Fast confirmatory analysis of rHuEPOs in plasma for horseracing doping control by coupling Evosep One & Q-Exactive HF

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1. Introduction

Recombinant human erythropoietins (rHuEPOs) are human therapeutic proteins prescribed to patients with severe kidney failure to increase the production of red blood cells through haematopoiesis. However, because of its indirect ability to enhance muscular oxygenation, its use has been digressed in sport competitions and is thus considered as a major doping agent by authorities such as the International Federation of Horseracing Authorities (IFHA), the International Federation for Equestrian Sports (FEI) or the World Anti-Doping Agency (WADA).
For doping control purposes, rHuEPOs are routinely screened in racing and sport horse plasma, and in case of suspicious high estimated EPO concentration, analysed by confirmatory analysis. The confirmatory approach usually involves antibody-based capture coupled to bottom-up proteomics targeting Human-specific EPO peptides to ensure method specificity[1]. Since rHuEPOs use is strictly banned by horseracing authorities at any time of the horse life, analytical methods always require extended sensitivity. Adaptation of described protocols employing narrow bore LC to nanoflow chromatography significantly improved detection sensitivity of rHuEPOs in horse plasma samples at the expense of analysis throughput. Indeed, run times of rHuEPOs confirmatory analysis underwent a two to five-fold increase by employing nanoLC with on-precolumn or on-column loading procedures, respectively. This constitute a major concern when working with large number of samples in clinics, forensics or drug testing and where reliability of the results is crucial. This application note highlights the compatibility of the Evosep One for targeted proteomics in the context of protein-based drug testing in complex samples such as horse plasma.

2. Methods

Blank plasmas were collected from post-race samples which were tested negative after screening by ELISA assay (Quantikine IVD EPO, R&D Systems, USA). Blank plasmas were spiked with required amounts of the highly glycosylated 55 kDa Darbepoetin alfa rHuEPO (Aranesp® Amgen, USA) prior to extraction when necessary. The rHuEPO was enriched from plasma samples by affinity purification with MAIIA EPO monolith membranes (MAIIA diagnostics, Sweden) according to manufacturer's recommendations. Briefly, 2

mL of plasma were completed with 1 mL of plasma buffer, 1 mL of Exposure Aid, 18 mL of Ultrapure water and filtered through a 0.22 µm syringe filter unit. Sample was loaded onto the membrane, washed with 1 mL of washing buffer and eluted using 50 µL of low pH desorption buffer and completed with 5 µL of adjustment buffer A. Eluates were concentrated on a 10 kDa MWCO membrane device (Merck Millipore, USA) and retentate was denaturated by heating the sample at 95 °C for 15 minutes in presence of TCEP 8 mM on a heated rotatory device. Proteins were digested by adding 2 µg of sequencing grade trypsin (Promega, USA) at 37 °C for 3.5 hours. Peptide mixture was dried under a nitrogen stream, resuspended in 30 µL of water/acetonitrile/formic acid (95/5/0.2) and 5 µL of the peptide mixture were loaded on an Evotip using standard procedure.

The Evosep One pre-formed 11.5 min gradient[2] ensured sufficient separation of peptides on a PepSep column (8 cm × 100 µm) packed with ReproSil C18 3 µm beads which was connected to a PepSep EASY-Spray compatible stainless-steel emitter. Peptides were sprayed at 2.5 kV with an EASY-Spray nanoelectrospray ion source (Thermo Scientific, USA) in positive mode coupled to a hybrid quadrupole-Orbitrap mass spectrometer (Q-Exactive HF, Thermo Scientific).

Data were acquired using an unscheduled parallel reaction monitoring method. Doubly charged rHuEPO-specific tryptic peptides T6 "VNFYAWK" and T17 "VYSNFLR" (*m/z* 464.24 and 449.74, respectively) were selected by the quadrupole within a 2 Th isolation window and fragmented in the higher collisional dissociation cell with a stepped normalized collision energy of 18 ± 3. Maximum fill time of the C-Trap was set to 500 ms and automatic gain control to 3 × 10^6. Spectra were acquired in profile mode at Rs = 30 000 and starting at m/z 140.

3. Results

The presence of rHuEPO was successfully confirmed in all twelve rHuEPO spiked plasmas (6 × 100 pg/mL and 6 × 1 ng/mL; *i.e.* 1.8 and 18 fmol/mL, respectively). Indeed, the two rHuEPO-specific proteotypic peptides T6 and T17 were identified with high retention time reproducibility (Figure 1 & 2) and at least four specific transitions without carry-over. As an example, two T17-VYSNFLR weak transitions y_4^+ and y_3^+ were detected in all six 1.8 fmol/mL spiked plasmas with averages of 8 and 6 points per peaks respectively, which is adequate for peak definition. These results demonstrate the reliability of the Evosep One for the targeted analysis of rHuEPOs in horse plasma.

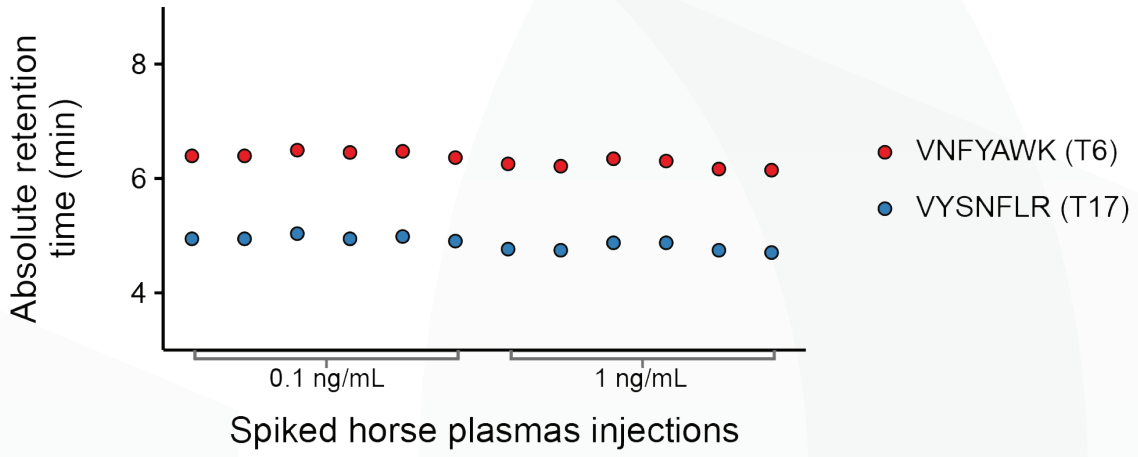

Figure 1: Retention time reproducibility analysis of the two rHuEPO targeted peptides T6 and T17 in spiked horse plasma.

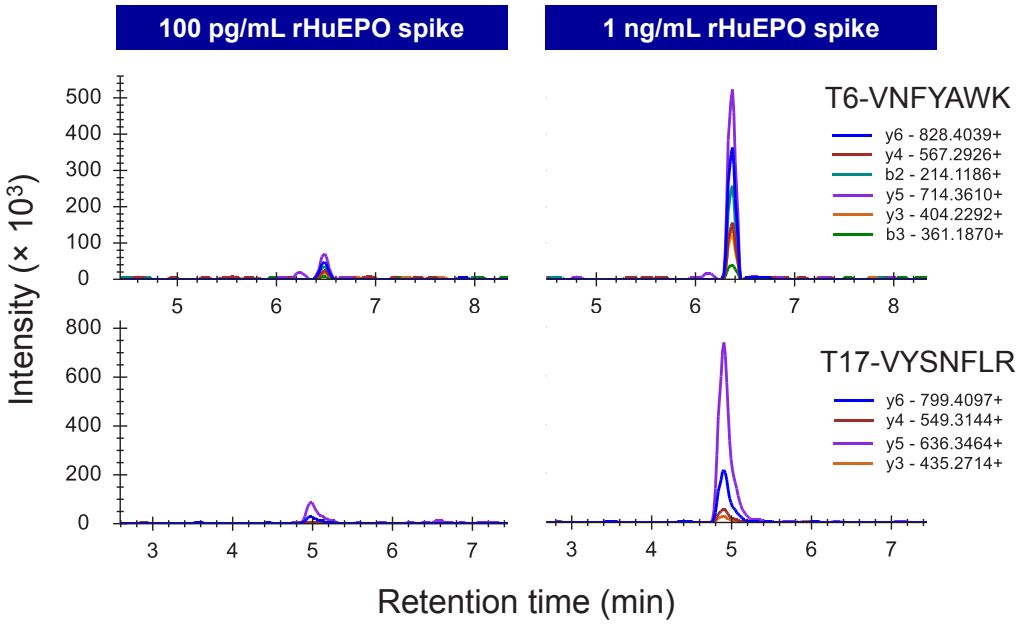

Figure 2: Example of rHuEPO peptides T6 (top) and T17 (bottom) in two spiked horse plasmas (100 pg/mL and 1 ng/mL).

In order to further validate these interesting results, we performed the re-extraction and analysis of a real post-race sample which was previously tested positive for rHuEPOs misuse by a validated nanoLC-HRMS analytical method. As shown on Figure 3, T6 rHuEPO specific peptide “VNFYAWK” was successfully identified in the post-race sample and the positive control sample (spiked at 250 pg/mL) whereas no specific signal was detected in the blank plasma (negative control), at the expected retention time. We can thus estimate that the concentration of rHuEPO in the post-race sample was close to 50 pg/mL (i.e. 0.9 fmol/mL).

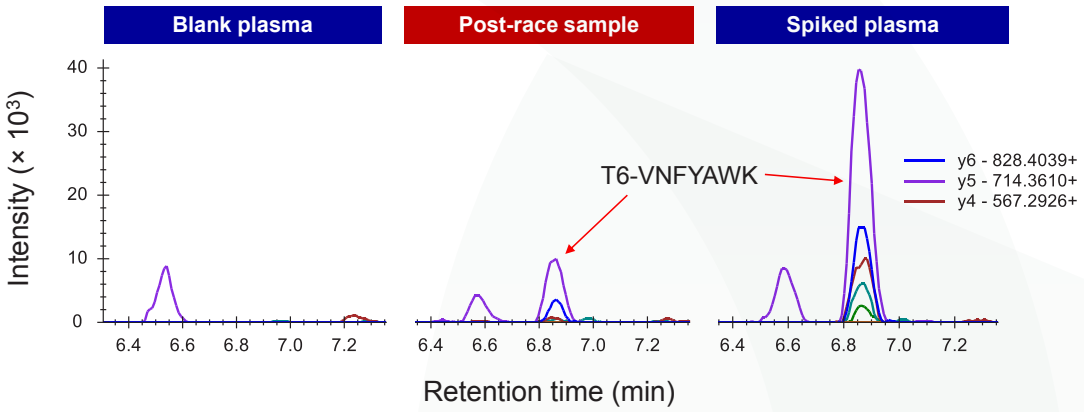

Figure 3: Detection of the fraudulous administration of rHuEPO in a post-race sample by means of T6-VNFYAWK target peptide transitions y_6^+, y_5^+ and y_4^+.

The high througput 11.5 minutes pre-formed gradient of the Evosep One is therefore suitable to detect a possible forbidden administration of rHuEPOs in horse with four to eight-fold time saving and unimpaired sensitivity, even at trace concentration.

4. Conclusion

By employing innovative sample processing and analysis strategy, the Evosep One offers new perspectives to drug testing laboratories thank to high sample throughput without compromising sensitivity. Indeed, the use of tip-based sample trapping, optimized elution and on-column peptide refocusing, the Evosep One provided consistent detection of rHuEPO in spiked plasmas and real rHuEPO administration case with low sample-to-sample overhead time and rapid gradients. These results illustrate the promising capabilities of the Evosep One innovative technology for the analysis of protein/peptide-based drugs in the context of drug testing and more generally for targeted proteomics.

References

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2. Bache, N. *et al.* A novel LC system embeds analytes in pre-formed gradients for rapid, ultra-robust proteomics. *Mol. Cell. Proteomics* mcp.TIR118.000853 (2018). doi:10.1074/mcp.TIR118.000853

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