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Screen and confirmation of PEG-epoetin β in equine plasma

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Methods have been developed to screen for and confirm darbepoetin alfa, recombinant human EPO, and methoxy polyethylene glycol-epoetin β (PEG-epoetin β) in horse plasma. All three methods screen samples with an enzyme-linked immunosorbent assay (ELISA) and confirm by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This report focuses on PEG-epoetin β. The ELISA assay was able to detect PEG-epoetin β at 0.02 ng/mL in 50 μL of horse plasma. Many samples had high background levels of immunoreactivity; however, introducing polyethylene glycol 6000 (PEG 6000) into the samples before the ELISA assay removed the high background and increased the apparent concentrations of PEG-epoetin β. In samples collected following the administration of 100 μg of PEG-epoetin β by the intravenous (IV), intramuscular (IM) and subcutaneous (SC) routes, PEG-epoetin β was detectable up to 72, 144, and 120 h, respectively. The samples were prepared for LC-MS/MS analysis by extraction with anti-rHuEPO-antibodies-coated Dynabeads followed by digestion with trypsin. The LC-MS/MS confirmation method used the multiple reaction monitoring (MRM) scan mode to monitor four precursor-product ion transitions of the EPO-derived peptide T_6. All four transitions of T_6 were detectable with S/N > 3. The limit of confirmation for PEG-epoetin β was 1.0 ng/mL in 2 mL of horse plasma. The method successfully confirmed the presence of PEG-epoetin β in a sample collected from a Mircera®-treated horse. Compared to PEG-epoetin β, better sensitivity was achieved for darbepoetin alfa and recombinant human EPO. Darbepoetin alfa was detected in horse plasma four days after IM administration of 100 μg.

Keywords: equine; doping; erythropoietin; recombinant human erythropoietin; darbepoetin alfa; PEG-epoetin β; Mircera®; immunoaffinity purification; liquid chromatography/mass spectrometry

Introduction

Recombinant human erythropoetin (rHuEPO), darbepoetin alfa (DPO) and methoxy polyethylene glycol epoetin β (PEG-epoetin β, Mircera) are erythropoiesis-stimulating agents which are prohibited in racehorses and humans because they enhance their performance by increasing red cell mass and delivering more oxygen to muscles.[1,2] The newest analog, PEG-epoetin β, is synthesized by pegylating rHuEPO β. This increases its molecular weight by ~30 kDa, increases its half-life, and decreases its urinary excretion.[3] All analogs are detectable in human urine and plasma using various ELISAs and they can be distinguished from each other by the isoelectric-focusing, double-blotting (IEF–DB) method[4] that has been widely applied in human doping control laboratories.[4,5] A method based on sarcosyl-page has good sensitivity for PEG-epoetin β, and it allows for the direct detection of all the EPO analogs in a single experiment.[6] This method has not been reported in equine. Doping control organizations greatly prefer unambiguous mass spectrometry-based methods, however until very recently they have not been available for equine or human tissue.

PEG-epoetin β has been detected in the plasma of human athletes.[7,8] No cases have been fully documented in competing racehorses. The IEF-DB test successfully detected rHuEPO (Eprex®) and DPO in horse urine for several hours after administration.[9] In our previous report[10] we detected PEG-epoetin β in the plasma of a treated horse using a chemiluminescent immunometric assay and confirmed it with an immuno-affinity procedure followed by the IEF-DB method.

The IEF-DB method is complex and is not designed to provide a mass spectrum, consequently there continues to be a need for an improved and unambiguous method for identifying rHuEPO and analogs in body fluids. Recent efforts to detect these compounds in horse plasma have been aided by the observation that trypsin digestion fragments of rHuEPO, DPO or PEG epoetin β include common peptides (T_6, VNFYAWK and T_{17}, VYSNFLR). Most importantly trypsin treatment of equine EPO does not yield T_6 or T_{17}, therefore finding T_6 or T_{17} in horse plasma is direct proof of doping with rHuEPO, DPO or PEG-epoetin β.[11,12] T_6 and T_{17} findings have been reported to racing authorities as positive cases of rHuEPO or DPO.[11]

In the first mass spectrometry-based study of EPO analogs in the equine, Guan *et al*.[11] showed that an immunoaffinity extraction procedure followed by trypsin digestion isolated a T_6 peptide (VNFYAWK) and a T_{17} (VYSNFLR) peptide which were then identified by LC-MS/MS. The reported detection limits for rHuEPO and DPO were 0.1 ng/mL, however Yu *et al*.[12] using a similar method, were not able to reproduce these detection levels. They developed a multidimensional nano LC and nanospray MS/MS method and reported that it detected 1.0 ng of PEG-epoetin β spiked into 5 mL of plasma; however no administration samples were tested.

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This report expands our previous administration studies[10] of PEG-epoetin β in horses, by administration via three routes, reporting pharmacokinetic data, and demonstrating that T_6 is detected by LC-MS/MS following immunoaffinity extraction and trypsin digestion. Furthermore the method does not require a nano LC system.

Materials and Methods

Materials

Mircera®, 250 µg/0.3 mL solution for injection in a pre-filled syringe, was obtained from Hoffmann-La Roche, Roche Diagnostics GmbH (Mannheim, Germany). Aranesp® (100 µg/mL) and Epogen® (10 000 U/mL) were obtained from Amgen (Thousand Oaks, CA, USA). Peptide VNFYAWK (human EPO peptide T_6) and VYSNFLR (human EPO peptide T_{17}) were synthesized by AnaSpec, Inc. (Fremont, CA, USA). Heavy peptide T_6-d_{10} was provided by Thermo Fisher Scientific (Ulm, Germany). Magnetic beads, Dynabeads® M-280 tosylactivated, were obtained from Invitrogen (Carlsbad, CA, USA). Human erythropoietin polyclonal antibody, rabbit IgG was purchased from R& D Systems, Inc. (Minneapolis, MN, USA). Sequencing grade modified trypsin and tris base were purchased from Promega (Madison, WI, USA). Igepal CA-630, sodium hydroxide, boric acid, albumin from bovine serum (BSA) and EDTA were obtained from Sigma (St Louis, MO, USA). Polyethylene glycol (PEG) 6000 was purchased from Fisher Scientific Inc. (Pittsburgh, PA, USA). Phosphate buffered saline tablets were purchased from VWR (West Chester, PA, USA). All the solutions were prepared in purified water (resistivity $>$ 18.2 MΩ/cm) obtained from a Millipore Elix® water purification system (Billerica, MA, USA).

The following buffers were prepared as described by Yu *et al*;[12] 0.1 M borate buffer, pH 9.5 (Buffer B); PBS (pH 7.4), plus 0.1% (w/v) BSA, 2 mM EDTA and 0.02% sodium azide (w/v) (Buffer C); 0.2 M Tris buffer (pH 8.5) plus 0.1% (w/v) BSA (Buffer D); washing buffer, 1% (w/v) Igepal CA-630 in PBS (pH 7.4). The elution buffer was 0.1% PEG 6000 (w/v) in PBS (pH 2.0).[11]

Animal experiments

Three healthy ~450 kg standard-bred horses were dosed with Mircera® at 100 µg via intravenous (IV), intramuscular (IM) and subcutaneous (SC) injection, respectively. Plasma samples (1 mL) were obtained from the contralateral vein and collected every 4 h until 12 h, then once a day until day 7 for the IV injection. For the IM injection, plasma sample was collected at hour 0, 2, 8, and then once a day until day 11. For the SC injection, plasma sample was collected at hour 0, 2, 8, and then once a day until day 7. A fourth healthy horse was dosed with IM DPO at 100 µg and plasma samples (2 mL) were collected on days 0–5. All samples were shipped on ice and stored at −80 °C at the laboratory.

Analytical methods

ELISA assay

The ELISA kit (STEMCELL Technologies, Vancouver, BC, Canada) was designed for quantitative measurements of natural and recombinant human EPO in biological fluids. We used it to quantitate Mircera® in horse plasma by constructing standard curves using Mircera® spiked in the sample diluent. The concentration range of the standards was 0.02–4.2 ng/mL. Fifty microliters of each sample and standard solution were loaded into the wells for the ELISA test. The samples pretreated with PEG 6000 were prepared as follows: 0.1 mL of sample and 0.1 mL of 50% (w/v) PEG-6000 solution were mixed, incubated at 37 °C for 15 min, centrifuged at 9300 g for 10 min[13], and 50 uL of the supernatant were transferred to the wells for ELISA testing.

Anti-rhEPO antibody coupling procedure

We used a procedure described by Yu *et al*.[12] Briefly 2 mL of magnetic beads were vortexed and 1 mL was transferred to a 2-mL Eppendorf® protein LoBind microcentrifuge tube (Fisher Scientific, Pittsburgh, PA, USA), which was placed in a magnetic particle concentrator (Dynal MPC®-S, Invitrogen, Carlsbad, CA, USA). After separation from the liquid the beads were washed with 1 mL of Buffer B. Human erythropoietin polyclonal antibody (R& D Systems, Inc. Minneapolis, MN, USA) was reconstituted in 1 mL of Buffer B and 0.5 mL was added to the magnetic beads. Following incubation (37 °C with shaking for 16–24 h) the coated beads were washed twice with Buffer C and incubated with 1 mL of Buffer D (4 h at 37 °C). Following separation the coated beads were washed with 1 mL of Buffer C and suspended in Buffer C.

Immunoaffinity extraction and trypsin digestion

We used a procedure described by Yu *et al*.[12] with several modifications. Briefly, coated magnetic beads (0.5 mL) were transferred to a 2-mL Eppendorf® protein LoBind microcentrifuge tube, separated from the liquid and washed with 2 mL of Buffer C. The suspended beads mixture was added to 2 mL of equine plasma sample, vortexed and incubated at 37 °C for 16–24 h with shaking. Next the beads were separated and washed four times with 2 mL washing buffer for 5 min at ambient temperature. The beads were then suspended in 1.0 mL washing buffer and transferred to a fresh 1.5-mL LoBind tube. Following separation, 1.0 mL of elution buffer (0.1% PEG 6000 in PBS) was added to elute the EPO. After separation the supernatant was transferred to an Amicon Ultra centrifugal filter tube (30K) and centrifuged for 30 min. Two mL of 50 mM ammonium bicarbonate (pH 7.8) were added to the retentate and centrifuged another 30 min. The retentate was transferred to a 1.5-mL LoBind tube and incubated at 80 °C for 10 min to stop the proteolytic activity of the proteases.[14]

Trypsin (20 µg) was reconstituted in 500 µL of 50 mM ammonium bicarbonate (pH 7.8) and 50 µL was added to the sample after cooling. The digestion was performed at 37 °C for 3 h. The digested sample was centrifuged and 10 µL of the supernatant was injected directly without acidification into the LC-MS/MS for analysis of peptides.

LC-MS/MS analysis

LC-MS/MS analysis was performed on an Applied Biosystems 4000 Q TRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) with an ESI source interfaced with a Shimadzu LC-20AD HPLC system and a CTC Analytics HTS PAL autosampler. Chromatographic separation was achieved on a Zorbax 300 SB-C18, 1 × 50 mm, 3.5 µm column (Agilent Technologies, Santa Clara, CA, USA) using H_2O (A) and acetonitrile (B) as mobile phase, both containing 0.1% (v/v) formic acid, programmed as follows: initial mobile phase 5% B for 3 min, increased to 50% B at 25 min, held

Table 1. Four transitions of peptide T_6 and T_{17} on LC-MS/MS

Peptide	Parent ion $[M+2H]^{2+}$	Product ion															
		m/z	EP	CE	CXP	m/z	EP	CE	CXP	m/z	EP	CE	CXP	m/z	EP	CE	CXP
T_6	**464.4**	**214.5**	8	22	10	**714.3**	10	20	10	**828.2**	10	22	10	**811.3**	10	25	10
T_{17}	**450.2**	**235.1**	10	25	10	**263.3**	10	20	12	**441.3**	10	18	20	**636.5**	10	20	20

EP: Entrance potential.
CE: Collision energy.
CXP: Collision cell exit potential.

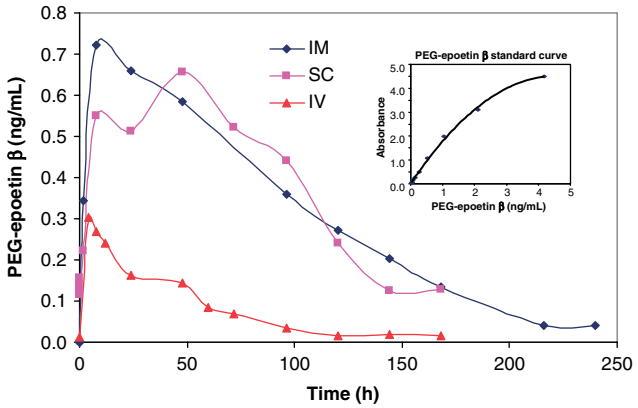

Figure 1. Plasma PEG-epoetin β concentration determined by ELISA following a single injection of 100 µg Mircera to three horses via IV, IM and SC, respectively. Inset: PEG-epoetin β standard curve; $y = -0.2370x^2 + 2.052x + 0.01208$; $R^2 = 0.9969$.

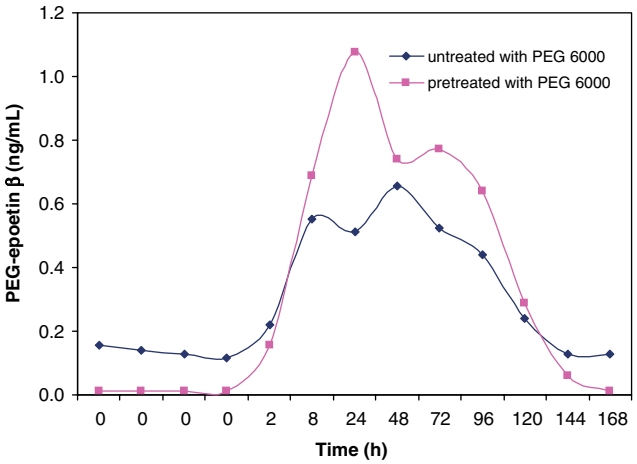

Figure 2. Comparison of PEG-epoetin β concentrations in horse plasma samples pretreated with PEG 6000 or not. The samples were collected post administration of a single SC injection of 100 µg Mircera to a horse and tested by ELISA.

for 5 min, then decreased to 5% B at 32 min. The flow-rate was 50 µL/min.

The mass spectrometer was operated in the turbo ion spray mode with positive ion detection. The turbo ion spray temperature was maintained at 350 °C and the ion spray voltage was set to 5000 V. Gas 1, gas 2 and the curtain gas were set to 40, 20 and 10 psi, respectively. Four transitions were used to monitor peptide T_6 and T_{17}. The product ions and corresponding compound-dependent parameters are listed in table 1. For all the transitions the declustering potential (DP) was 50 V.

Results

Concentrations of PEG-epoetin β following administration of Mircera® and the effects of PEG 6000

The concentrations of PEG-epoetin β at different times following administration of Mircera® via IV, IM, and SC at 100 µg are shown in Figure 1. For the IV injection, the concentration of PEG-epoetin β immediately reached the highest level in the 4-h post-administration sample, then gradually declined back to the baseline level at 120 h. For the IM injection, the concentration of EPO was increasing from 2, to 8 and 24 h post-administration, then gradually declined over the subsequent days but was still readily detected at 240 h. For the two horses that received IV and IM injections, the time zero samples showed no interference. For the horse that received Mircera SC, the EPO concentration was highest at 48 h, then it decreased gradually until it reached the basal value at 144 h. The data also showed that at time zero before drug administration, when the actual plasma concentration of EPO should be zero, the assay detected a signal. This interference disappeared when the samples were pre-treated with PEG 6000. Figure 2 shows that the PEG decreased the background at time zero while increasing peak concentrations at the later times.

LC-MS/MS

The sensitivity of the LC-MS/MS method was examined by testing equine plasma samples (2 mL) spiked with PEG-epoetin β at various concentrations. Both T_6 and T_{17} were analyzed by monitoring four transitions (Table 1). For T_6 detection, we used the same precursor-product ion transitions as Yu *et al.*[12] The results suggest that T_6 has better sensitivity than T_{17} (data not shown). We defined the minimum requirements for confirmation as all four transitions of T_6 being detectable with S/N > 3. With these criteria the limit of confirmation of PEG-epoetin β was 1.0 ng/mL in 2 mL plasma (Figure 3B). No peak was detectable for blank horse plasma (Figure 3A).

The method was used to confirm the presence of PEG-epoetin β in plasma obtained from a research horse dosed SC with 100 µg of Mircera®. The samples collected post-administration at hour 8 and day 1, 2, 3, and 4 were combined for a total volume of approximately 3.2 mL. The sample was subjected to immunoaffinity purification followed by trypsin digestion before analysis by LC-MS/MS. All four transitions for T_6 were detectable with satisfactory S/N ratios (Figure 4). For peptide T_{17} only two transitions, 450.2/235.1, and 450.2/263.3 were detectable (data not shown).

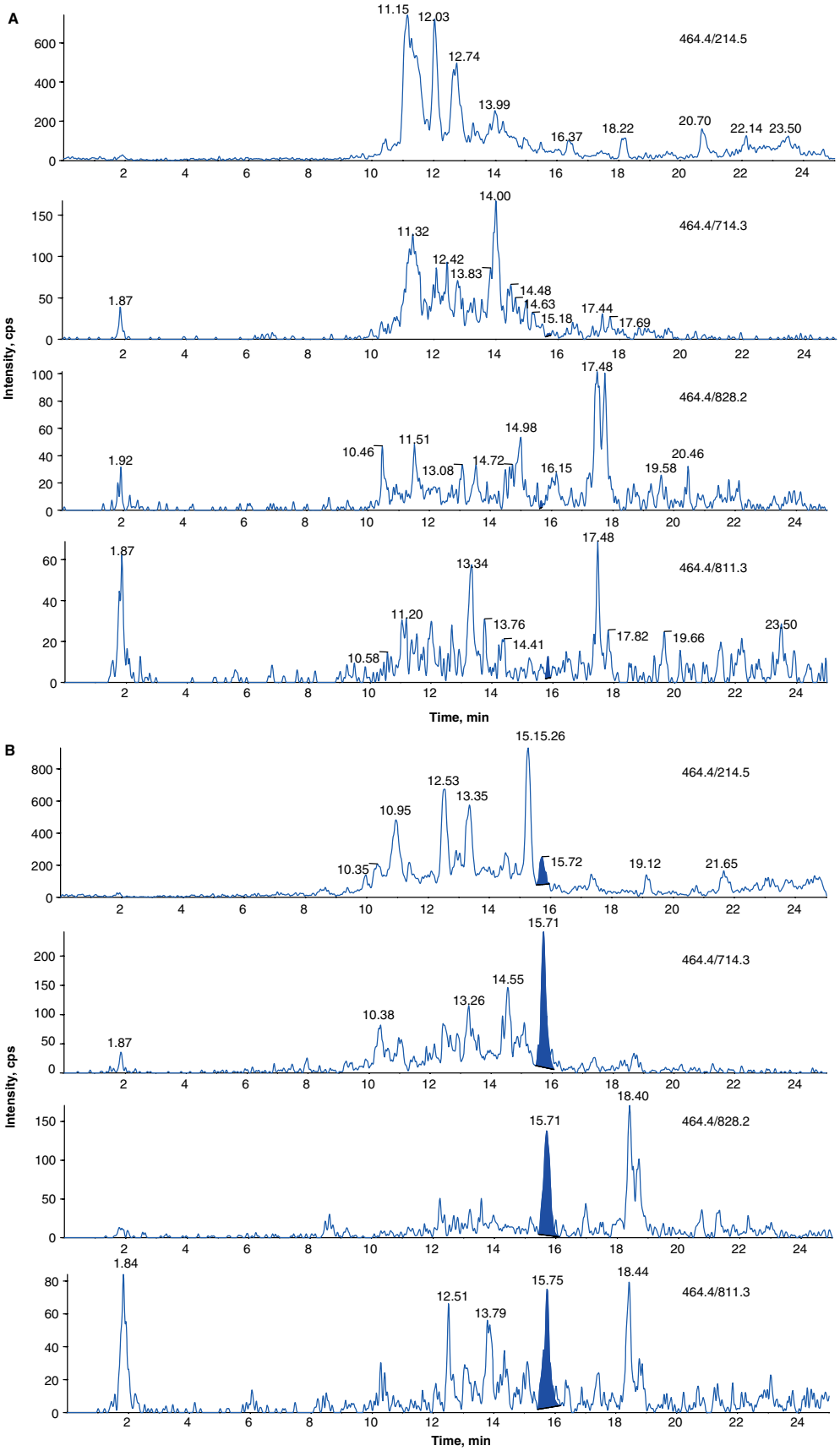

Figure 3. MRM chromatograms of a pooled blank horse plasma (A) and blank horse plasma (2 mL) spiked with PEG-epoetin β at 1.0 ng/mL (B).

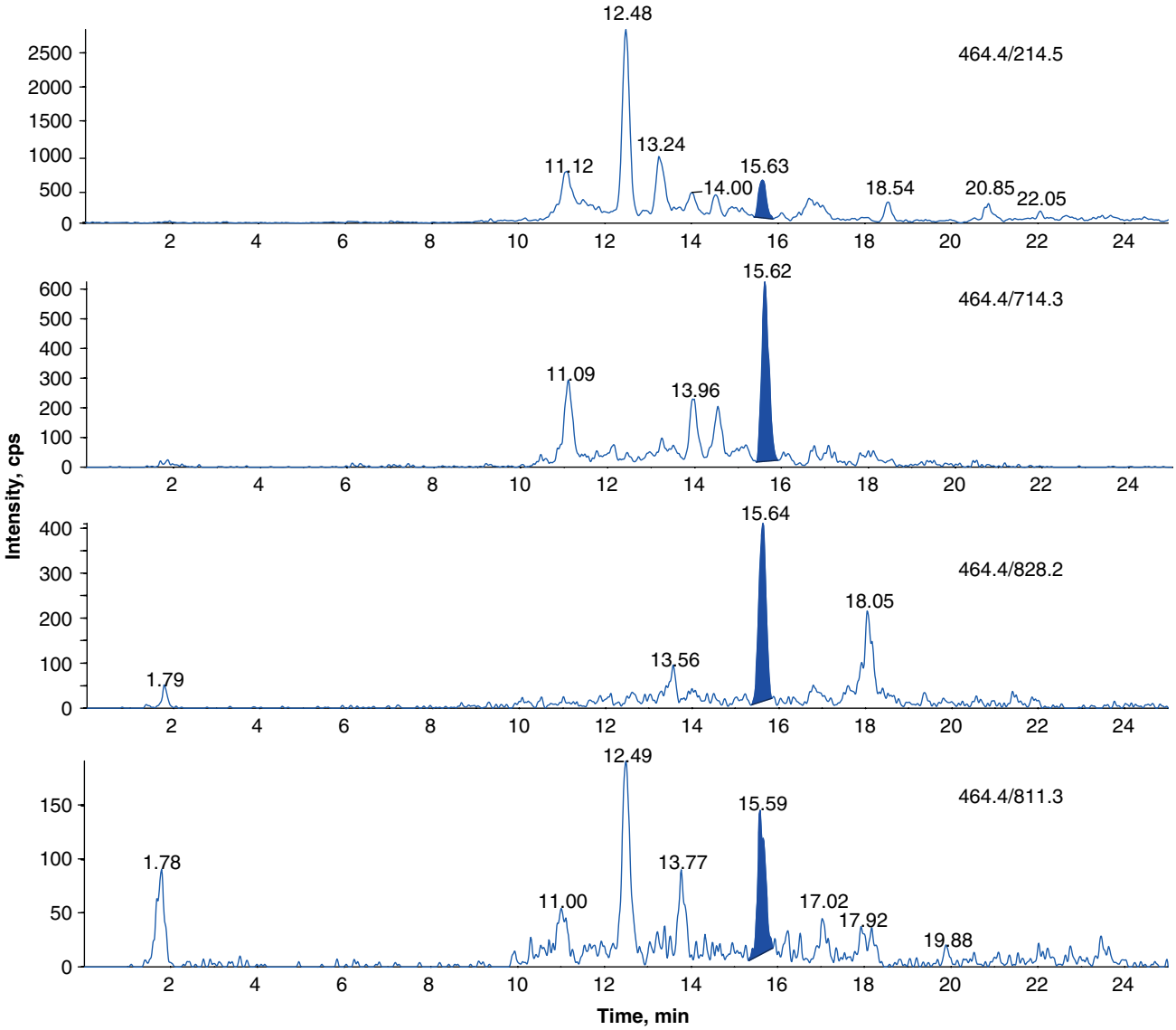

Figure 4. MRM chromatograms of combined horse plasma samples (hour 8 and day 1, 2, 3, and 4, total volume 3.2 mL) collected post-administration of Mircera at 100 µg via SC.

Discussions

PEG 6000 is a nonionic polymer that has been widely used in protein purification as a fractional precipitating agent.[13,15] This study shows that when it was added to the equine samples before the ELISA reagents were added, it greatly reduced the interferences for PEG-epoetin β testing. Samples that appeared to contain up to 0.16 ng of PEG-epoetin β/mL before treatment contained essentially zero following the addition of PEG 6000. Ten control horse plasma samples were also tested by ELISA and four of them exhibited a relatively high signal. When the samples were treated with PEG 6000 the signal dropped to zero. The apparent concentrations of PEG-epoetin β in the post-administration samples were up to 100% higher following the PEG 6000 treatment, which is similar to the finding reported by Van Maerken *et al.*,[13] who proposed that PEG 6000 can strongly enhance the solubility of the CERA. The results suggest that the specificity and sensitivity of the ELISA assay for PEG-epoetin β can be improved by PEG 6000. This might be helpful to reduce the number of false positive cases when using ELISA as a screen method for PEG-epoetin β. We plan further investigations of this finding.

Two publications focus on the detection and confirmation of rHuEPO and/or DPO in horse plasma by LC-MS/MS.[11,12] Only one of these publications reported detecting PEG-epoetin β in spiked plasma samples, however no administration samples were tested.[12] In the current study, we achieved better sensitivity for PEG-epoetin β than Yu *et al.*[12] We report a limit of confirmation of 1.0 ng/mL in 2 mL plasma (Figure 3). Among the four transitions, 464.4/714.3 showed the strongest signal while 464.4/811.3 was the weakest. The peak area counts for 464.4/214.5 and 464.4/828.2 were similar but the latter peak was more distinguishable. A retention time shift was also observed during LC-MS/MS analysis, therefore an internal standard T_6-d_{10} (HeavyPeptide™ rhEPO Confirmation DEMO Kit, Thermo Fisher Scientific, Ulm, Germany) was tried. The preliminary data suggested that T_6-d_{10} helps to locate the T_6-d_0 peak, but the deuterated analog cannot be used at a high concentration because it will interfere with T_6-d_0 detection. The experimental results also indicated that increasing

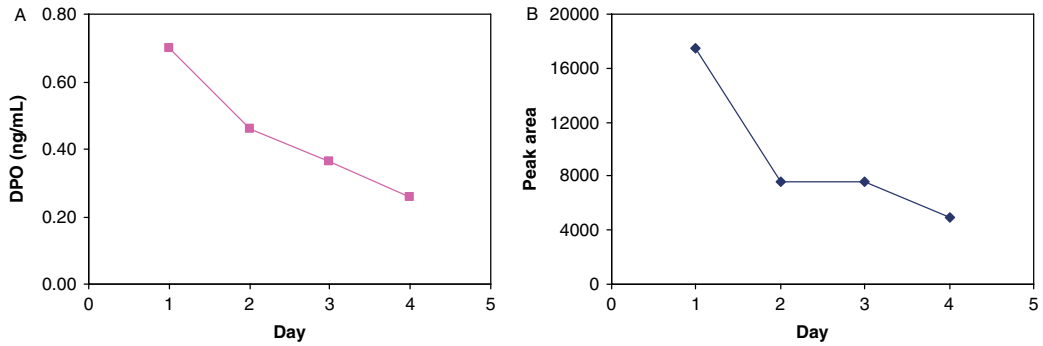

Figure 5. Comparison of DPO testing by ELISA assay (A) and LC-MS/MS method (B). The samples were collected post administration of DPO at 100 µg by IM. For the LC-MS/MS method the peak area of 464.4/714.3 was used.

the sample volume made it possible to confirm PEG-epoetin β at a lower concentration (data not shown).

In the current study the ELISA assay can detect PEG-epoetin β at 0.02 ng/mL in 50 µL of horse plasma and the lowest concentration that can be confirmed by the LC-MS/MS method is 1.0 ng/mL in 2 mL of horse plasma. This indicates that the LC-MS/MS method is not able to confirm the whole range of concentrations that ELISA screens can detect. We successfully detected PEG-epoetin β in the SC administration horse by combining high plasma concentration samples (hour 8, 24, 48, 72, and 96), for a total volume of 3.2 mL. Based on the ELISA data (Figure 1), we postulated that LC-MS/MS can confirm the presence of drug in plasma samples collected at a single time point over the higher concentration range if the volume is sufficient.

The current method was also applied to DPO administration horse plasma samples. We confirmed T_6 up to 4 days post-administration of 100 µg DPO by IM. The results of the ELISA and LC-MS/MS analysis are directly compared in Figure 5A and 5B. The LC-MS/MS data are semi-quantitative since only the peak area of the 464.4/714.3 transition was used for the y-axis in Figure 5B. Under the same experimental conditions better sensitivity was achieved for DPO and rHuEPO than was reported by Yu *et al*.[12]

Compared to the results of Yu *et al*.,[12] our method achieved better detection of T_6. This is unexpected since their instruments were equipped with nano-LC which normally achieves lower detection thresholds than conventional LC-MS/MS. We suspect that the modifications we made in the immunoaffinity extraction and trypsin digestion procedure account for the increased sensitivity. We used 1.0 mL of 0.1% PEG 6000 in PBS as elution buffer, the same as Guan *et al*.,[11] whereas Yu *et al*.,[12] used 250 uL of 0.01% PEG 6000. We added extensive buffer exchanges as described in the methods. Guan *et al*.,[11] also performed buffer exchanges however the experimental details differed from ours. Further the details of the trypsin digestion step were different.

In the work of Guan *et al*.,[11] it was proposed that antibody-coated magnetic beads can be stored for future use after they are washed with 2.0 mL of Buffer C for 5 min at ambient temperature. We have not evaluated the possibility of re-using the antibody-coated magnetic beads yet because we are not sure that we would reuse the beads unless there was a very large number of positives.

A major advantage of the present method is that it can detect the characteristic T_6 peptide in plasma from horses treated with any one of these three doping agents, PEG-epoetin β, rHuEPO or DPO. Furthermore the method only requires a conventional LC-MS/MS instrument. It does not require a nano LC systems which is inherently complex and requires highly trained and costly personnel to operate. Accordingly, the method is relatively easy to perform and can be implemented by a large number of laboratories.

We propose a simple immunoassay to screen equine plasma samples for rHuEPO, DPO and PEG-epoetin β. Samples that screen positive are confirmed by the LC-MS/MS method described herein. This method is capable of detecting and confirming the T_6 that arises from PEG-epoetin β in horse plasma. The method also has demonstrated utility for detecting the T_6 that arises from rHuEPO and DPO in horse plasma. The current method can detect EPO abuse in equine sports but not in humans since T_6 is also produced by human endogenous EPO.

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