Low pg/mL Detection of rHuEPOs in Horse Plasma Employing High-Resolution MS

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Application Note 585

Key Words
Q Exactive, Orbitrap, targeted peptide quantitation, EPO, rHuEPO, doping control, nanoLC

Goal
Demonstrate detection of rHuEPOs in horse plasma samples at low pg/mL levels using high-resolution mass spectrometry.

Introduction
Recombinant human erythropoietin (rHuEPO) is a 30–34 kDa glycoprotein and a common doping agent banned by equestrian and racing authorities. To differentiate between equine endogenous EPO and rHuEPOs, methods employing selected-reaction monitoring (SRM) or isoelectric focusing (IEF) with double-blotting have been developed. However, the short confirmation-time window of the compound in plasma continues to present a challenge for doping control laboratories in horse racing. In this study, a new sample preparation technique using small anti-EPO monolith membranes contained in disposable columns (anti-EPO monolith columns) was combined with nanoLC-MS/MS analysis on a benchtop high-resolution Thermo Scientific™ Orbitrap™-based mass spectrometer. The approach enabled the confirmation and quantitation of the rHuEPO target peptides in a single analytical run, with high specificity, sensitivity, and resolution.

Experimental
Source of Chemicals
Acetonitrile and water used were Fisher Chemical™ Optima™ LC/MS grade. Sequencing-grade trypsin was purchased from Promega (Charbonnieres-les-Bains, France). Formic acid, tris (hydroxymethyl)-aminomethane (TRIS), NaCl, NaN_3, tris(2-carboxyethyl)phosphine (TCEP), ammonium bicarbonate, ethylenediaminetetraacetate (EDTA), and pepstatine A were obtained from Sigma-Aldrich®. The rHuEPO used was Aranesp® from Amgen®.

Sample Preparation
Simulated plasma matrix
A matrix was created by mixing several proteins, such as γ-globulin and rHuEPO, at different concentrations.

Plasma preparation
To prepare the plasma sample, 36 mL of pH neutral buffer (20 mM Tris, 0.1 M NaCl, 0.02% NaN_3) were added to 4 mL of horse plasma. EPO monolith columns containing immobilized monoclonal anti-EPO antibody (EPO purification kit, MAIIA Diagnostics) were conditioned and the samples were allowed to pass completely through the column. rHuEPO target molecules were eluted by centrifugation. The eluent (100 µL) was completed with 400 µL of denaturant buffer before sample filtration by centrifugation with a 10 kDa molecular weight cut off (Millipore).[1]

Trypsin digestion
Samples were reduced with TCEP (7 mM) in EPAB buffer (50 mM ammonium bicarbonate pH 7.9, 10 mM EDTA, 1 µM pepstatin A) at 95 °C for 15 min. Trypsin (2 µg) was added to reach a final volume of 100 µL in EPAB buffer. The protein solution was digested at 37 °C for 210 min. Before LC-MS/MS analysis, the peptides were dried and then dissolved in 300 µL water/methanol (70:30 v/v, 0.2% FA).

Heavy peptide spiked plasma preparation
To confirm retention times and build calibration curves, custom Thermo Scientific™ HeavyPeptides™ AQUA Ultimate $^{13}C/^{15}N$-labeled forms of the two human-specific peptides T6 (VNFYAWK*) and T17 (VYSNFLR*), were spiked in extracted plasma at concentrations ranging from 10 amol to 100 fmol.

Thermo SCIENTIFIC

LC-MS/MS

Using online reversed-phase chromatography, 5 µL of samples were separated with a Thermo Scientific™ EASY-nLC™ system equipped with a trap column (100 µm i.d. x 2 cm) and C18 packed-tip column (100 µm i.d. x 15 cm, Nikkyo Technos Co. Ltd). Peptides were separated using an increasing amount of acetonitrile with 0.2% formic acid (5–40% over 48 min) in water with 0.2% formic acid at a flow rate of 300 nL/min. The LC eluent was electrosprayed directly from the analytical column. A voltage of 1.7 kV was applied via the liquid junction of the nanospray source.

The chromatography system was coupled to a Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer (Figure 1). A method consisting of alternating selected-ion monitoring (SIM) scans at 140,000 resolving power and targeted MS^2 scans at 35,000 resolving power was applied to detect and quantify the two specific peptides previously described as proteotypic variants of the rHuEPOs used in equine doping control: T6 (VNFYAWK) and T17 (VYSNFLR)[2] (Figure 2). The mass spectrometer settings are listed in Table 1.

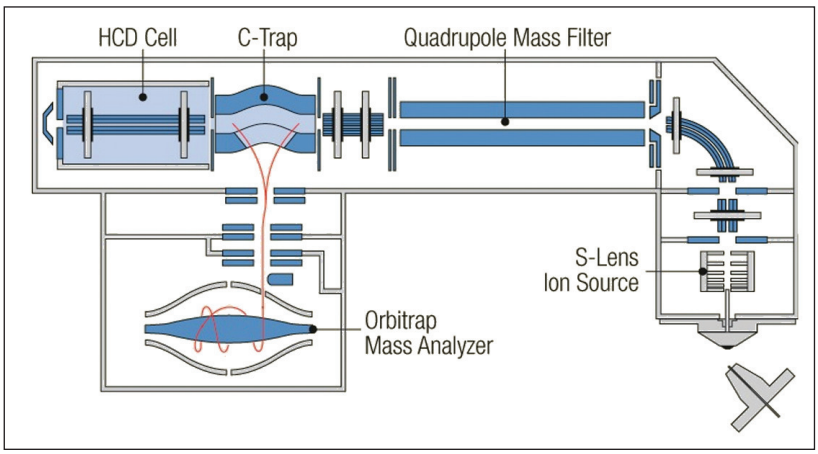

Figure 1. Q Exactive hybrid quadrupole-Orbitrap mass spectrometer

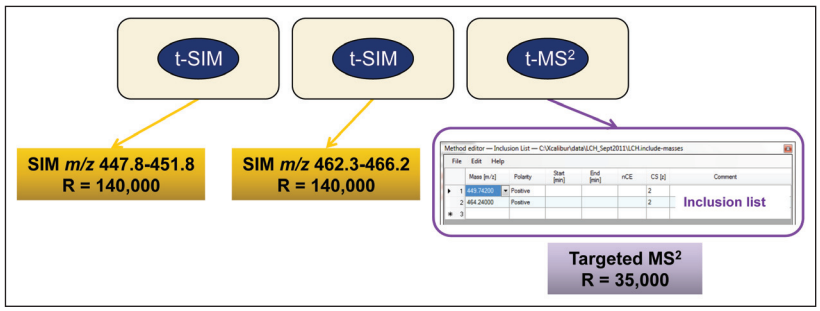

Figure 2. Q Exactive mass spectrometer SIM/targeted MS^2 method

Table 1. Q Exactive mass spectrometer parameter settings

Source	nano-ESI
Capillary Temperature	300 °C
S-Lens RF Level [%]	70
Source Voltage [kV]	1.7
Resolution Settings	
SIM	140,000 (*m/z* 200)
Targeted MS^2	35,000 (*m/z* 200)
Target Value	
SIM	1.00×10^6
Targeted MS^2	1.00×10^6
Maximum Injection Times	
SIM	500 ms
Targeted MS^2	500 ms
Normalized Collision Energy	15%
Isolation Window	4 *m/z* (SIM and targeted MS^2)

Data Processing

Thermo Scientific™ Pinpoint™ software version 1.2 was used to process the data. Chromatograms were extracted with 5 ppm mass tolerance for the Q Exactive mass spectrometer data. A possible alignment error of 1 minute was allowed. Peak areas were computed on smoothed data using seven points.

Results and Discussion

High-Resolution Targeted Quantitation

Detection of rHuEPOs is traditionally performed using SRM on a triple-stage quadrupole mass spectrometer and requires the detection of at least three fragment ions from each peptide. Two peptides, T6 (VNFYAWK) and T17 (VYSNFLR),[1,2] are traditionally used for rHuEPOs detection. These peptides have been selected due to their specificity for rHuEPOs used as a doping agent and because their size of seven amino acids renders them easy to detect by LC/MS.

Triple-stage quadrupole technology is known to provide sensitivity, robustness, and efficiency for high-throughput analyses. However, in complex matrices like plasma, limits of detection and quantitation are often correlated to the presence of interferences. These interferences can to some extent be overcome by applying gas-phase separation such as high-field asymmetric waveform ion mobility spectrometry (FAIMS), which brings an additional dimension of selectivity and allows detection of rHuEPO down to 100 pg/mL.[1]

In this study, we evaluated the use of high-resolution mass spectrometry technology combined with nanoLC to improve the detection limit of rHuEPOs in plasma without need for FAIMS. The Q Exactive mass spectrometer features Orbitrap mass analyzer technology. This analytical platform, in which a quadrupole is used to select precursors for identification via MS/MS experiments or targeted quantitation, has been optimized to address the analytical challenges of MS-based quantitative analysis with high sensitivity, throughput, and robustness.[3] In this work, two SIM scans and two targeted MS^2 scans were used alternately to detect and confirm the presence of EPO, in a total cycle time of less than 2 s when measuring 200 amol of neat peptide standard (Figure 3). Unlike SRM-based quantitation high-resolution quantitation does not require selection of fragment ions during method development. A normalized collision energy of 15% was used to fragment the two rHuEPO peptides after a very simple optimization process.

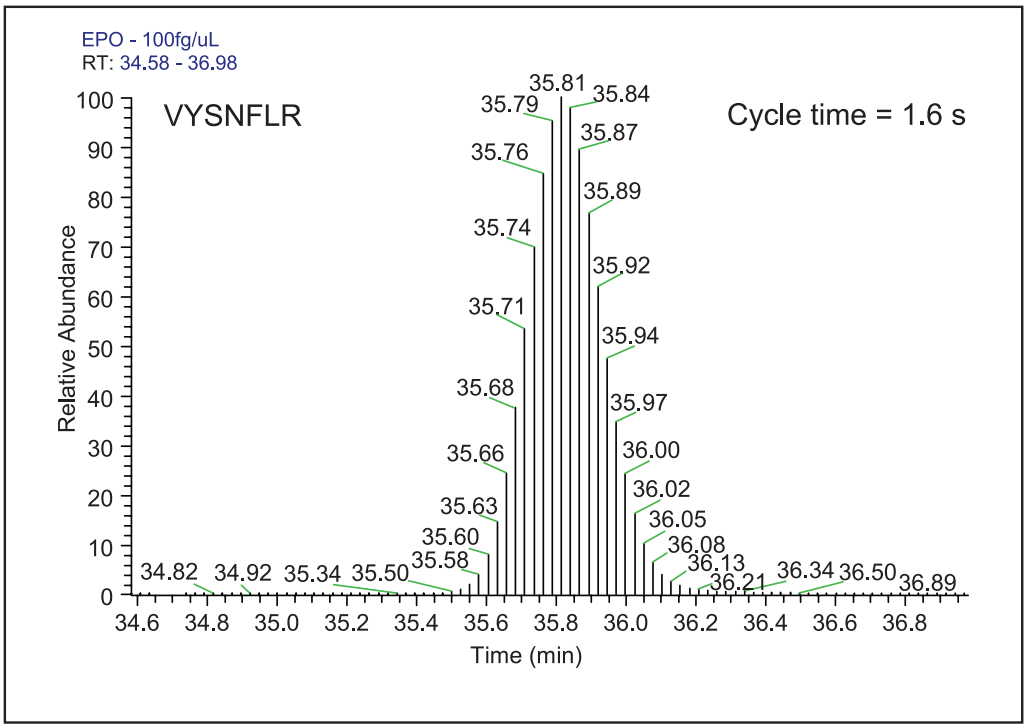

Figure 3. Chromatogram of peptide T17 demonstrating the cycle time of the Q Exactive mass spectrometer

Determination of the Limit of Detection (LOD) of rHuEPO in Plasma

Plasma samples spiked with concentrations of rHuEPO ranging from 25 pg/mL to 250 pg/mL were extracted, digested, and used to determine the linearity and detection limit of the Q Exactive mass spectrometer for peptides T6 (VNFYAWK) and T17 (VYSNFLR). Good detection was observed at 250 pg/mL by targeted MS^2; the concentration used as a positive control in a doping confirmatory sequence. The observed LOD was 25 pg/mL (CV = 8%, n = 3) for both peptides, with three to seven confirmatory transitions respectively (Figure 4). 25 pg/mL was the lowest limit tested and is taken de facto as the LOD. Our assessment of the data indicates that the LOD is likely to be at this level or only slightly lower. This result represents a four-fold improvement over previously published triple-stage quadrupole SRM methods.[1] Linearity of detection was also proven to be excellent in this concentration range, with $R^2 > 0.99$ when using the signal from both the precusor ion detected in the SIM scan, and the respective detected product ions.

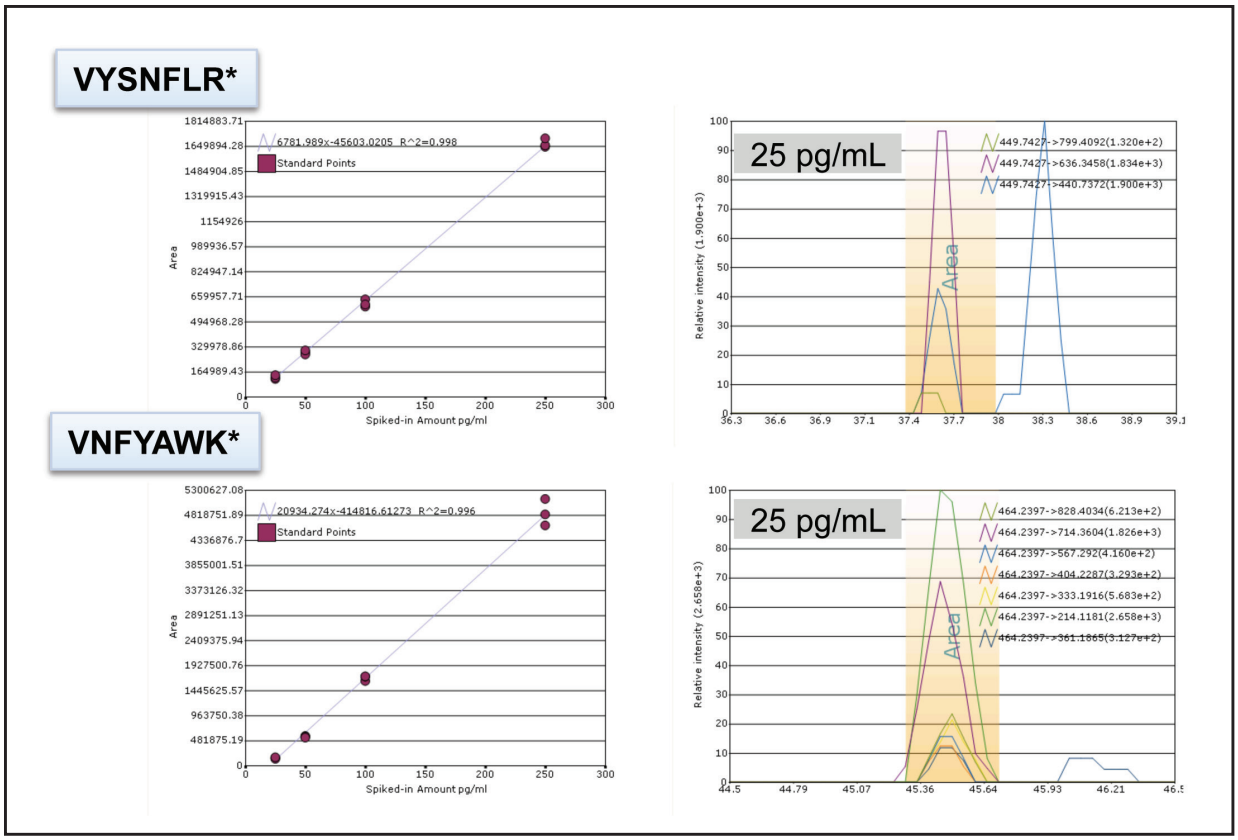

Figure 4. Linearity of the extracted rHuEPO peptides T6 (VNFYAWK) and T17 (VYSNFLR), and extracted ion chromatograms (XICs) of both peptide fragments at the limit of detection (25 pg/mL) obtained on the Q Exactive mass spectrometer

Quantitation of Spiked Heavy Peptides T6 and T17 in Extracted Plasma

To assess limits of sensitivity and dynamic range of the Q Exactive mass spectrometer, heavy surrogates of peptides T6 and T17 were used. Plasma samples containing rHuEPO were extracted, digested, and spiked with different concentrations of the heavy peptides ranging over four orders of magnitude (10 amol to 100 fmol).

The Q Exactive method was slightly modified to accommodate the mass changes associated with the $^{13}C/^{15}N$ incorporation. Targeted MS^2 was triggered alternately for the four peptides resulting in a maximum cycle time of 1.2 s. Good linearity was observed over four orders of magnitude ($R^2 > 0.97$). The two peptides could be detected down to 10 amol with CVs equal to 11% and 8%, respectively (n = 3, Figure 5).

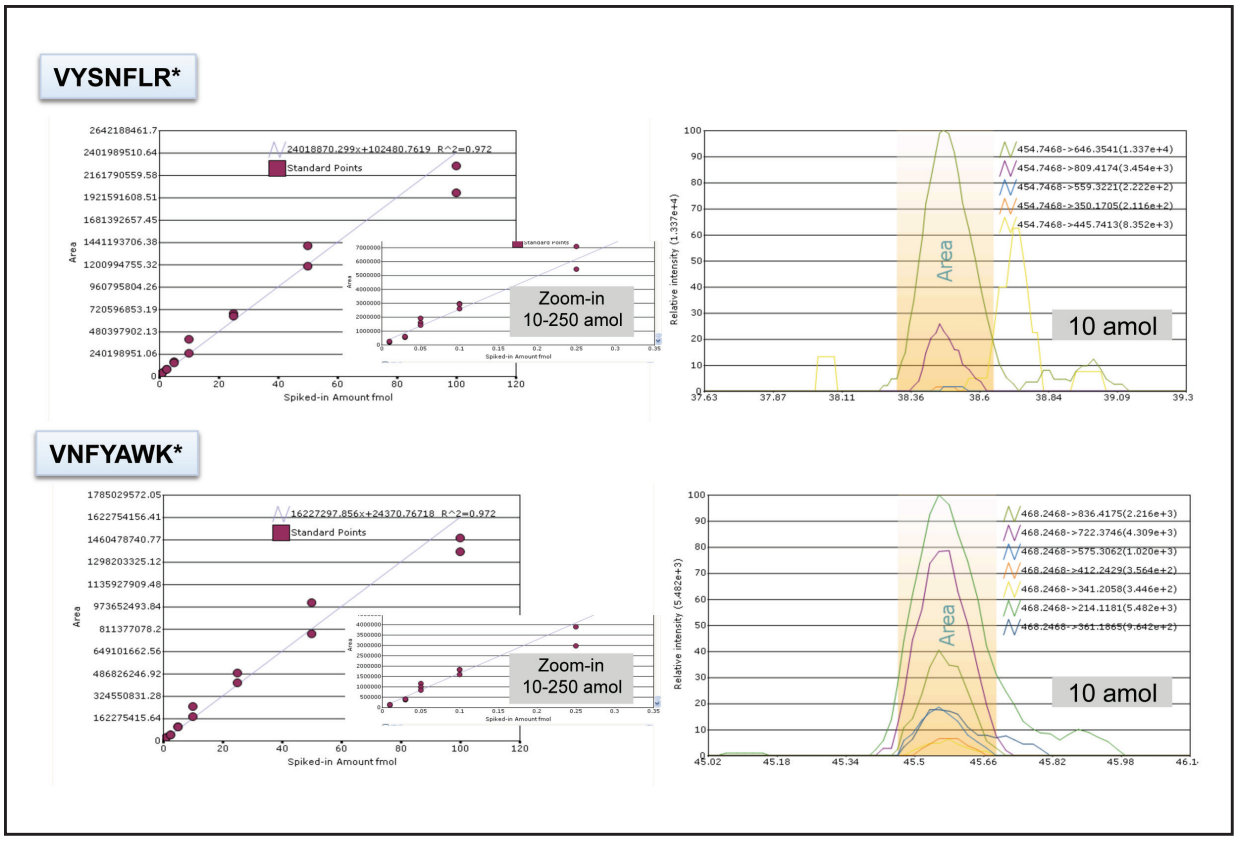

Figure 5. Linearity of the heavy T6 (VNFYAWK*) and T17 (VYSNFLR*) peptides, and extracted ion chromatograms (XICs) of both peptide fragments at 10 amol in plasma obtained on the Q Exactive mass spectrometer

Conclusion

The high-resolution capability of the Q Exactive mass spectrometer was successfully applied to detect two peptides of rHuEPO in plasma, enabling a powerful workflow that combines targeted peptide quantitation with identity validation. The workflow does not require the selection of product ions during method development; fragments resulting from higher-energy collisional dissociation (HCD) fragmentation can be selected for sequence confirmation and quantitation. The results demonstrate:

- By alternating two SIM and two targeted MS^2 scans, a total cycle time of less than 2 s is possible.
- Quantification and identity confirmation are concomitant, with sensitive quantification enabled by either SIM or targeted MS^2 scans, where rich HCD fragmentation spectra are also used to verify the target.
- Target sensitivity for rHuEPO detection for the purposes of doping control in horse racing can be reached. rHuEPO can be detected at 25 pg/mL (CV = 8%, n = 3), a four-fold improvement over previously published triple-stage quadrupole mass spectrometer methods. Therefore, the limit of detection obtained enables rHuEPO detection in horse plasma beyond 48 h after administration.
- Linearity over four orders of magnitude and 10 amol level sensitivity in an extracted plasma matrix can be achieved using heavy T6 and T17 peptides.

In summary, these results show the promise of high-resolution mass spectrometry for the identification and quantitation of peptide- and protein-based drugs in doping control.

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