Detection of rHuEPOs in Horse Plasma Samples at Low pg/mL Levels Employing High-Resolution MS Strategies

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Overview

Purpose: Improve the detection and quantification limits for the analysis of recombinant human erythropoietin (rHuEPOs) in horse plasma for doping control.

Methods: Evaluate a combination of high-resolution selected ion monitoring (SIM) and selected reaction monitoring (SRM) for the detection of 2 rHuEPO peptides.

Results: High-resolution mass spectrometry allows the detection of rHuEPOs in plasma at 25 pg/mL which represents a fourfold improvement over previously published methods which use a triple quadrupole mass spectrometer (ref. 1). Sensitivity and linearity of the quantification method were demonstrated over four orders of magnitude down to 10 amol of spiked surrogate heavy peptides.

Introduction

Recombinant human erythropoietin (rHuEPO) is a 30-34 kDa glycoprotein banned by racing authorities. To differentiate between horse endogenous EPO and rHuEPOs, methods employing LC-MS/MS or isoelectric focusing (IEF) with double-blotting have been developed. However, the short confirmation-time window of the compound in plasma remains a problem for doping-control laboratories in horse racing. In this study, a new sample preparation technique using disposable anti-EPO monolith columns was combined with nano-LC-MS/MS analysis on a high-resolution Orbitrap mass analyzer. The approach enabled the confirmation and quantification of the rHuEPO target peptides in a single analytical run, with high specificity, sensitivity and resolution.

Methods

Sample Preparation

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

Plasma preparation 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 (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 on a 10 kDa MWCO (Millipore, Bedford, MA, USA) by centrifugation.

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 3 h 30 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 Thermo Scientific $^{13}C/^{15}N$ labeled forms of the two human-specific peptides T6 (VNFYAWK*) and T17 (VYSNFLR*) were spiked in extracted plasma to confirm retention times and build calibration curves. Final concentrations are displayed in Table 1.

Table 1. Heavy peptide dilution curves.

Dilution from original peptide concentration (5 pmol/uL)	Stock heavy peptide concentration (fmol/uL)	Heavy peptide concentration in extracted plasma (fmol/uL)	Injection volume (uL)	Heavy peptide on column (fmol)
100000	0.05	0.002	5	0.01
100000	0.05	0.005	5	0.025
100000	0.05	0.01	5	0.05
10000	0.5	0.02	5	0.1
10000	0.5	0.05	5	0.25
10000	0.5	0.1	5	0.5
1000	5	0.2	5	1
1000	5	0.5	5	2.5
1000	5	1	5	5
100	50	2	5	10
100	50	5	5	25
100	50	10	5	50
100	50	20	5	100

LC-MS/MS

5 µL of samples were separated by online reversed-phase chromatography using a Thermo Scientific Easy-nLC system equipped with a trap column (100 µm ID x 2 c,) and C18 packed tip column (100 µm ID x 15 cm, Nikkyo Technos Co. Ltd). Peptides were separated using an increasing amount of acetonitrile (5%-40% over 48 minutes) and 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) and a Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer for comparison.

Q Exactive™ mass spectrometer method 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)(ref.2)--a method alternating SIM scans at 140,000 resolving power and targeted MS/MS scans at 35,000 resolving power (for sequence confirmation) was applied. Other settings were: AGC target value of 1E6, isolation windows of 4 amu, normalized collision energy of 15% (after optimization) and maximum ion injection time of 500 ms.

FIGURE 1. Q Exactive benchtop Orbitrap LC-MS/MS instrument layout.

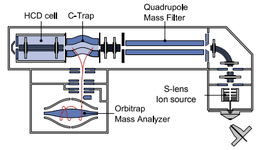
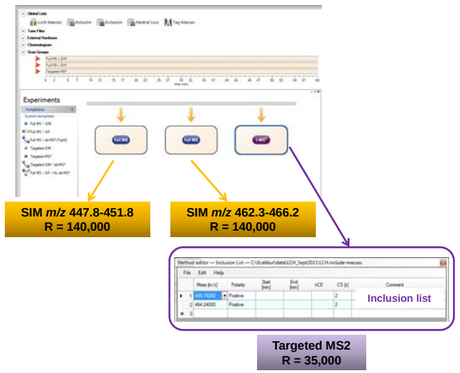


FIGURE 2. Q Exactive mass spectrometer method



TSQ Vantage™ instrument method *The* Q1 and Q3 peak widths were 1.20 *m/z*, Q3 scan width was 0.05 *m/z*, and scan time was 25 ms. The collision energy (eV) was optimized for each selected transition and is displayed in Table 2. The Argon collision gas pressure was 1.5 mTorr.

Data Analysis

Data was processed with Thermo Scientific Pinpoint software v.1.2. Chromatograms were extracted with 10 ppm mass tolerance for the Q Exactive data.

Table 2 List of SRM transitions monitored on the triple quadrupole instrument.

Peptides	Charge state	Precursor ion (m/z)	Fragment ions (m/z)	Collision energy (eV)	Fragment type
T6 (VNFYAWK)	[M+2H]2+	464.24	119.99	38	Extra
			130.09	43	Y_amine1
			135.97	35	Extra
			158.58	32	Y_amine2
			214.12	14	b2
			333.19	20	y2
			404.23	16	y3
			567.29	16	y4
			714.36	12	y5
			828.40	15	y6
T17 (VYSNFLR)	[M+2H]2+	449.74	119.66	38	Extra
			135.60	27	Y_water2
			201.95	29	Extra
			235.14	16	a2
			263.14	13	b2
			440.74	12	b6
			636.35	15	y5
			799.41	15	y6

Results

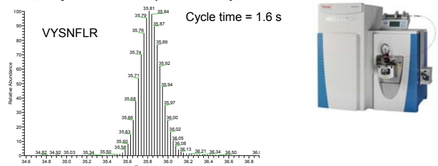
High resolution verses triple quadrupole-based quantitation

Detection of rHuEPOs is traditionally performed using SRM on a triple quadrupole mass spectrometer and requires the detection of at least three fragment ions from the peptide. Triple quadrupole technology is known to provide sensitivity, robustness and efficiency for high-throughput analysis. However, in complex matrices like plasma, limits of detection and quantification are often linked to the presence of interferences which 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 (ref.1).

In this study, we evaluated the use of new high resolution mass spectrometry to improve the detection limit of rHuEPOs in plasma without need for FAIMS. Two instrument platforms were compared--the high-resolution Q Exactive mass spectrometer and the TSQ Vantage triple quadrupole mass spectrometer--using the same LC conditions. The list of SRM transitions and collision energies were optimized for the QqQ-based quantitative analysis (Table 2). High resolution-based quantification 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.

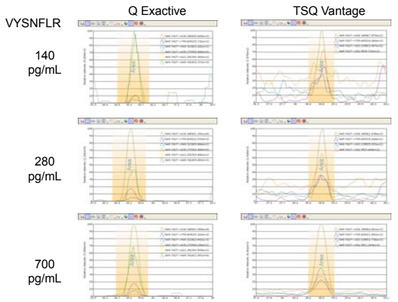
The Q Exactive mass spectrometer is based on Thermo Scientific Orbitrap mass analyzer technology. This analytical platform, in which a quadrupole is used to select precursors for identification via MS/MS experiments or targeted quantitation via SIM scans, has been optimized address the analytical challenges of MS-based quantitative analysis with high sensitivity, throughput and robustness (ref.3). In this work, two SIM scans and two confirmatory MS/MS scans where used alternatively, resulting in a total cycle time of less than two seconds (Figure 3).

FIGURE 3. Q Exactive mass spectrometer cycle time.



A simulated plasma matrix spiked with recombinant Human EPO was used to compare the sensitivity of the two quantitative approaches. Figure 4 shows that, at low concentrations of spiked protein, the Q Exactive mass spectrometer produces a better signal-to-noise ratio and a higher number of confirmatory fragments (five versus four) compared to the TSQ Vantage mass spectrometer.

FIGURE 4. Quantitative comparison using a simulated plasma matrix.

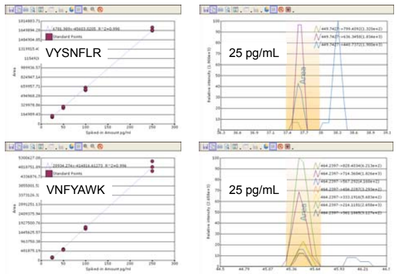


Determination of the limit of detection of rHuEPO in plasma

Real plasma samples spiked with rHuEPO 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/m; the concentration used as a positive control in a doping confirmatory sequence. Limit of detection was 25 pg/mL (%CV = 8%, n=3) for both peptides with three to seven confirmatory transitions respectively (Figure 5). Finally excellent linearity (R>0.99) was observed with %CVs between 1% and 8% for all concentrations (n=3).

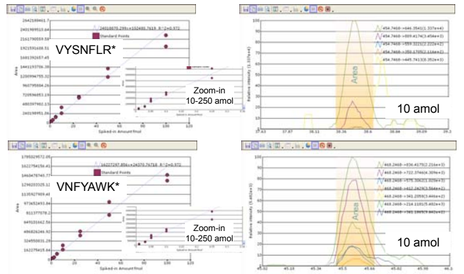
FIGURE 5. Detection limits of rHuEPO peptides T6 (VNFYAWK) and T17 (VYSNFLR) extracted from plasma.



Quantification of spiked heavy peptides T6 and T17 in extracted plasma

In order 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. Dilution curve parameters and concentrations of the heavy peptides loaded on columns are described in Table 1. The Q Exactive mass spectrometer method was slightly modified to accommodate the mass changes associated with the $^{13}C/^{15}N$ incorporation, i.e. targeted MS2 was triggered alternately for the four peptides resulting in a maximum cycle time of 1.2 s. A good linearity was observed over four orders of magnitude (R > 0.97) and the two peptides could be detected down to 10 amol with &CVs equal to 11% and 8% respectively (n=3, Figure 6).

FIGURE 6. Calibration curves of Heavy T6 (VNFYAWK*) and T17 (VYSNFLR*) peptides on the Q Exactive mass spectometer.



Conclusion

- High-resolution mass spectrometry using the Q Exactive mass spectrometer was successfully applied to detect two peptides of rHuEPO in plasma with better sensitivity and robustness than obtained using a triple quadrupole mass spectrometer.
- High resolution-based quantitation does not require selection of fragment ions during method development and results in data points for fragment confirmation.
- A method alternating two SIM and two confirmatory MS/MS scans was developed resulting in a total cycle time of less than two seconds.
- The presence of rHuEPO was confirmed in horse plasma samples with a limit of detection of 25 pg/mL (CV=8%, n=3), which represents a four-fold improvement over previously published triple quadrupole mass spectrometer methods (ref 2). The target sensitivity for rHuEPO detection for the purposes of doping control in horse racing was reached.
- Linearity over four orders of magnitude and 10-amol level sensitivity in an extracted plasma matrix were demonstrated using heavy T6 and T17 peptides.
- The limit of detection obtained with this new technology allows an increase in detection time beyond 48 h after administration for rHuEPOs in horse plasma. Studies are in progress using plasma samples obtained from animals.
- These results show the promise of the use of high-resolution mass spectrometry dedicated to peptide and protein based drugs in doping control.

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