were used to fragment the molecular ions. Two isotope peaks of a 5+ charge state product ion were monitored for human ACTH [1–39] (m/z 876.045 and m/z 876.244) and murine ACTH [1-39] (m/z 884.258 and m/z 884.458) in a Parallel Reaction Monitoring (PRM) acquisition mode.

Results

Figure 2 shows chromatographic peaks of human ACTH [1-39] at 5 pg/mL LLOQ (Fig. 2a) spiked in surrogate matrix, endogenous human ACTH [1-39] (Fig. 2b) present in K2EDTA human plasma, and murine ACTH [1-39] (Figure 2c) as the internal standard. Figure 3 presents a representative calibration curve and Table 1 summarizes QC performance from two runs, demonstrating good accuracy and precision.

Figure 2: Chromatographic peak shape of a surrogate matrix sample fortified with 5 pg/mL ACTH[1-39] (a), endogenous ACTH [1-39] in a human plasma sample (b), and murine ACTH [1-39] as internal standard (c).

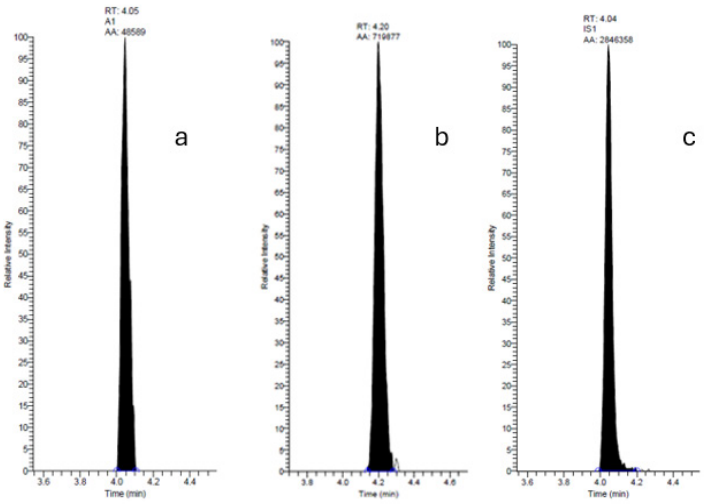


Figure 3: A representative calibration curve with duplicate points was analyzed twice — with one replicate at the beginning of the batch and another replicate at the end.

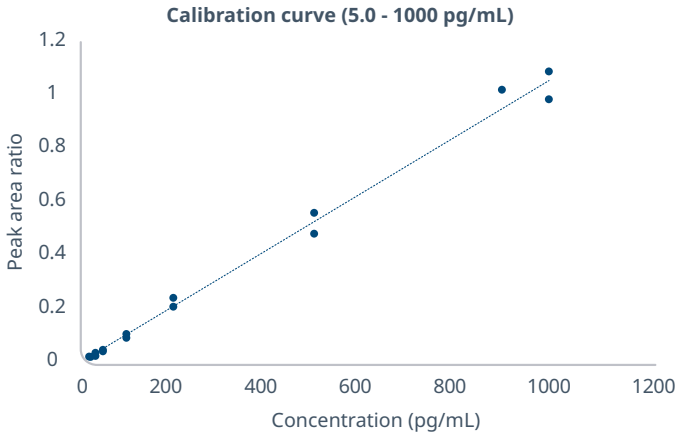


Table 1: Precision and accuracy table for one ACTH [1-39] assay in two qualification experiments, with QC samples prepared in both surrogate matrix and human plasma.

NOMINAL CONCENTRATION (PG/ML)	5.0	15	50	700
QC set 1	4.864	14.22	38.6	799
QC set 2	4.244	13.65	39.34	717.3
QC set 3	4.833	14.72	50.78	818.8
QC set 4	4.186	18.08	48.87	697.1
QC set 5	4.684	15.53	40.94	795.6
QC set 6	5.915	14.7	59.8	728.1
QC set 7	5.998	12.54	54.4	627.5
QC set 8	6.056	13.87	52.2	592.8
QC set 9	5.488	13.07	53.7	597.5
QC set 10	5.121	11.73	49.7	614.1
QC set 11	4.198	13.89	50.3	555.3
QC set 12	4.058	12.25	48.9	692
Average	**4.97**	**14.0**	**49.0**	**686.3**
CV%	**15.0**	**12.1**	**13.1**	**13.0**
Accuracy%	**99.4**	**93.5**	**97.9**	**98.0**
Deviation%	**-0.6**	**-6.5**	**-2.1**	**-2.0**

Conclusion

This work demonstrates that incorporating an online SEC dimension into a nano–flow LC–MS workflow provides a practical and highly effective solution to one of the most persistent challenges in peptide hormone quantitation: maintaining system robustness while achieving ultra–sensitive detection in complex biological matrices. By selectively removing high–molecular–weight, proteinaceous material prior to trapping and analytical separation, the three–column architecture enables stable performance over large injection sequences thus eliminating the progressive backpressure increases that frequently compromise two–column nano–LC systems.

The resulting assay for human ACTH [1-39] achieves pg/mL–level sensitivity with excellent accuracy, precision, and chromatographic consistency, fully supporting quantitative work in pharmacology, endocrinology, and translational research. Beyond ACTH, the same architecture has been successfully leveraged for several additional endogenous peptide hormones, underscoring its adaptability and value as a generalizable platform for challenging low abundance analytes.

For laboratories seeking to expand the capabilities of LC–MS–based peptide bioanalysis, this approach illustrates a clear path forward: improved robustness without sacrificing sensitivity, streamlined sample processing, and high–confidence quantitation across extended study runs. As peptide biomarkers and therapeutic peptides continue to grow in importance, platforms that pair high analytical selectivity with operational durability will play an increasingly central role, ensuring reliable data generation even at the limits of detection.

Connect with IQVIA Laboratories Biosciences experts to discuss your analyte, matrix, and performance targets and move forward with confidence.

Reference

1. B. R. Jones, L. Shan, A. Spytko, R. Luo, A. Ayala, J. Wang & S. Lowes (26 Dec 2025): Improving sensitivity and selectivity of human ACTH[1–39] quantitation using online size exclusion chromatography and antibody-free LC-HRMS, Bioanalysis, DOI: 10.1080/17576180.2025.2608756

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