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Differentiation and Identification of Recombinant Human Erythropoietin and Darbepoetin Alfa in Equine Plasma by LC−MS/MS for Doping Control

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Recombinant human erythropoietin (rhEPO) and darbepoetin alfa (DPO) are protein-based drugs for the treatment of anemia in humans by stimulating erythrocyte production. However, these agents are abused in human and equine sports due to their potential to enhance performance. This paper describes the first method for differentiation and identification of rhEPO and DPO in equine plasma by liquid chromatography coupled to tandem mass spectrometry (LC−MS/MS). The method comprised analyte extraction and enrichment by immunoaffinity separation with anti-rhEPO antibodies, dual digestion by trypsin and peptide-N-glycosidase F (PNGase F), and analysis by LC−MS/MS. Two unique deglycosylated tryptic peptides, ^{21}EAENITTGCAEHCSLNENITVPDTK^{45} (T_5) from rhEPO and ^{77}GQALLVNSSQVNETLQLHVDK^{97} (T_9) from DPO, were employed for differentiation and identification of rhEPO and DPO via LC retention times and major product ions. The limit of identification was 0.1 ng/mL for DPO and 0.2 ng/mL for rhEPO in equine plasma, and the limit of detection was 0.05 ng/mL for DPO and 0.1 ng/mL for rhEPO. Analyte carryover problem encountered was solved by adding 20% acetonitrile to the solvent of the sample digest to increase solubility of the peptides. This method was successfully applied to identification of DPO in plasma samples collected from a research horse following DPO administration and from racehorses out of competition in North America. Thus, it provides a powerful tool in the fight against blood doping with rhEPO and DPO in the horse racing industry.

Erythropoietin (EPO), a protein hormone produced primarily by the kidney, stimulates red blood cell production.[1] Recombinant human EPO (rhEPO) was genetically engineered, and is approved for the treatment of anemia in humans and cancer patients under chemotherapy.[2] An analogue of rhEPO, darbepoetin alfa (DPO) has the same pharmacological effect as rhEPO but longer duration of action.[3] The ability of these protein-based drugs to stimulate erythrocyte production has led to their abuse as blood doping agents in human [4–7] and equine sports.[8] Doping with rhEPO and DPO in horse racing was suggested by the immunoassay detection of anti-rhEPO antibodies in racehorses (unpublished result), and by the actual confirmation of rhEPO/DPO by liquid chromatography coupled to tandem mass spectrometry (LC−MS/MS) in noncompetition plasma samples collected from racehorses.[8] The abuse of these drugs in racehorses is of great concern to the regulators of the horse racing industry, and thus, useful methods for their detection, differentiation and confirmation are continuously in demand. Enzyme-linked immunosorbent assay (ELISA) developed for detection of rhEPO [9–11] can be used for fast screening of equine plasma and urine samples for detection of rhEPO and DPO, but not for confirming the presence of rhEPO or DPO in a test sample due to possible cross-reactivity with other proteins. Isoelectric focusing with Western blotting was developed for detection of rhEPO and DPO in human urine[12–16] and officially adopted by the International Olympic Committee. The method was also applied to analyses of equine and canine urine samples for the detection of rhEPO and DPO following drug administration.[17,18] However, the method does produce false positive

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results in some instances.[19–22] Its major drawback is lack of mass spectral data or "fingerprints" that are required for confirmation of a positive finding in equine forensic drug testing. Furthermore, the method is not amenable to detection of rhEPO or DPO in plasma samples.

Mass spectrometry is the technique of choice for specific identification of banned substances in doping control analyses,[23] and LC−MS is the preferred technique for identification of protein-based drugs such as rhEPO and DPO. LC−MS methods were attempted by other investigators,[24–27] but they lack adequate sensitivity to detect or identify rhEPO or DPO when present at extremely low concentrations in suspect plasma and urine samples obtained from racehorses. Our previous LC−MS/MS method for identification of rhEPO/DPO in equine plasma was not capable of differentiating between them though it was able to confirm the presence of rhEPO/DPO in plasma.[8] In this paper, we present a new LC−MS/MS method for differentiation between rhEPO and DPO at extremely low concentrations in equine plasma, which is applicable to and very useful in doping control analysis of real-world plasma samples collected from racehorses.

MATERIALS AND METHODS

Chemicals. Peptide-N-glycosidase F (PNGase F) (500,000 U/mL) was purchased from New England BioLabs Inc. (Ipswich, MA) and stored at –20 °C; fresh PNGase F working solution (20,000 U/mL) was prepared by dilution of the stock solution with 50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM Na_2EDTA, and 50% glycerol. Other chemicals and reagents used were described in detail elsewhere[8] and are listed here: DPO and rhEPO reference standards from Amgen (Thousand Oaks, CA), polyclonal anti-rhEPO antibodies (purified rabbit IgG) from R&D Systems (Minneapolis, MN), Dynabeads M-280 tosylactivated magnetic beads (2×10^9 beads/mL, or 30 mg/mL) from Invitrogen (Carlsbad, CA), trypsin (sequencing grade modified) from Promega (Madison, WI), Igepal CA-630, polyethylene glycol 6000, and bradykinin fragment 2–9 all from Sigma (St. Louis, MO).

Sample Preparation. Sample preparation consisting of the following procedures was described in detail in a previous paper:[8] linkage of anti-rhEPO antibodies to magnetic beads, immunoaffinity separation of rhEPO and DPO from equine plasma, and buffer exchange of rhEPO or DPO eluate in preparation for enzymatic digestion.

Dual Digestion by Trypsin and PNGase F. To rhEPO or DPO standard or extract (0.1–5 ng) in 81 μL of ammonium bicarbonate buffer (50 mM, pH 7.8) in a 1.5 mL plastic microcentrifuge vial (Thermo Fisher Scientific, Pittsburgh, PA) was added 10 μL of trypsin (20 μg/100 μL) in the bicarbonate buffer. The vial was briefly shaken by vortex. The mixture was incubated at 37 °C for 3 h, following which the vial was heated in a water bath at 80 °C for 10 min to denature trypsin. After the vial was removed from the water bath and allowed to cool to ambient temperature, 5 μL of PNGase F (20,000 U/mL) was added. The vial was briefly shaken and incubated in a water bath at 37 °C for 1 h. Following the incubation, 4 μL of 10% formic acid was added to terminate the digestion. Twenty microliters of acetonitrile was added to the digest to aid dissolution of deglycosylated tryptic peptides.

LC−MS/MS Analyses. All LC−MS/MS analyses except mentioned otherwise were conducted in a Finnigan LTQ linear ion trap mass spectrometer with an electrospray ionization (ESI) source interfaced to a Surveyor Plus liquid chromatograph with an online degasser and a Surveyor Plus autosampler (Thermo Fisher Scientific, San Jose, CA). A high resolution and high mass accuracy Finnigan LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) operated at a resolving power of 30,000 (at m/z 400) and coupled to the Surveyor Plus liquid chromatograph was used to verify identity of product ions of the deglycosylated tryptic peptides from rhEPO and DPO. The LTQ mass spectrometer was calibrated monthly, and the LTQ Orbitrap was weekly using calibration standard as per instructions in the instrument manuals.

LC separations were carried out on a wide-pore Zorbax 300SB-C_{18} column (50 × 1.0 mm I.D., 3.5 μm) with a Zorbax StableBond guard column (17 × 1.0 mm I.D., 5 μm) (Agilent, Wilmington, DE) maintained at 26 °C, and using gradient elution with mobile phase A (H_2O/acetonitrile/formic acid, 95/5/0.1, v/v/v) and B (H_2O/acetonitrile/formic acid, 5/95/0.1, v/v/v). Injection volume was 20 μL for all analyses. For LC-MS profiling of deglycosylated tryptic peptides from rhEPO and DPO, a gradient was programmed as follows: 0% B (0 – 1 min) was increased to 62% B (1 – 32 min) and to 80% B (32 – 33 min), held at 80% B (33 – 36 min), decreased to 0%B (36 – 37 min), and held at 0% B (37 – 43 min). The flow programming was employed as listed below: 50 μL/min (0 – 33 min) was increased to 100 μL/min (33.0 – 33.5 min), held at 100 μL/min (33.5 – 42.0 min), decreased to 50 μL/min (42.0 – 42.5 min), and held at 50 μL/min (42.5 – 43.0 min). For LC−MS/MS differentiation and identification of rhEPO and DPO, a different gradient was used as follows: 0% B (0 – 1 min) was increased to 33% B (1 – 23 min) and to 80% B (23.0 – 23.5 min), held at 80% B (23.5 – 29.0 min), decreased to 0%B (29.0 – 29.5 min), and held at 0% B (29.5 – 35.0 min). The relevant flow programming is listed below: 50 μL/min (0 – 23.5 min) was increased to 100 μL/min (23.5 – 24.0 min), held at 100 μL/min (24.0 – 34.0 min), decreased to 50 μL/min (34.0 – 34.5 min), and held at 50 μL/min (34.5 – 35.0 min).

Optimization of ESI source parameters of the LTQ instrument was also described in detail elsewhere [8]. The sheath gas flow was 20 (arbitrary units), auxiliary gas flow was 12 (arbitrary units), and the ion transfer capillary temperature was 325 °C. For peptide mapping of rhEPO and DPO following dual digestion by trypsin and PNGase F, data dependent scans were conducted as previously described [8]. As to differentiation and identification of rhEPO and DPO in equine plasma, two signature deglycosylated tryptic

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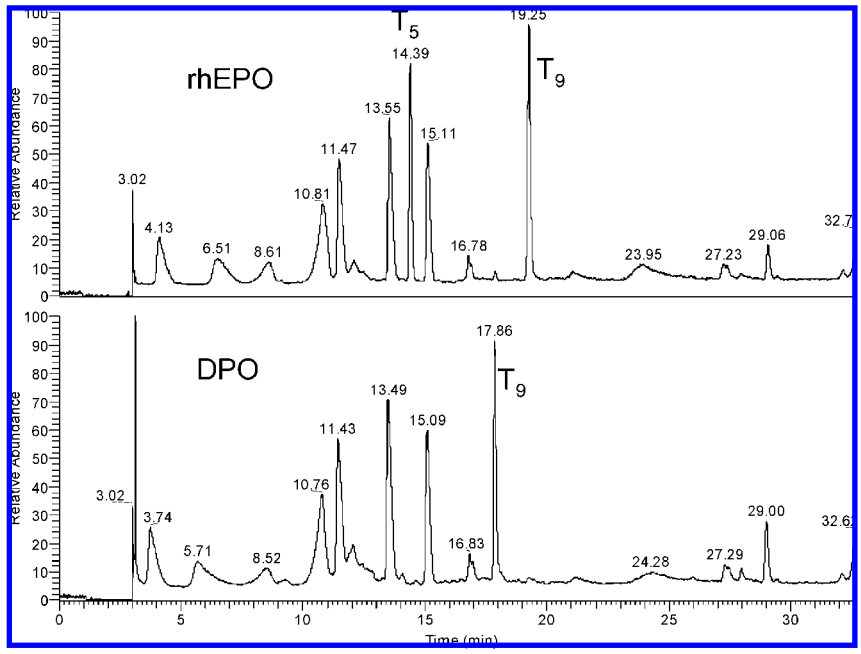

Figure 1. LC−MS chromatograms of digests of rhEPO and DPO (50 μg/mL each) from dual digestion by trypsin and PNGase F indicating differentiation between DPO and rhEPO by the deglycosylated tryptic peptides, T_9 from DPO and T_5 and T_9 from rhEPO.

peptides, ^{21}EAENITTGCAEHCSLNENITVPDTK^{45} (T_5) from rhEPO and ^{77}GQALLVNSSQVNETLQLHVDK^{97} (T_9) from DPO with triply charged precursor ions at m/z 897.8 and 766.4, respectively, were monitored in MS/MS in the respective time window of 8 – 17.5 and 17.5 – 23.0 min of an LC-MS analysis. Scan range for product ions of the m/z 897.8 and 766.4 precursor ions was m/z 400 – 1200, and 700 – 1100, respectively. Parameters for MS/MS experiments were: Wideband Activation enabled, 25% normalized collision energy, 0.25 of the Q value, 30 ms of activation time, and 1.5 of Isolation Width.

SEQUEST Searches. SEQUEST searches were conducted with Bioworks (v. 3.3.1, Thermo Fisher Scientific, San Jose, CA) to examine specificity of product ions of the deglycosylated tryptic peptides for identification of rhEPO and DPO. Raw data files from the targeted MS/MS experiments on the triply charged peptides from rhEPO and DPO reference standards were used for SEQUEST search that was performed on an individual chromatographic peak confined with scan numbers. SEQUEST search parameters used were 2.0 amu for precursor peptide mass tolerance and 1.0 amu for fragment ion tolerance. The protein databases searched were reference human protein FASTA database (March 2007) included in Bioworks and an equine protein FASTA database (April 2004) in Bioworks.

RESULTS AND DISCUSSION

Differentiation between rhEPO and DPO. Differentiation between rhEPO and DPO was based on the difference in amino acid sequence of rhEPO and DPO. Although rhEPO and DPO are analogous, there is a slight difference in their amino acid sequence, e.g. A30N, H32T, P87V, W88N, and P90T in DPO.[3] The five variant amino acids in DPO are located in the two regions that relate to two tryptic peptides (T_5 = ^{21}EAENITTGCNETCSLNENITVPDTK^{45} with a disulfide bond between ^{29}Cys and ^{33}Cys, and T_9 = ^{77}GQALLVNSSQVNETLQLHVDK^{97}). Theoretically, these two tryptic peptides could be employed for differentiation between DPO and rhEPO. Practically, however, they are glycopeptides with glycosylation at ^{24}Asn, ^{30}Asn, ^{38}Asn, ^{83}Asn and ^{88}Asn, which poorly ionize under positive electrospray ionization conditions, and thus, cannot be detected at low concentrations. The same is true of the two relevant glycopeptides from rhEPO (T_5 = ^{21}EAENITTGCAEHCSLNENITVPDTK^{45} with a disulfide bond between ^{29}Cys and ^{33}Cys, and T_9 = ^{77}GQALLVNSSQPWEPLQLHVDK^{97}). Experimentally, the four glycopeptides were not observed from tryptic digests of rhEPO and DPO, but several tryptic peptides without glycans and common to both rhEPO and DPO were detected (S-Figure 1, Supporting Information Figure 1). The absence of those glycopeptides from detection could be explained by the known fact that glycans with sialic acid or other acid attachments in glycopeptides weaken ionization of the latter in positive-ion mode detection. Thus, differentiation between rhEPO and DPO could not be achieved, with desired sensitivity, at glycopeptide level. To overcome this problem glycans were removed from the glycopeptides such that they would favorably ionize and be detected.[24,28] Following deglycosylation with PNGase F, the T_5 from rhEPO and the T_9 peptides from rhEPO and DPO were experimentally observed (Figure 1). The identity of the T_5 and the T_9 was confirmed by isotopic peak distribution of the triply charged peptides and their product ions (Figures 2 and 3). It should be noted that following deglycosylation, ^{24}Asn and ^{38}Asn in the T_5, ^{83}Asn in the T_9 of rhEPO, and ^{83}Asn and ^{88}Asn in the T_9 of DPO were deaminated to aspartic acid residues by the amidase − PNGase F.[29] However, the T_5 of DPO was not observed even after deglycosylation because glycosylation at ^{30}Asn in the T_5 was resistant to deglycosylation by PNGase F if the disulfide bond adjacent to the ^{30}Asn was not reduced and alkylated.[24] In this study, neither reduction of disulfide bonds nor alkylation of

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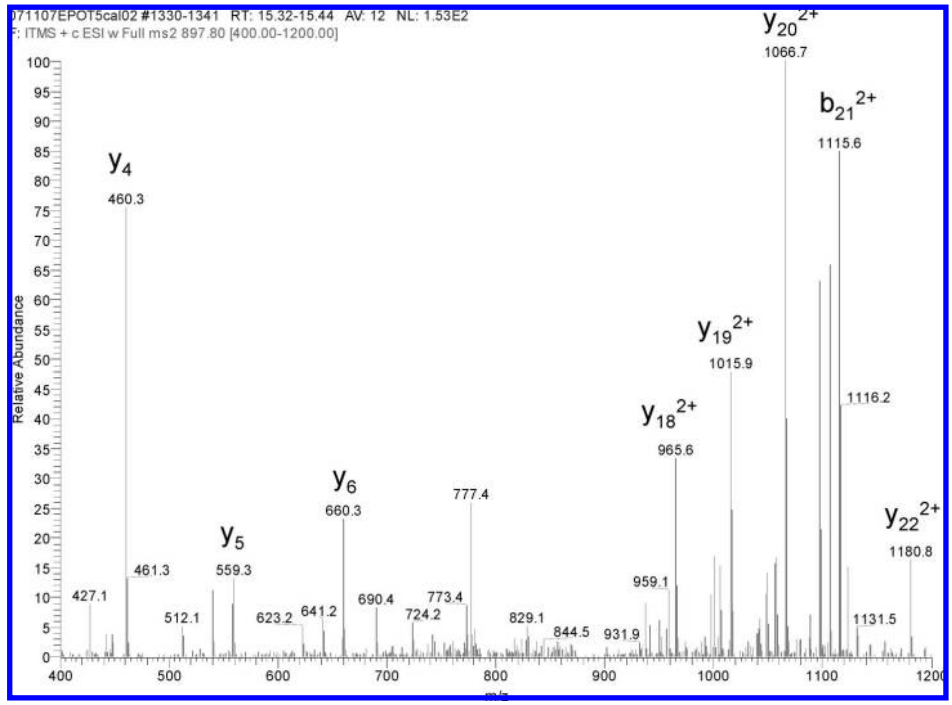

Figure 2. Product ion spectrum of the triply charged (deglycosylated) T_5 at *m/z* 897.8 from rhEPO (5 ng/120 μL). Unannotated are the two predominant peaks at *m/z* 1097.6 and 1106.6 for the $(b_{21} - 2H_2O)^{2+}$ and $(b_{21} - H_2O)^{2+}$ ions, respectively.

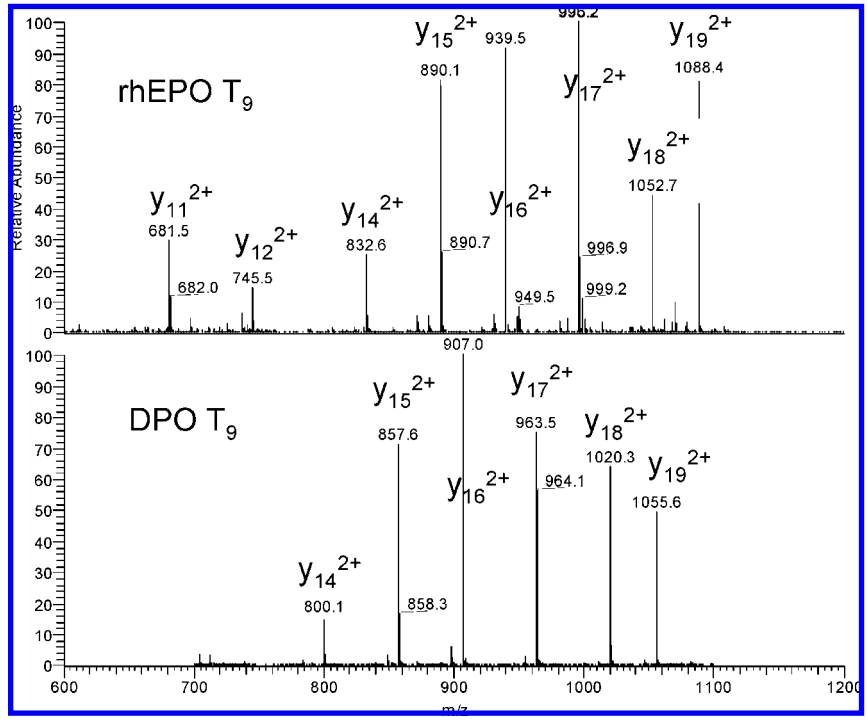

Figure 3. Product ion spectra of the triply charged (deglycosylated) T_9 at *m/z* 788.1 from rhEPO and at *m/z* 766.4 from DPO (5 ng/120 μL each) indicating totally different product ion profiles.

cysteine residues in the T_5 was performed for the purpose of both simplicity of the method and prevention from possible loss of the analyte. Even though the T_5 of DPO was not detected, the difference between rhEPO and DPO was still evident. The T_5 and T_9 of rhEPO are different in both amino acid sequence and molecular weight from the relevant tryptic peptides of DPO, and thus, they were the specific peptides for differentiation and identification of rhEPO and DPO. Additionally, the chromatographic retention time (t_R) of the T_9 of rhEPO was different from that of DPO (19.25 vs 17.86 min in Figure 1), which was useful in their differentiation and identification.

Product Ion Spectra of the Unique Peptides. Product ion spectra of the deglycosylated T_5 of rhEPO (Figure 2) and T_9 of rhEPO and DPO (Figure 3) are essential to identification of rhEPO and DPO. Product ions observed for the triply charged (deglycosylated) T_5 of rhEPO were *m/z* 460.3 (y_4), 559.3 (y_5), 660.3 (y_6), 965.6 (y_{18}^{2+}), 1015.9 (y_{19}^{2+}), 1066.7 (y_{20}^{2+}), 1097.6 ($(b_{21} - 2H_2O)^{2+}$), 1106.6 ($(b_{21} - H_2O)^{2+}$), 1115.6 (b_{21}^{2+}), and 1180.8 (y_{22}^{2+})

(Figure 2). The abundant triplet peaks at m/z 1097.6, 1106.6 and 1115.6 were related to b_{21}^{2+} product ion rather than y_{21}^{2+} ion (predicted m/z = 1124.8), which was verified by agreement between experimental accurate masses of those peaks and the predicted (see S-Table 1, Supporting Information Table 1). The predominant b_{21}^{2+} and y_4 product ions (Figure 2) originated from the preferential cleavage [30–32] at the N-terminal side of proline residue in the T_5. For the triply charged (deglycosylated) T_9 of rhEPO, product ions observed were m/z 257.0 (b_3, not shown in Figure 3), 370.2 (b_4, not shown), 483.2 (b_5, not shown), 582.3 (b_6, not shown), 681.5 (y_{11}^{2+}), 745.5 (y_{12}^{2+}), 832.6 (y_{14}^{2+}), 890.1 (y_{15}^{2+}), 939.5 (y_{16}^{2+}), 996.2 (y_{17}^{2+}), 1052.7 (y_{18}^{2+}), and 1088.4 (y_{19}^{2+}) (Figure 3). The triply charged (deglycosylated) T_9 of DPO generated product ions of m/z 257.0 (b_3, not shown in Figure 3), 370.2 (b_4, not shown), 483.2 (b_5, not shown), 582.3 (b_6, not shown), 800.1 (y_{14}^{2+}), 857.6 (y_{15}^{2+}), 907.0 (y_{16}^{2+}), 963.5 (y_{17}^{2+}), 1020.3 (y_{18}^{2+}), and 1055.6 (y_{19}^{2+}) (Figure 3). All the b-ion product ions except b_{21}^{2+} of the T_5 were weak in intensity, but all the y-ion product ions of the three peptides were predominant. The identity of most of the doubly charged y-ion product ions was verified by accurate mass measurements (S-Table 1, Supporting Information Table 1) and isotopic peak distributions in the product ion spectra obtained with the Orbitrap instrument of high resolving power and high mass accuracy. It should be emphasized that the y-ion product ions of the deglycosylated T_9 of DPO were distinctly different in mass from those of rhEPO (Figure 3), which was essential to differentiation and identification of rhEPO and DPO.

Sample Carryover Problem and Solution. Some (large) peptides tend to stick to surfaces in the LC flow path and consequently are partially lost during an analysis and carried over to the next analysis, causing interferences in the latter analysis. In the early phase of this method development, carryover of the deglycosylated T_9 of rhEPO and DPO was so severe (at least 15%) that detection of the T_9 peptides from rhEPO and DPO reference standards at concentrations below 500 ng/mL was impossible, and that concentration was far higher than the desired limit of detection. Washing and rinsing of outside and inside of the sample injection needle with isopropanol and acetonitrile failed to eliminate the carryover problem. Since adsorptive behavior of peptides is inversely related to their solubility in a solvent of the sample digest, the percentage of acetonitrile in the solvent was evaluated for its effect on elimination of carryover problem. The addition of 20% acetonitrile to the solvent of the sample digest completely eliminated the carryover problem without causing any noticeable broadening of chromatographic peaks of the analytes under the LC elution condition. After the carryover problem was solved, rhEPO and DPO reference standards at 0.5 ng/mL were detected. It was assumed that the carryover took place in the stainless steel sample loop of the autosampler that was out of the LC flow path by design. Other investigators solved the carryover problem by replacement of the stainless steel sample loop with a PEEK sample loop;[33] in this study the addition of 20% acetonitrile to the solvent of the sample digest was totally effective in eliminating the problem.

Duration of Deglycosylation Incubation. Overnight incubation for deglycosylation of glycopeptides from rhEPO and DPO and other proteins was performed by other investigators.[24,28] In this study, 1 h, 2 h and overnight incubations for deglycosylation were compared, and the result indicated that incubation for overnight led to remarkably lower yield of the deglycosylated peptides than did 1 and 2 h incubations that resulted in equal yield of the peptides. Thus, incubation for 1 h was chosen and used in the present study.

Method Evaluation. Differentiation and identification of rhEPO and DPO in equine plasma were conducted by using the unique and discriminating deglycosylated tryptic peptides, T_5 of rhEPO and T_9 of DPO, via LC retention time and major product ions. The deglycosylated T_9 of rhEPO could not be used for identification of the latter in equine plasma because the signal intensity from the T_9 for rhEPO spiked to equine plasma severely decreased in comparison with that for rhEPO reference standard (the cause was unknown). As shown in Figure 4, rhEPO spiked to blank plasma and recovered by immunoaffinity separation resulted in a chromatographic peak at t_R of 15.66 min for the T_5, as did DPO at t_R of 19.87 min for the T_9. The identity of those two chromatographic peaks was verified by the relevant MS/MS spectrum (not shown). In contrast, blank plasma did not give rise to a chromatographic peak at or around the t_R of the T_5 or T_9 and, thus, did not cause any interference to the identification of rhEPO and DPO (Figure 4).

Criteria proposed in this study for the identification of rhEPO were presence of the major product ions from the deglycosylated T_5, m/z 460.3 (y_4), 1066.7 (y_{20}^{2+}), and 1115.6 (b_{21}^{2+}) with S/N > 3 in the product ion spectrum obtained from LC−MS/MS analysis; for identification of DPO, the presence of major product ions from the deglycosylated T_9, m/z 857.6 (y_{15}^{2+}), 907.0 (y_{16}^{2+}), 963.5 (y_{17}^{2+}), 1020.3 (y_{18}^{2+}), and 1055.6 (y_{19}^{2+}) with S/N > 3. The criteria also included a match (±0.5 min window) of the t_R of the T_5 or T_9 from a suspect plasma sample to that of the corresponding peptide from rhEPO or DPO reference standard spiked to blank equine plasma. Under these criteria, the limit of identification (at which an analyte can be identified) of this method was 0.2 ng/mL for rhEPO and 0.1 ng/mL for DPO in plasma (S-Figure 2, Supporting Information Figure 2). The limit of detection was 0.1 ng/mL for rhEPO and 0.05 ng/mL for DPO (S/N > 3 for the chromatographic peak of the T_5 or the T_9 in LC−MS/MS chromatogram).

rhEPO and DPO in equine plasma can be quantified with external calibration. The quantification range was 0.2–2.5 ng/mL for rhEPO, and 0.1–2.5 ng/mL for DPO, with coefficients of variation (r^2) greater than 0.98 (S-Figure 3, Supporting Information Figure 3).

Specificity for Identification of rhEPO and DPO. Specificity of the present method for identification of rhEPO and DPO originates from the strategies taken in sample preparation and analysis, as described in our previous method.[8] BLAST search against the Nonredundant Protein Database of NCBI (August 2007) was conducted to examine the specificity of the deglyco-

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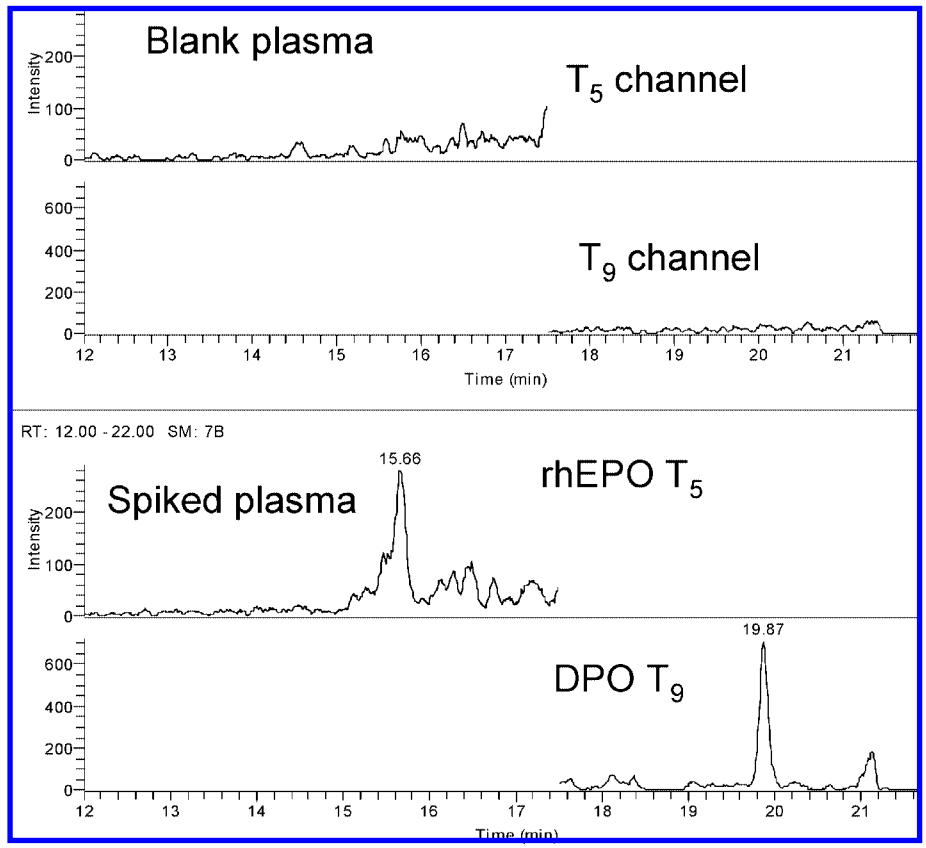

Figure 4. LC−MS/MS chromatograms of blank equine plasma (top two panels) and rhEPO and DPO (0.5 ng/mL each) spiked to blank plasma (bottom two panels) showing the chromatographic peaks of the T_5 from rhEPO (t_R = 15.66 min) and T_9 from DPO (t_R = 19.87 min) and the absence of interferences from blank plasma. The following product ions were employed to reconstruct the chromatograms: *m*/*z* 460.3 (y_4), 660.3 (y_6), 965.6 (y_{18}^{2+}), 1015.9 (y_{19}^{2+}), 1066.7 (y_{20}^{2+}), 1106.6 (($b_{21} - H_2O$)$^{2+}$) and 1115.6 (b_{21}^{2+}) from the triply charged T_5 at *m*/*z* 897.8; and *m*/*z* 800.1 (y_{14}^{2+}), 857.5 (y_{15}^{2+}), 963.7 (y_{17}^{2+}), 1020.4 (y_{18}^{2+}) and 1055.8 (y_{19}^{2+}) from the triply charged T_9 at *m*/*z* 766.4.

sylated T_5 and T_9 for identification of rhEPO and DPO, and the search result (S-Table 2, Supporting Information Table 2) indicated that the T_5 of rhEPO was present in EPO molecules of only a few species including the human, chimpanzee and gorilla but not the horse. No exact match to the deaminated form of the T_5 or the T_9 of DPO or the deaminated T_9 was found. The search result suggests the unique specificity of the present method at amino acid sequence level for identification of rhEPO and DPO in equine plasma.

In exploring the possibility that other proteins may also give rise to a set of major product ions of the deglycosylated T_5 from rhEPO or T_9 from DPO, SEQUEST search of experimental product ion spectrum of the deaminated T_5 and T_9 (Figures 2 and 3) against human and equine protein FASTA databases available was performed to determine rate of false positive identification for rhEPO and DPO. Although a few proteins were found as possible matches to the product ion spectra (S-Table 3, Supporting Information Table 3), manual examination of the search results revealed that those proteins were all false matches because the product ions predicted from the candidate tryptic peptide of the proteins did not match all of the major experimental product ions of the T_5 or T_9. In essence, no exact protein match to the product ion spectrum of the T_5 or T_9 was found in the databases. Although rhEPO and hEPO were expected to be among the protein matches to the experimental product ions of the T_5, it did not happen because of two possible causes: change in amino acid sequence of the deglycosylated T_5 due to conversion of the ^{24}Asn and ^{38}Asn to the relevant Asp residues from deglycosylation, and intact or unreduced disulfide bond between ^{29}Cys and ^{33}Cys. As for the effectiveness of SEQUEST search for protein identification, its usefulness and suitability are well established and recognized.[34] In this study, SEQUEST search correctly matched the experimental product ion spectrum of the T_6 and T_{17} of rhEPO to hEPO (S-Table 3, Supporting Information Table 3), suggesting correct implementation of the SEQUEST searches performed. Thus, it is concluded that no other human or equine protein gives rise to the set of major product ions of the deglycosylated T_5 or T_9, and those product ions are unique and specific for identification of rhEPO or DPO without any false positive result.

Preliminary Applications. The present method was successfully applied to analyses of DPO administration samples and real-world samples collected from racehorses either pre- or postcompetition for differentiation and identification of rhEPO and DPO. DPO was identified in plasma sample collected 168 h post its administration to a horse (intravenous dose of 0.37 μg/kg) and detected 192 h post administration. This result is consistent with the result by our previous LC−MS/MS method for plasma samples from a similar administration.[8] DPO was also identified in a suspect plasma sample that was collected from a noncompetition testing of racehorses, screened by ELISA, and submitted

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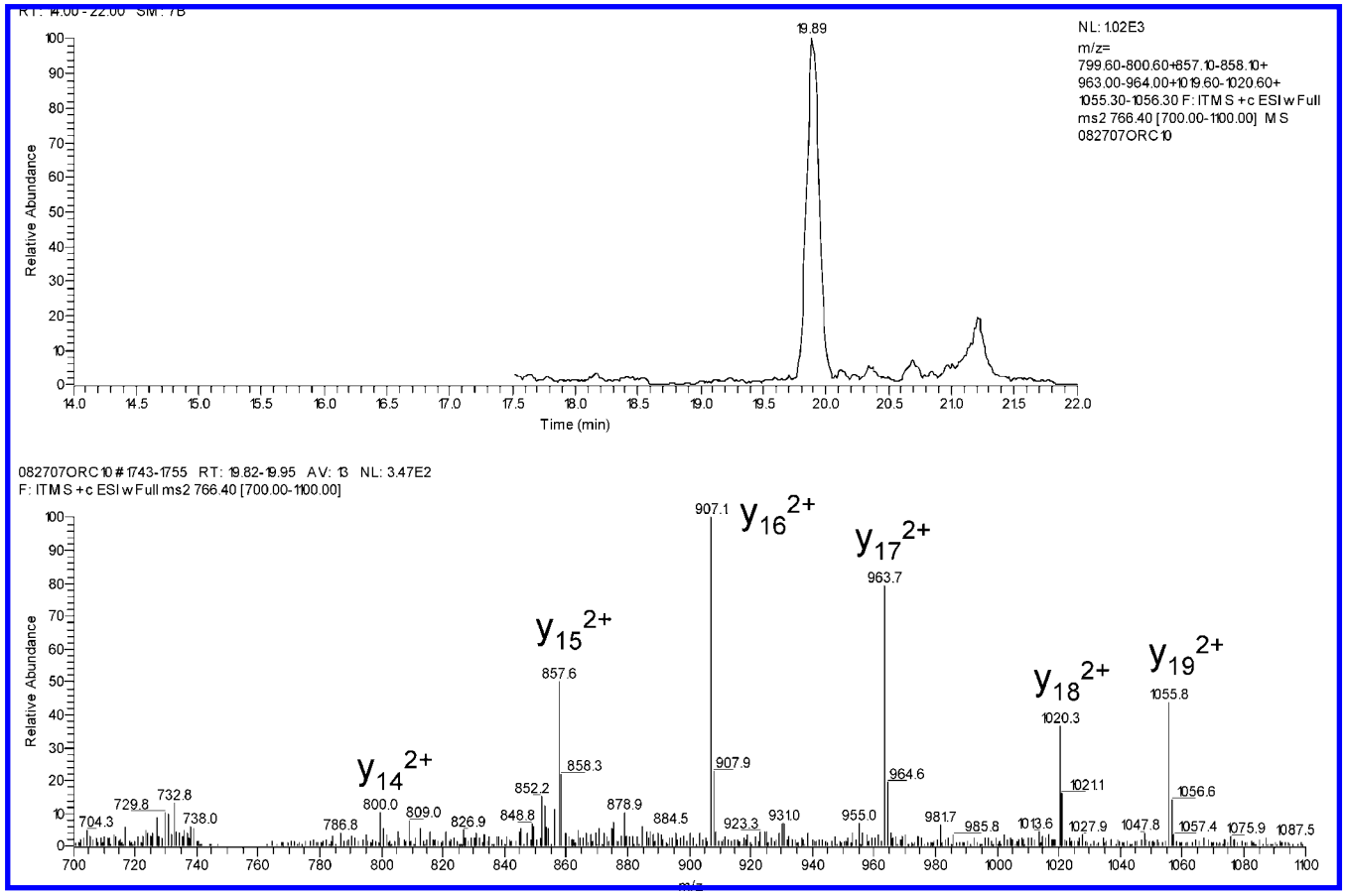

Figure 5. LC−MS/MS chromatogram (top panel) and product ion spectrum (bottom panel) indicating identification of DPO (t_R = 19.89 min) in a serum sample (~0.34 ng/mL) collected from a racehorse during noncompetition in North America. The precursor ion for the LC−MS/MS was *m*/*z* 766.4.

to our laboratory by a horse racing jurisdiction in North America. While the initial ELISA screening indicated that the sample was suspected for rhEPO/DPO, the present method confirmed, with certainty, the presence of DPO in the suspect sample (Figure 5). These results along with several other identification results for racehorse samples support the applicability and validity of the current method for analysis of real-world equine plasma samples.

CONCLUSION

A very selective and sensitive LC−MS/MS method was developed for differentiation and identification of rhEPO and DPO in equine plasma. The differentiation and identification were achieved using two signature deglycosylated tryptic peptides, T_5 from rhEPO and T_9 from DPO, via LC retention times and major product ions. The limit of identification was 0.1 ng/mL for DPO and 0.2 ng/mL for rhEPO in equine plasma, and the limit of detection was 0.05 ng/mL for DPO and 0.1 ng/mL for rhEPO. This method was successful in identifying DPO in plasma samples collected from research horses post DPO administration and from pre-, post- or noncompetition racehorses in North America. To our knowledge, this is the first method capable of definitively identifying rhEPO and DPO in real-world equine plasma samples. Thus, it provides a powerful tool in the fight against blood doping with rhEPO and DPO in the horse racing industry.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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