Application Note: 408

Developing a Method to Protect the Integrity of Racing Using Targeted SRM: Detection and Quantitation of rhEPO/DPO in Horse Plasma

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Key Words

- TSQ Quantum Access™
- HeavyPeptide Labeled Standards
- Proteotypic Peptides
- SRM Method
- Targeted Protein Analysis

Overview

Purpose: To develop a method for the detection and confirmation of rhEPO/DPO in horse plasma using a targeted protein assay and labeled internal standards.

Methods: Combined immunoaffinity separation, enzymatic digestion, and mass spectrometry has been used to confirm the presence of rhEPO in horse plasma[1]. The use of an SRM method for targeted protein detection enabled measurements of retention times, ion ratios, and labeled internal standards to confirm and quantify the presence of rhEPO in horse plasma.

Results: Using labeled internal standards, rhEPO was detected, quantified and confirmed in administered horse plasma 72 hours following administration, simulating real world situations.

Introduction

Recombinant human erythropoietin (rhEPO)[2] and Darbepoetin-alpha (DPO)[3] are genetically engineered protein-based drugs used for the treatment of anemia by stimulating red blood cell production. The ability of these agents to stimulate red blood cell production has led to use and abuse by human and equine athletes and, thus, violates the rule of fair competition resulting in their classification as banned substances by the horse racing industry. In addition, continued administration to horses can result in anemia.[3] Despite the negative aspects of rhEPO for horses, a reliable, verifiable, and legally defensible method for identification and confirmation of rhEPO/DPO has been elusive due to the very low concentrations administered. Sample collection is typically acquired only after competition, which could be in excess of 72 hours following administration. Testing of rhEPO/DPO is further confounded by the complexity of the matrices in which the drug is typically found–plasma and urine.

Methods

All experiments were performed using a Thermo Scientific TSQ Quantum Access triple quadrupole mass spectrometer equipped with a Thermo Scientific Surveyor™ MS Pump and MicroAS Autosampler (Thermo Fisher Scientific, San Jose, CA) operated in mSRM mode monitoring six diagnostic peptides that differentiate rhEPO and DPO from equine EPO. (Scheme 1). In addition to the six diagnostic peptides, four stable isotope labeled internal standards for the T_4, T_6, T_{11}, and T_{17} rhEPO proteotypic peptides were used for absolute quantification and additional confirmation of the presence of rhEPO/DPO (Thermo Biopolymers, Thermo Fisher Scientific, Ulm, Germany). Method development was performed using neat rhEPO/DPO protein digests. (Amgen, Inc., Thousand Oaks, CA).

HPLC separations were achieved using a Hypersil Biobasic™ C18 100×0.5 mm column and a binary solvent system consisting of A) 0.1% formic acid and B) MeCN (0.1% formic acid). A gradient profile of 2-40% B in 12 minutes was used at 60 µL/min.

Sample preparation included immunoaffinity separation using rabbit and mouse IgG antibodies linked to magnetic beads. Following separation, the resulting protein was filtered and enzymatically digested with an enzymatic or proteolytic cleavage from which a set of diagnostic peptides representing rhEPO/DPO was chosen as candidate biomarkers for confirmation of the presence of rhEPO/DPO in horse plasma.[1]

Two different sets of samples were prepared and analyzed. The first set was a controlled spiking experiment in which a known quantity of rhEPO was spiked into 1 mL of digested horse plasma to determine detection efficiency. The second sample set was plasma extracted as a function of time following rhEPO administration (iv) of 8000 IU. The time points for extraction ranged from 0 hr to 72 hours. Each of the time point samples was spiked with 10 fmol/µL of the labeled peptide standards.

Results and Discussion

Scheme 1 shows the basis of identification for rhEPO/DPO in equine plasma. The results of enzymatic digestion produced multiple diagnostic markers that can be used to increase the confidence of the presence of the foreign substance in the equine athlete. In addition, the method described enables detection of rhEPO or DPO due to the conserved sequence for each protein over the targeted peptides. Figure 1 shows summed SRM chromatograms for (1A) DPO and (1B) rhEPO using the same SRM transitions. Clearly, the retention times are closely identical for both samples indicating the experimental method is robust for either drug.

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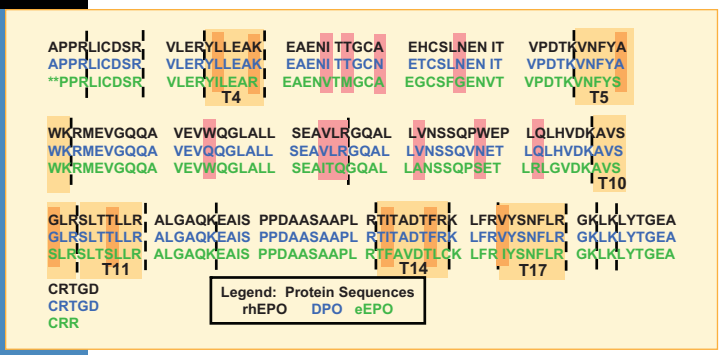
Figure 2 shows the summed SRM chromatograms for the four targeted rhEPO peptides and the labeled analogues. The labeled peptide can be used to confirm the correct elution time as well as the ion ratio provided more than one transition was used to monitor each peptide. A level of 500 amol on column was used to test the detection capabilities of the approach used, which would equate to a concentration of ca. 1.7 ng/mL. Note that the responses of T_4, T_{11}, T_{17} markers were greater than 10000 counts, indicating lower levels of detection to be about 10x lower (or 0.2 ng/mL) without requiring nanoliter flow rates, which simplifies the experiment and increases the robustness of the method.



Scheme 1. Comparison of protein sequences for rhEPO, DPO, and equine EPO. The dashed lines represent sites of enzymatic cleavages and the red boxes highlight non-conserved sequence sites between rhEPO/DPO and equine EPO. The targeted peptides are marked with a gold box.

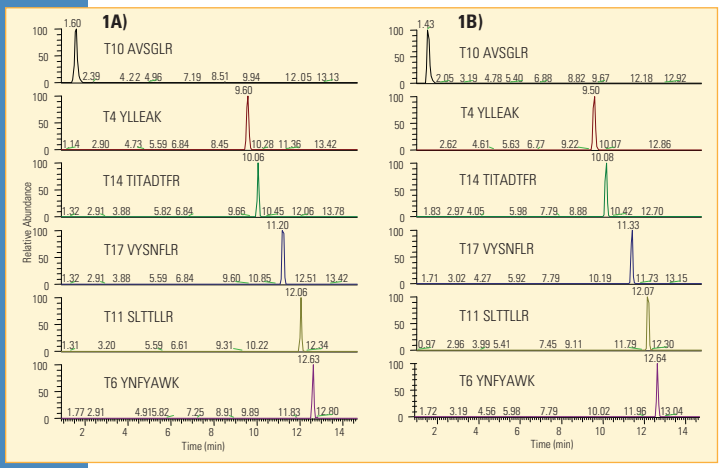


Figure 1: SRM chromatographic traces for each of the targeted peptides for 1A) DPO and 1B) rhEPO enzymatic digest using identical experimental method.

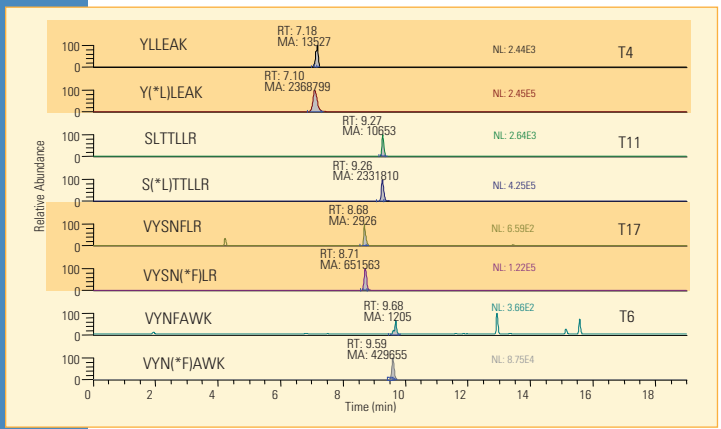


Figure 2: SRM responses for four targeted rhEPO peptides and the corresponding stable isotope labeled peptide. The measured response is for a total of 500 amol on column for the unlabeled rhEPO and 100 fmol for the labeled rhEPO peptides.

In addition to establishing the correct retention times for targeted peptides, the stable-isotope labeled peptides can be used for correct ion ratio determination as an additional means of verification. Figure 3 shows comparative full-scan product ion spectra for the (3A) unlabeled and (3B) labeled T_{11} peptide. Note the y-series detected for each, providing sequencing information and site determination for the stable isotope labeled residue such as the a_2/b_2 fragments as well as the y_6 for the unlabeled peptide. The two product ions used for detecting the T_{11} peptide were the y_4 and y_5 ions. The calculated abundance ratios for the unlabeled and labeled peptides were ca. 25%. The insets to the right of Figure 3 show the measured ion abundance for each SRM transition at 500 amol level. The calculated ratio is within experimental error to be used as an additional means of confirmation for the targeted peptide elution.

Figure 4 shows the quantification curve calculated for the controlled rhEPO spiking of horse plasma. The values show excellent agreement between theoretical and experimentally determined levels based on the integrated peak area ratios between the unlabeled and labeled targeted rhEPO peptides. The %CVs for each was less than 20% at 500 amol level indicating excellent capabilities to quantify the presence of rhEPO in plasma. While a positive confirmation would only require one diagnostic peptide to be present, this method yields four proteotypic peptides that could be used unequivocally to increase the confidence in a positive determination.

The second sample set was used to test the entire workflow. A female horse (500 kg) was administered rhEPO intravenously using 8000 IU (0.08 mg/kg) for four days. Following the injection on the fourth day, blood was withdrawn at 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 24, 48, and 72 hour intervals. Samples for each time point were processed using the method outlined previously, reducing complexity of the resulting protein digest mixture. The protocols of most horse racing commissions require the saliva, urine, and/or blood sample to be taken from the winning horse following completion of a race. The 72 hour time window represents a possible maximum duration between the final doping and racing while maintaining a pharmacological effect following administration of rhEPO/DPO. The 8000 IU dose is also an estimate of the dose required to induce the desired biological effects of increasing oxygen carrying capacity for equine athletes. The proposed protocol must enable a reduction of sample loss through the number of sample purification, filtering, reconstitution, and digestion steps prior to mass spectral analysis. Figure 5 shows the summed SRM chromatograms for the four targeted rhEPO peptides with their stable-isotope labeled internal

standards. Three of the four peptides showed a positive response with little signal attributed to the T_6 peptide at the 72 hour time point. Although the response for the T_4 peptide does not appear to be measurable, closer inspection shows an integrated peak area over 2000 counts observed to have the same retention time as that for the labeled T_4 peptide at 7.27 minutes.

Comparison of chromatographic retention times for the administered rhEPO study with the spiked rhEPO study (Figure 2) showed excellent chromatographic reproducibility, with retention times that shifted less than 6-10 seconds, enabling an additional means of confirmation for the presence of rhEPO in the extracted horse plasma. Based on the integrated peak area ratios for the three detected rhEPO biomarkers, a total of ca. 0.05 ng/mL was present in the horse plasma following a 72-hour delay between rhEPO administration and sample collection. Comparison of LC-MS/MS results with those measured using ELISA show similar levels (0.04 ng/mL–data not presented) indicating excellent agreement between the two methods.

Using a stable-isotope labeled internal standard provides two clear advantages: identification of the correct retention times, as shown above, and determination of the correct ion ratio for the monitored product ions. Figure 3 demonstrates the consistency of the ion ratios measured following CID for both full scan MS/MS detection as well as SRM analysis for the T_{11} labeled and unlabeled rhEPO peptides. The same measurements can be used to confirm the presence of rhEPO at each time point. Figure 6 shows the measured ion abundance for the y_4 and y_5 fragment ions for the unlabeled and labeled T_4 peptides at the time points of 72, 10, and 0.5 hrs following the final rhEPO administration. The measured ion ratios for the unlabeled T_4 peptides were consistently between 20 and 25% while the ratio for the labeled T_4 peptide was consistently between 30 and 35%. The slight increase in the ratio for the labeled peptide was observed for the three other pairs of signature peptides (see Figure 3).

Figure 7 shows the calculated rhEPO concentration in the extracted horse plasma samples for T_4 and T_6 peptides. The levels were calculated using the integrated area ratios between the targeted rhEPO peptide and their corresponding labeled internal standards. The calculated concentration for two targeted peptides agree with those obtained using two different labeled standards to monitor the concentration of rhEPO in the test sample. In addition to mass spectral determination, ELISA was also used to A) predict the presence of rhEPO and B) calculate the level of rhEPO in plasma at each time point. The ELISA results nicely corresponded with those calculated using the targeted SRM approach; in fact, the levels estimated at 48 and 72 hours agreed well (0.06 and 0.04 ng/mL, respectively), increasing the confidence in the calculated concentrations.

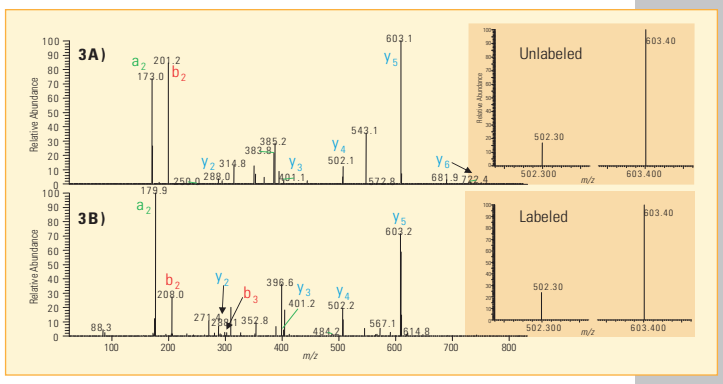


Figure 3: Comparative Quantitation Enhanced Data-Dependent™ MS/MS spectra for 3A) unlabeled and 3B) labeled T_{11} peptides at 50 fmol on column. The inset shows the measured ion intensity for the SRM transitions for the unlabeled and labeled T_{11} peptides at 500 amol on column.

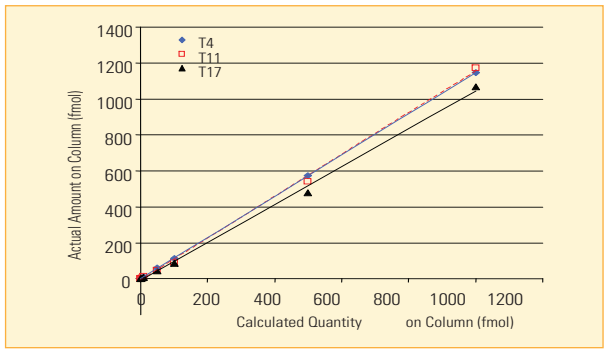


Figure 4: Quantification curve for neat rhEPO analysis. The calculated levels were determined using area ratios of the labeled analogues.

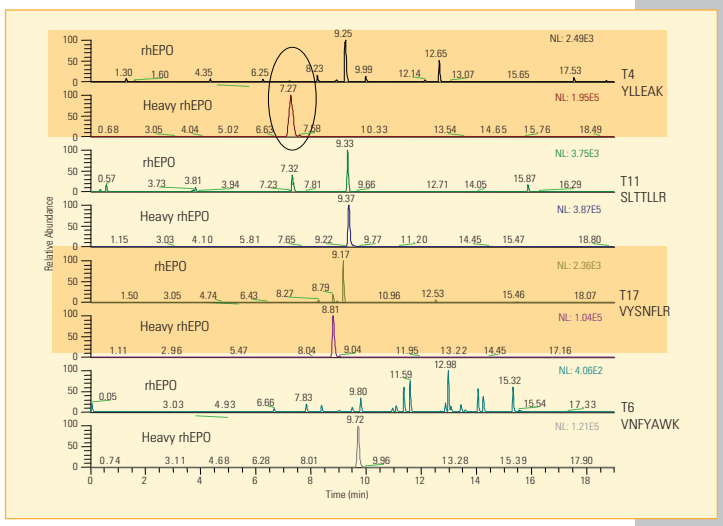


Figure 5: Summed SRM chromatograms for the four targeted rhEPO peptides and their labeled derivatives for the horse plasma extraction sample collected 72 hours following rhEPO administration.

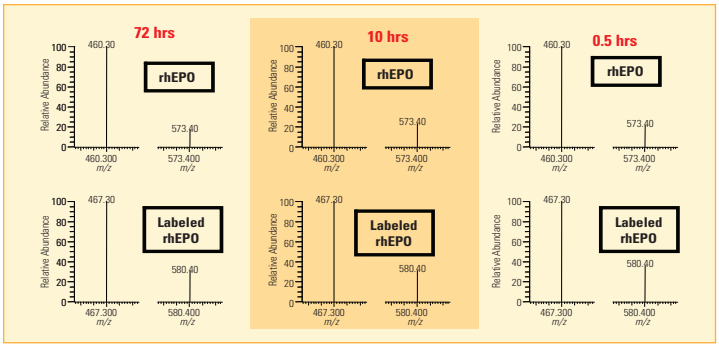


Figure 6: Comparative ion abundance ratios for the T_4 peptide at three different time points for plasma collection. The top row is the measured ion abundance for the unlabeled peptide and the bottom row is the response from the labeled peptide.

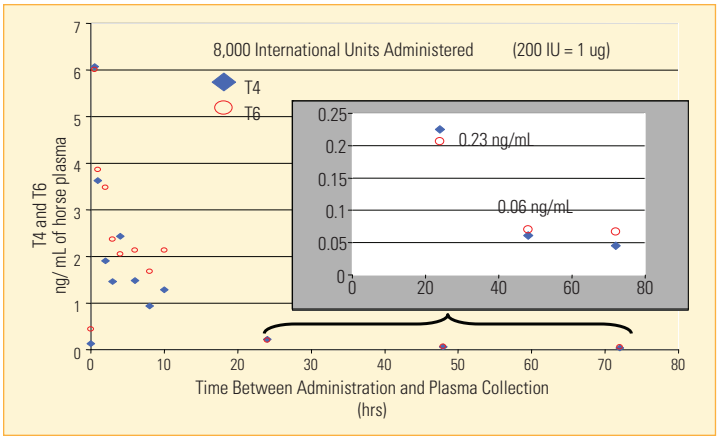


Figure 7: Calculated rhEPO levels in extracted horse plasma as a function of time delay between administration and sample collection. The calculated levels were based on the area ratios of T_4 and T_6 targeted rhEPO peptides and the labeled internal standards.

Conclusions

The approach presented here provides a sensitive and selective method for preparing and analyzing horse plasma for the presence of rhEPO or DPO. The advantages of this method include the ability to use up to six diagnostic peptides to confirm or refute the presence of either illegal protein-based drug.

The use of stable-isotope labeled analogues provides further means of confirming the presence of diagnostic peptides based on chromatographic retention times and ion ratios.

The sensitivity demonstrated enabled detection up to 72 hours following the last administration of rhEPO, increasing the confidence that the described method is useful in the racing industry to maintain a level field of competition.

Of particular interest is the measured sensitivity that was achieved using microspray, increasing the analysis time while simplifying the experimental method and thus, enabling more laboratories the option of employing rhEPO/DPO screening.

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