LC–MS/MS Method for Confirmation of Recombinant Human Erythropoietin and Darbepoetin α in Equine Plasma

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LC−MS/MS Method for Confirmation of Recombinant Human Erythropoietin and Darbepoetin α in Equine Plasma

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Recombinant human erythropoietin (rhEPO) and darbepoetin α (DPO) are protein-based drugs for the treatment of anemia by stimulating red blood cell production. Consequently, they are abused in human and equine sports. To deter their abuse in the horse racing industry, a sensitive and reliable method for confirmation of these agents in equine plasma has been in urgent need. Such a method by LC−MS/MS is described in this paper. The method involved analyte enrichment by immunoaffinity separation using anti-rhEPO antibody linked to magnetic beads, digestion by trypsin, and analysis by LC−MS/MS. Two specific proteotypic peptides, ^{46}VNFYAWK^{52} and ^{144}VYSNFLR^{150} from rhEPO and DPO were employed for confirmation of the analytes based on chromatographic retention times and major product ions. The limit of confirmation of this method was 0.2 ng/mL, and the limit of detection was 0.1 ng/mL for rhEPO and DPO in equine plasma. This method was successful in confirming the presence of rhEPO and DPO in plasma samples collected from research horses to which rhEPO or DPO was administered and from racehorses following competition and in noncompetition samples in North America. To our knowledge, this is the first LC−MS method with adequate sensitivity and specificity in providing unequivocal confirmation of rhEPO and DPO in equine plasma samples. This method provides a powerful enforcement tool that was lacking in the fight against the abuse of rhEPO and DPO in the horse racing industry.

Erythropoietin (EPO) is a hormone protein produced mainly in the kidney. It stimulates red blood cell production by promoting the proliferation and differentiation of erythroid progenitor cells (ancestors of red blood cells).[1] Genetically engineered recombinant human EPO (rhEPO) is indicated for the treatment of anemia in humans.[2] Darbepoetin α (DPO) (NESP, or Aranesp) is a second-generation rhEPO with 5 of its 165 amino acids intentionally modified to increase duration of action while retaining the pharmacological effect of rhEPO.[3] The ability of these agents to stimulate red blood cell production has led to their abuse as blood doping agents in human endurance sports[4−7] and horse racing.[8] It has been demonstrated in humans that rhEPO provides a significant erythropoietic benefit in trained individuals as evidenced by increases in hemoglobin, hematocrit concentrations, maximal oxygen uptake, and exercise endurance time.[9−12] Deaths in endurance athletes were attributed to rhEPO-induced erythrocytosis.[13] Despite the lack of comparable studies on the effect of rhEPO and DPO on performance in the horse, anecdotal information and the suspicion of horse trainers turning losers into winners suggest improvement in the performance of the horse by rhEPO. The abuse of these protein-based drugs in racehorses is of great concern to regulators of the horse racing industry, because like many other banned substances, the use of these agents in racehorses during competition violates the rule of fair competition. A second compelling reason to ban the use of rhEPO and DPO is the potentially harmful effects on the health of the horse. Recombinant human EPO and DPO are foreign proteins and, when injected into a horse, produce anti-rhEPO antibody that may cross-react with endogenous EPO causing inhibition of erythropoieses[14] and death of some horses.[15,16]

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Concerns of the international sports community over the abuse of rhEPO and DPO in human athletes led to the evaluation of various methods for the detection and confirmation of these drugs. Initially, methods by enzyme-linked immunosorbent assay (ELISA) were developed for detection of rhEPO.[12,17,18] ELISA can be used for fast screening of equine plasma and urine samples for detection of rhEPO and DPO. However, they are good only for detection, not for confirming the presence of rhEPO or DPO in a test sample due to possible cross-reactivity with other proteins.

An electrophoretic method combining Western blotting was developed for the detection of rhEPO and DPO in human urine, based on the principle that the rhEPO molecule is less negatively charged than the endogenous human EPO molecule.[19−23] This method was officially adopted by the International Olympic Committee. In testing human athletes for rhEPO and DPO, the method is used in combination with blood tests to measure markers of enhanced erythropoiesis.[24] The same method was also used for the analysis of equine and canine urine samples for the detection of rhEPO and DPO following drug administration.[25,26] However, the method occasionally produced false positive results.[27] Its major drawback is the lack of mass spectral data or "fingerprints" that are required for confirmation of a positive finding. Furthermore, the method is not amenable to detection of rhEPO or DPO in plasma samples.

Liquid chromatography−mass spectrometry (LC−MS) methods were reported for the characterization of tryptic digests of rhEPO and DPO standards[28−30] and the intact proteins.[31] Capillary electrophoresis−mass spectrometry (CE−MS) methods were also reported for separation of glycoforms of rhEPO and DPO standards[32,33] and for quality control analysis of rhEPO in biotech products.[34−36] Nevertheless, none of the reported LC−MS or CE−MS methods has been shown to be adequately sensitive for detection or confirmation of the presence of rhEPO or DPO in "real-world" racehorse samples.[29] The difficulty with LC−MS detection and confirmation of rhEPO and DPO in plasma samples arises from the fact that they are hormone protein-based drugs, and thus, their effective dose and plasma concentration are very low, e.g., ∼1 ng/mL or 29 fmol/mL in plasma. Unlike small-molecule drugs, protein-based drugs such as rhEPO and DPO are difficult to separate from plasma due to the presence of abundant proteins. The extremely low concentration of rhEPO or DPO in plasma makes confirmation very difficult. However, plasma was still the preferred test sample because the concentration of rhEPO or DPO in plasma was higher than that in urine. Despite the inherent difficulties, the aim of the present study was to develop a sensitive and reliable LC−MS/MS method for unequivocal confirmation of the presence of rhEPO and DPO at very low concentrations in equine plasma. This LC−MS method has been successfully used to confirm rhEPO/DPO in equine plasma samples. The equine racing industry does not require quantification to substantiate confirmation of illegal drug(s) in test samples.

MATERIALS AND METHODS

Chemicals. rhEPO and DPO standards used in this study were donated by Amgen Inc. (Thousand Oaks, CA). The rhEPO was a human serum albumin-free epogen (epoetin α) with protein content of 1.91 mg/mL in 100 mM NaCl plus 20 mM sodium citrate (pH 7.0) and was stored in aliquots of 100 μL at −70 °C. DPO standard was also a human serum albumin-free product with protein content of 500 μg/mL (in polysorbate solution) and was stored at 4 °C according to the manufacturer's recommendation. An intermediate rhEPO solution of 100 μg/mL and DPO solution of 20 μg/mL in water were freshly prepared by dilution of the stock solutions with HPLC-grade water. Aqueous working standard solutions of rhEPO and DPO were prepared by consecutive 1/10 dilutions of the two intermediate standard solutions.

Polyclonal anti-rhEPO antibody (purified rabbit IgG) and monoclonal anti-rhEPO antibody (clone 9C21D11, purified mouse IgG1) were purchased from R&D Systems (Minneapolis, MN). Magnetic beads, Dynabeads M-280 tosylactivated, (concentration: 2×10^9 beads/mL, approximately 30 mg/mL) were purchased from Invitrogen (Carlsbad, CA). Igepal CA-630 ("for molecular biology" grade), polyethylene glycol 6000 (ultra grade), and bradykinin fragment 2−9 were purchased from Sigma (St. Louis, MO). Trypsin (sequencing grade modified) was purchased from Promega (Madison, WI). HPLC-grade acetonitrile and water were purchased from Fisher Scientific (Pittsburgh, PA). All chemicals used in this study were of ACS reagent grade or better, and water was HPLC grade.

Buffers used for linking anti-rhEPO antibody to the magnetic beads and subsequent immunoaffinity separation of rhEPO and DPO were prepared according to Invitrogen's manual and other references[37,38] and are only mentioned here: phosphate-buffered saline (PBS, pH 7.4); borate buffer, 0.1 M, pH 9.5 (buffer B in the

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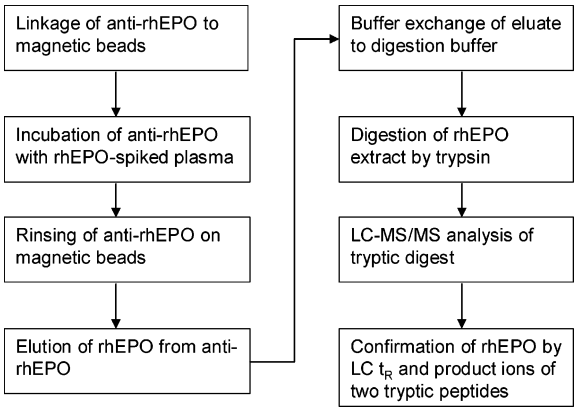

Figure 1. Flow chart showing the steps in immunoaffinity separation of rhEPO and DPO from equine plasma and LC−MS/MS confirmation.

Invitrogen's manual); PBS (pH 7.4) plus 0.1% (w/v) bovine serum albumin (BSA), 2 mM EDTA, and 0.02% (w/v) sodium azide (buffer C); Tris buffer (0.2 M, pH 8.5) plus 0.1% (w/v) BSA (buffer D); washing buffer, 1% (w/v) Igepal CA-630 in PBS (pH 7.4); elution buffer, 0.1% PEG 6000 in PBS (adjusted to pH 2.0 with concentrated HCl). All the buffers were stored at 4 °C.

Devices. The magnetic particle concentrator (MPC-L) was purchased from Invitrogen. Ultrafree-CL centrifugal filter devices (2 mL sample capacity) with 0.22 μm membrane pore size and Centricon centrifugal filter devices (2 mL capacity) with molecular weight cutoff of 30 kDa were purchased from Millipore (Billerica, MA). A Sorvall Legend Mach 1.6 R centrifuge with fixed angel rotors (Thermo-Kendro Laboratory Products Inc., Asheville, NC), an Isotemp incubator (model no. 637 D), and a Labquake shaker (Barnstead/Thermolyne, model no. 400110) for 360° rotation of tubes (at 8 rpm) were purchased from Fisher Scientific (Pittsburgh, PA).

Linkage of Anti-rhEPO Antibody to Magnetic Beads. A brief summary of the process for immunoaffinity separation of rhEPO and DPO from equine plasma and LC−MS/MS confirmation is depicted in Figure 1. Linkage of anti-rhEPO antibody to magnetic beads was carried out as previously reported.[37,38] The detailed procedure for the linkage is available in the Supporting Information.

Immunoaffinity Separation of rhEPO and DPO from Equine Plasma. rhEPO and DPO were extracted from equine plasma by anti-rhEPO antibody linked to magnetic beads, using the reported procedure.[37,38] The detailed procedure for the immunoaffinity separation is available in the Supporting Information.

Buffer Exchange of rhEPO or DPO Eluate in Preparation for Tryptic Digestion. Since the eluates from the immunoaffinity separation, containing phosphate buffer (pH 2) and PEG 6000, were not suitable for subsequent tryptic digestion of rhEPO or DPO, buffer exchange to ammonium bicarbonate (50 mM, pH 7.8) suitable for tryptic digestion was performed using the Centricon centrifugal filter device with molecular weight cutoff of 30 kDa. The molecular weight of rhEPO and DPO are 30.4 and 35 kDa, respectively (according to Amgen Inc.), and thus, they can be retained by the filter. The detailed procedure for buffer exchange is available in the Supporting Information.

Tryptic Digestion of rhEPO and DPO. rhEPO or DPO extract was digested by trypsin as previously reported,[39] for LC−MS/MS analysis. To each rhEPO or DPO extract in ammonium bicarbonate buffer (50 mM, pH 7.8), 10 μL of trypsin in the ammonium bicarbonate (20 μg/100 μL) was added. The mixture was briefly mixed by vortex and then incubated in a water bath at 37 °C for 3 h. The digestion was terminated by adding 4 μL of 10% formic acid in water. The digested protein was immediately analyzed by LC−MS/MS or stored at −70 °C until analyzed.

Table 1. LC Mobile Phase Gradients

time, min	mobile phase A (%)[a]	mobile phase B (%)[b]	flow rate (μL/min)
Gradient 1 (for LC−MS Profiling of DPO Digest)			
0	96	4	50
1.0	96	4	50
30.0	38	62	50
31.0	20	80	50
31.5	20	80	100
34.0	20	80	100
35.0	96	4	100
40.0	96	4	100
40.5	96	4	50
41.0	96	4	50
Gradient 2 (for LC−MS/MS Confirmation of rhEPO and DPO)			
0	100	0	50
1.0	100	0	50
19.0	73	27	50
19.5	20	80	50
20.0	20	80	100
23.0	20	80	100
23.5	100	0	100
28.0	100	0	100
28.5	100	0	50
29.0	100	0	50

[a] Mobile phase A: H_2O/acetonitrile/formic acid (95/5/0.1, v/v/v).
[b] Mobile phase B: H_2O/acetonitrile/formic acid (5/95/0.1, v/v/v).

LC−MS/MS Analysis. LC−MS/MS analyses were carried out on a Finnigan LTQ linear ion trap mass spectrometer with an electrospray ionization (ESI) source interfaced with a Surveyor Plus liquid chromatograph with an online degasser and a Surveyor Plus autosampler (Thermo Fisher Scientific, San Jose, CA). LC separations were performed on a wide-pore Zorbax 300SB-C_{18} column (50 mm × 1.0 mm i.d., 3.5 μm particle size) with a Zorbax StableBond guard column (17 mm × 1.0 mm i.d., 5 μm) (Agilent, Wilmington, DE) maintained at 26 °C.

The LTQ linear ion trap instrument was operated in positive ion mode and calibrated monthly with a calibration mixture of caffeine, L-methionyl-arginyl-phenylalanyl-alanine (MRFA), and Ultramark 1621 (Thermo Electron), according to the instrument manual. The ESI source parameters were automatically optimized by introducing 1.0 μg/mL of bradykinin fragment 2−9 at 5 μL/min into the LC flow of 45 μL/min of mobile phases A and B (20/80, see Table 1). The ion transfer capillary temperature of the mass spectrometer was 325 °C for optimal sensitivity of rhEPO and DPO measurements at the LC flow rate of 50 μL/min. For MS/MS experiments, the collision gas was helium (dampening gas). A normalized collision energy of 25% was used for fragmentation of peptide precursor ions. Activation Q of 0.25 and activation time of 30 ms were employed to effect activation of precursor ions. An isolation width of 1.5 was used, and wideband activation was

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Table 2. Tryptic Peptides Predicted from Simulated Tryptic Digestion of Human and Equine EPOs and Actually Detected Following Tryptic Digestion of DPO

	human EPO					equine EPO		
fragment	start−end	sequence	$[M + 2H]^{2+}$ (calcd)[a]	note	t_R, min[b]	start−end	sequence	$[M + 2H]^{2+}$ (calcd)
T_1	1−4	APPR				1−3	PPR	
T_2	5−10	LICDSR	353.9	T2−T20		4−9	LICDSR	353.9
T_3	11−14	VLER				10−13	VLER	
T_4	15−20	YLLEAK	368.9	detected	3.00	14−19	YILEAR	382.9
		EAENITTGC					EAENVTMG	
T_5	21−45	AEHCSLNE	1346.0	glycopeptide[c]		20−44	CAEGCSFG	1295.4
		NITVPDTK					ENVTVPDTK	
T_6	46−52	VNFYAWK[d]	464.5	detected	13.55	45−51	VNFYSWK	472.5
T_7	53−53	R				52	R	
		MEVGQQAV					MEVEQQAV	
		EVWQGLALL					EVWQGLAL	
T_8	54−76	SEAVLR	1264.5	detected	26.63	53−91	LSEAILQGQ	2120.4
		GQALLVNSS					ALLANSSQP	
							SETLR	
T_9	77−97	QPWEPLQLHVDK	1180.8	glycopeptide		92−96	LHVDK	
T_{10}	98−103	AVSGLR	301.9			97−102	AVSSLR	316.9
T_{11}	104−110	SLTTLLR	402.5	detected	11.86	103−109	SLTSLLR	395.5
T_{12}	111−116	ALGAQK	294.3			110−115	ALGAQK	294.3
		EAISPPDA					EAISPPDAA	
T_{13}	117−131	ASAAPLR	733.8	glycopeptide		116−130	SAAPLR	733.8
T_{14}	132−139	TITADTFR	463.0	detected	3.00	131−139	TFAVDTLCK	499.26
T_{15}	140−140	K						
T_{16}	141−143	LFR				140−142	LFR	
T_{17}	144−150	VYSNFLR[d]	450.0	detected	9.20	143−149	IYSNFLR	457.0
T_{18}	151−152	GK				150−151	GK	
T_{19}	153−154	LK				152−153	LK	
T_{20}	155−162	LYTGEACR	457.0			154−161	LYTGEACR	457.0
T_{21}	163−165	TGD				162	R	
T_2−T_{20}	5−10	LICDSR	539.6	detected	1.82			
	155−162	LYTGEACR	(triply charged)					

[a] The m/z value for the $[M + 2H]^{2+}$ is the calculated average (not the monoisotopic value). [b] Those tryptic peptides marked with retention time (t_R) are the ones detected in the actual enzymatic digest of DPO (250 μg/mL). [c] The glycopeptides were not observed under the LC−MS conditions used in this study. [d] The unique tryptic peptides, ^{46}VNFYAWK^{52} and ^{144}VYSNFLR^{150}, were used for confirmation of rhEPO and DPO in equine plasma.

enabled. Data acquisition and analysis were accomplished with Xcalibur software version 1.4 (Thermo Fisher Scientific).

For peptide profiling of tryptic digest of DPO standard, data-dependent scan functionality of the mass spectrometer was employed, and three scan events were set: scan event 1, MS full scan in the range of m/z 310−1500; scan event 2, UltraZoom scan (width: 4 Th) of the most abundant doubly or triply charged ion from scan event 1 to obtain its isotopic peak distribution; scan event 3, MS/MS scan of the most abundant doubly or triply charged ion from scan event 1. For confirmation of rhEPO or DPO in equine plasma, two specific proteotypic peptides, ^{46}VNFYAWK^{52} and ^{144} VYSNFLR^{150} from both rhEPO and DPO with doubly charged precursor ions of m/z 464.5 and 450.0, respectively, were monitored in MS/MS mode in which the scan range for product ions was m/z 200−850.

RESULTS AND DISCUSSION

Simulation of Tryptic Digestion of rhEPO. The bottom-up approach for fragment peptides from enzymatic digestion of proteins was chosen in this study for confirmation of rhEPO and DPO in equine plasma. Since the amino acid sequences of human and equine EPOs were known,[40−44] simulation of tryptic digestion of the proteins was carried out, and the resulting proteotypic peptides are summarized in Table 2. Although human and equine EPOs share 84% similarity in amino acid sequence, major and relevant proteotypic peptides from them are different by at least one amino acid residue, such as peptides T_4, T_5, T_6, T_8, T_9, T_{10}, T_{11}, T_{14}, and T_{17} from rhEPO versus their counterparts from equine EPO (Table 2). The difference in masses of these relevant tryptic peptides from human and equine EPOs is significant and, thus, can be distinguished from one another by mass spectrometry. DPO amino acid sequence is similar to that of rhEPO and, upon tryptic digestion, produces the same tryptic peptides, T_4, T_6, T_8, T_{10}, T_{11}, T_{14}, and T_{17} (except T_5 and T_9), as those of rhEPO.

LC−MS Profiling of Proteotypic Peptides from rhEPO and DPO. In this study, the disulfide bonds of rhEPO and DPO were not reduced or alkylated to avoid the possible loss of the analytes. Releasing of the peptides T_2 and T_{20} from the T_2−T_{20} cluster (linked together by the disulfide bond between 7- and 161-

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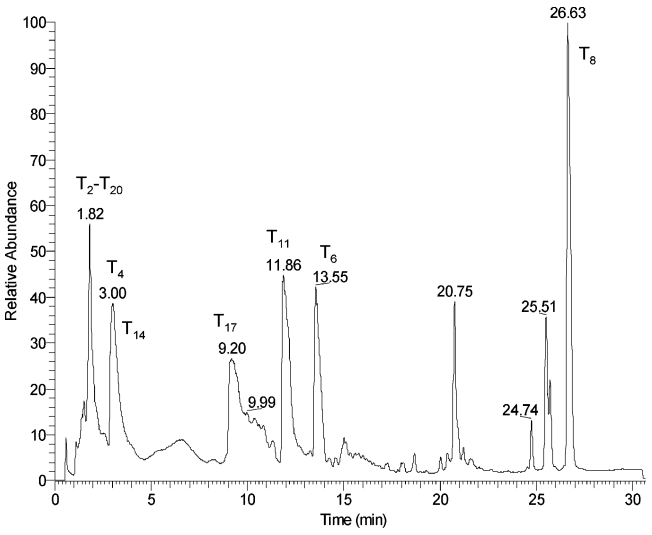

Figure 2. LC−MS chromatogram of tryptic digest of DPO (250 μg/mL). T_2−T_{20}, T_4, T_{14}, T_{17}, T_{11}, T_6, and T_8 are detailed in Table 2.

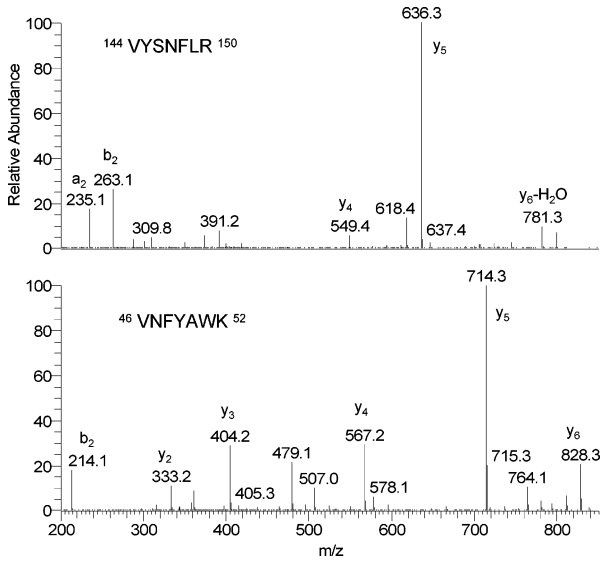

Figure 3. Product ion spectra of the doubly charged tryptic peptides T_{17} (^{144}VYSNFLR^{150}) and T_6 (^{46}VNFYAWK^{52}) specific for confirmation of rhEPO and DPO. Unannotated are the y_2 at *m*/*z* 283.3, b_3 at *m*/*z* 373.3, b_6 at *m*/*z* 724.3, and y_6 at *m*/*z* 799.4 from signature peptide T_{17} (top panel) and b_3 at *m*/*z* 361.2, and y_6−NH_3 at *m*/*z* 811.4 from signature peptide T_6 (bottom panel).

cysteines) would not add further information for confirmation of rhEPO and DPO because the tryptic peptides T_2 and T_{20} from rhEPO/DPO are the same as those from equine EPO. This consideration also applied to the disulfide bond between 29- and 33-cysteines in the tryptic peptide T_5 from rhEPO/DPO, because it is a glycopeptide and, therefore, would not be a useful diagnostic peptide. Tryptic digests of rhEPO and DPO were analyzed by LC−MS with data-dependent UltraZoom scan and MS/MS acquisition. In the LC−MS chromatogram of DPO digest (Figure 2), all the major proteotypic peptides, T_4, T_6, T_8, T_{11}, T_{14}, and T_{17}, were observed (Table 2). The identities of these peptides were further confirmed using charge state detection via isotopic peak distribution obtained from UltraZoom scans and by y-ion series and b-ion series in their product ion spectra. However, the glycopeptides, T5, T9, and T13, were not detected because they were known to exhibit low sensitivity under positive ESI condition. In a similar experiment, the peptides T_4, T_6, T_8, T_{11}, T_{14}, and T_{17} were also observed from rhEPO digest.

Selection of Proteotypic Peptides for Confirmation of rhEPO and DPO. Among the observed proteotypic peptides from rhEPO/DPO, T_6, T_8, T_{11}, and T_{17} were initially chosen as diagnostic peptide markers for confirmation of rhEPO/DPO, because their amino acid sequences are long enough to make them unique and their retention on the LC column was adequate for separation. Results from further experiments showed that T_8 and T_{11} were not detectable when the concentration of rhEPO or DPO was below 100 ng/mL. These peptides could not be used as markers for confirmation because their concentrations in plasma were lower than 100 ng/mL. However, peptides T_6 and T_{17} were still detectable by MS/MS as low as 0.1 ng/mL (20 pg of protein digest injected). Thus, these peptides (T_6 and T_{17}) were selected as the peptide markers for confirmation of rhEPO and DPO in equine plasma.

The product ion spectra of the doubly charged T_6 (^{46}VNFYAWK^{52}) and T_{17} (^{144}VYSNFLR^{150}) from an authentic DPO standard are shown in Figure 3. For the T_6 peptide marker, all the y-ion series product ions (including y_1-ion at *m*/*z* 147, not shown) were observed together with the b_{2}-, b_3- at *m*/*z* 361.2, (b_4−NH_3)- at *m*/*z* 507.0, (b_6−NH_3)- at *m*/*z* 764.1, and (y_6−NH_3)-ion at *m*/*z* 811.4. The T_{17} peptide marker generated the y_{2}- at *m*/*z* 283.3, y_4-, (y_5−H_2O)- at *m*/*z* 618.4, y_5-, y_6- at *m*/*z* 799.4, a_2-, b_2-, b_3- at *m*/*z* 373.3, and b_6-ion at *m*/*z* 724.3. The product ions of these two tryptic peptides are unique, specific, and essential for confirmation of rhEPO and DPO.

Optimization of Tryptic Digestion and LC−MS Conditions. rhEPO and DPO are hormone protein-based drugs and, thus, are pharmacologically effective even at very low plasma concentrations. To confirm their presence at such low plasma concentrations, tryptic digestion and LC−MS conditions were optimized to provide a sensitive analytical method. Trypsin of different pretreatments was compared for digestion of rhEPO/DPO; the sequencing grade modified trypsin (Promega) showed far less undesirable chymotrypsin-like activities than did the unmodified trypsin (Sigma). Incubation of DPO and trypsin at 37 °C for 22 h did not result in remarkably higher peptide yields than those for 3 h (data not shown).

The linear ion trap and ESI source parameters were optimized by tuning with bradykinin fragment 2−9. This tuning procedure optimized parameters for signal intensity of the doubly charged bradykinin fragment 2−9 at *m*/*z* 453 that was close in *m*/*z* value to the two target peptides (*m*/*z* 450.0 and 464.5) used for confirmation of rhEPO and DPO. The temperature of the ion transfer capillary was manually optimized. In addition, the maximum injection time of 50 ms for MS/MS experiments was compared with that of 100 ms, and the former resulted in better sensitivity. An isolation width of 1.5 for precursor ions in MS/MS experiments was chosen because the isolation width of 1.2, 1.4, 1.6, and 2.0 did not significantly improve signal intensity.

Extraction of rhEPO and DPO from Equine Plasma. The complexity of plasma proteome presents a great challenge to studies of low-abundant proteins. For example, in equine plasma as in human and other species, there are many abundant proteins that render recovery of low concentrations of rhEPO and DPO from such complex matrices extremely difficult. It is not uncom-

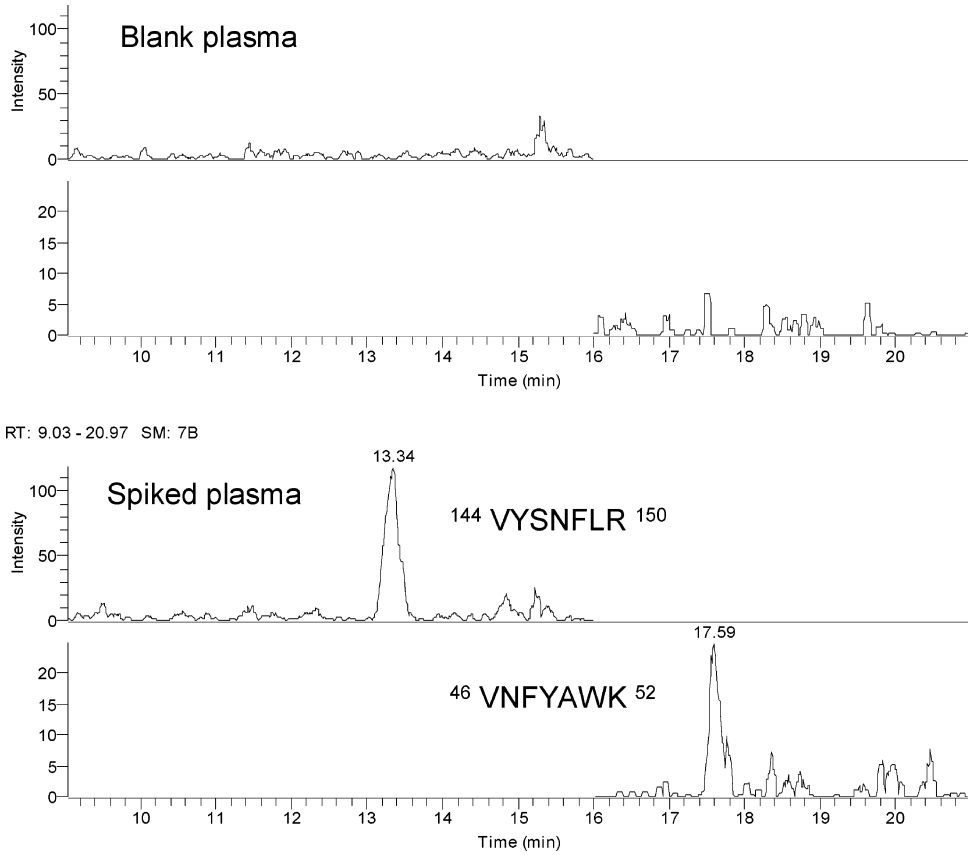

Figure 4. LC−MS/MS chromatograms of blank equine plasma (top two panels) and those of DPO spiked at 0.5 ng/mL into blank plasma (bottom two panels) showing the two chromatographic peaks of the two unique proteotypic peptides and the absence of interferences from blank plasma. The chromatograms were reconstructed using the product ions from T_{17}, *m/z* 235.1, 263.1, 636.3, and 799.4, and those from T_6, *m/z* 214.1, 567.2, 714.3, and 828.3.

mon for abundant proteins such as albumin and immunoglobins to prevent proteins of interest, at physiological concentration, from being recovered and studied. One of the approaches to overcoming this challenge was depletion of the top 6−12 most abundant proteins by immunoaffinity.[45] The drawback of this approach is that the depletion usually results in loss of low-abundant target proteins or analytes.[17,46] Immunoaffinity columns presently available cannot process more than 200 μL of plasma sample, and the depletion method is only suitable for studies involving a few plasma samples but not applicable to processing of a large number of samples as in an extensive equine doping control program. In this study, the procedure for immunoaffinity separation of human EPO from human plasma reported by Skibeli et al.[37] and Wognum et al.[38] was tried for recovery of rhEPO and DPO from equine plasma, and the initial result was encouraging. The procedure was then modified to optimize recovery of the analytes. Modifications to the procedure included the addition of buffer exchange steps after elution of the analytes from the anti-rhEPO antibody to eliminate the detergent (PEG 6000) in the eluate because it was not conducive to the subsequent tryptic digestion and LC−MS analysis. The modifications also included elimination of adding PEG 6000 to plasma samples to precipitate immunoglobins because addition of PEG 6000 to plasma samples resulted in decreased recovery of rhEPO and DPO. In addition, monoclonal and polyclonal anti-rhEPO antibodies were compared for recovery of rhEPO and DPO from equine plasma. The use of polyclonal instead of monoclonal anti-rhEPO antibody improved by nearly 2-fold the recovery of rhEPO and DPO from plasma. For this reason, polyclonal anti-rhEPO antibody was used in this study. As shown in Figure 4, DPO spiked into blank plasma and recovered by immunoaffinity separation and analyzed by LC−MS resulted in two chromatographic peaks at the retention times of 13.3 and 17.6 min for the two specific peptides T_{17} and T_6, respectively. The relevant MS/MS spectra (not shown) verified the identities of the two chromatographic peaks. In contrast, blank plasma did not give rise to any chromatographic peak around the retention times of the two specific peptides (Figure 4).

Extraction efficiency of the immunoaffinity separation procedure was approximately 77% for rhEPO and 50% for DPO at 1 ng/mL in equine plasma. This estimation was achieved by comparing the chromatographic peak area of the T_6 from rhEPO/DPO spiked and extracted with that from an authentic standard. This result suggested that the anti-rhEPO antibody used for the immunoaffinity separation had stronger affinity for rhEPO than DPO, which is reasonable in view of the specific binding between an antigen and an antibody. To date, the anti-rhEPO antibody linked to the magnetic beads has been used 20 times without any

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Table 3. BLAST Database Search Results for the Two Unique Proteotypic Peptides T_6 and T_{17} Indicating Their Inherent Specificity for Confirmation of DPO and rhEPO in Equine Plasma[a]

^{46}VNFYAWK^{52} (T_6)			^{144}VYSNFLR^{150} (T_{17})		
gi number	protein	species	gi number	protein	species
23379788	EPO	*Saguinus oedipus*	55629086	predicted protein	*Pan troglodytes*
23379786	EPO	*Macaca* sp.	8393316	EPO	*Rattus norvecus*
23379784	EPO	*Pongo pygmaeus*	31230	unnamed protein	*Homo sapiens*
23379782	EPO	*Gorilla gorilla*	204061	EPO	
23379780	EPO	*Pan troglodytes*	119527	EPO precursor	
48927343	EPO precursor	*Spalax galili*	112293295	EPO	*Macaca mulatta*
48927341	EPO precursor	*Spalax judaei*	109894633	EPO	*Microtus oeconomus*
48927339	EPO precursor	*Spalax carmeli*	2622288	unknown	
48927337	EPO precursor	*Spalax golani*			
67078424	EPO	*Ovis aries*			
31230	unnamed protein	*Homo sapiens*			
8393316	EPO	*Rattus norvecus*			
21389309	EPO	*Mus musculus*			
112293295	EPO	*Macaca mulatta*			
165877	EPO				
164446	EPO				
55629086	predicted protein	*Pan troglodytes*			
55742715	EPO	*Sus scrofa*			
27806897	EPO	*Bos taurus*			

[a] Only the results of ungapped 100% matches for T_6 or T_{17} are listed. The database searching program used was BLAST version 2.2.14 (NCBI). The database searched was the All Non-Redundant GenBank CDS (translations + PDB + SwissProt + PIR + PRF excluding environmental samples) accessed on August 21, 2006.

noticeable loss of immunoaffinity activity or presence of carry-over effect.

Method Evaluation. Confirmation of the presence of rhEPO and DPO in equine plasma was conducted by using the product ions of the two unique and discriminating proteotypic peptides, T_6 and T_{17}, together with the LC retention times. Minimum criteria proposed in this study for the confirmation are the presence of the major product ions from T_{17}, m/z 235 (a_2), 263 (b_2), and 636 (y_5) with S/N > 3 and the major product ions from T_6, m/z 214 (b_2) and 714 (y_5) (S/N > 3). The two sets of the b_2- and y_5-product ions are sufficient to verify the presence of the two 7-residue peptides (T_{17} and T_6). More product ions present from the two specific peptides are helpful for the confirmation. The minimum criteria also include the following: the retention times of the two specific peptides from a suspect plasma sample must be within 1.0 min (±0.5 min) window of the retention times of the two peptides from rhEPO or DPO standard spiked into blank equine plasma. Under these criteria, the limit of confirmation of this method was 0.2 ng/mL for rhEPO and DPO in plasma (see S-Figures 2 and 3 in the Supporting Information). The limit of detection (LOD) was 0.1 ng/mL.

Quantification of rhEPO and DPO in equine plasma was attempted using external calibration because appropriate internal standard was not available and, thus, could not be achieved with good precision. The major product ions from T_{17} used for semiquantification were m/z 235.1, 263.1, 636.3, and 799.4; or the major product ions from T_6 were m/z 214.1, 714.3, and 828.4. The correlation coefficient (r^2) for the calibration curve generated using peptide T_{17} was better than that using T_6, but on occasion peptide T_6 was preferred. For quantification, an $r^2 > 0.99$ is desired. However, in this method, it was less than 0.99 but better than 0.96. For this reason, this method is only suitable for semiquantification in estimating concentrations of rhEPO and DPO in equine plasma. The semiquantification range was 0.1−2.5 ng/mL (See S-Figures 4 and 5 in the Supporting Information). Semiquantification of rhEPO and DPO in plasma is sufficient for regulatory purposes because these proteins are not naturally produced in the horse and, therefore, should not be present in a racehorse during competition. Thus, it should be emphasized that confirmation, not quantification, of rhEPO and DPO in equine plasma was the specific purpose of this study.

Although this method was not precise for quantification, it was reproducible for confirmation. The two chromatographic peaks relating to the two peptides for confirmation were consistently present even at the lowest concentration of rhEPO/DPO (0.1 ng/mL) in all experiments conducted.

Specificity for Confirmation of DPO and rhEPO. The specificity of this method for confirmation of rhEPO and DPO in equine plasma resulted from the approaches taken in sample preparation and analysis. Specifically, the antibody-based immunoaffinity separation used in this study provides highly selective separation of the analyte(s) from plasma proteins. Second, the filter membrane with molecular weight cutoff of 30 kDa used for the buffer exchange procedure ensures that the analyte(s) retained by the membrane is the intact rhEPO or DPO molecule rather than their peptide fragments. Third, the LC column together with the mobile phase gradient separates from other peptides the two signature peptides (T_{17} and T_6) used for confirmation of rhEPO or DPO. Furthermore, MS selection of the doubly charged peptide precursor ions and MS/MS detection of y-ion series and b-ion series product ions from the two signature peptides provide unambiguous confirmation of the presence of rhEPO or DPO in plasma. Finally, the two proteotypic peptides chosen were very specific for rhEPO or DPO, as indicated by the results (Table 3) from BLAST search of the nonredundant protein database of The National Center for Bioinformatics (NCBI). Most of the proteins containing the amino acid sequence of either of the two signature peptides were EPOs of some species except the horse, indicating

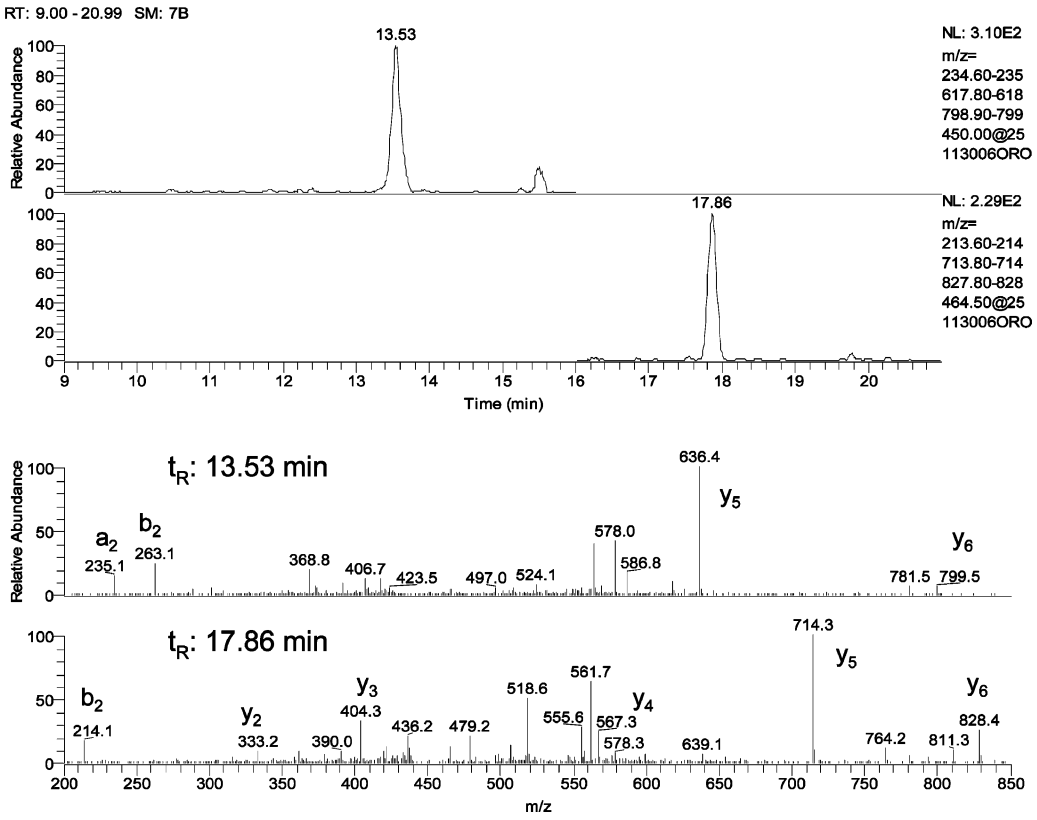

Figure 5. LC−MS/MS chromatograms (top two panels) and product ions spectra (bottom two panels) showing confirmation of the presence of DPO/rhEPO (~0.9 ng/mL) in a serum sample from a racehorse by using ^{46}VNFYAWK^{52} (t_R = 17.86 min) and ^{144}VYSNFLR^{150} (t_R = 13.53 min).

that the two signature peptides were conserved sequences in EPOs (Table 3). The remaining few unknown proteins were not from the horse. Thus, this method is very specific for confirmation of the presence of rhEPO and DPO in equine plasma, although it does not distinguish between rhEPO and DPO. The distinction is not necessary since neither rhEPO nor DPO is naturally produced by the horse and can only be present in the horse following an exogenous administration that would constitute a blatant violation of the rules of horse racing in any jurisdiction in North America.

Analysis of Administration Samples and Racehorse Samples. This method was successfully used in the analysis of plasma samples collected post rhEPO or DPO administrations to research horses. rhEPO was confirmed in equine plasma samples up to 24 h (see S-Figure 7 in the Supporting Information) post a single intravenous administration of 4000 international units (IU) of epoetin α to a horse weighing 474 kg (8.4 IU/kg) and up to 48 h post a single administration of 16 000 IU of epoetin α (34 IU/kg). DPO was confirmed in equine plasma samples up to 120 h (see S-Figure 8 in the Supporting Information) post the last intravenous dose of DPO (25 μg weekly for 7 weeks to a horse weighing 507 kg, i.e., 0.049 μg/kg). These results are in agreement with those by Egrie and Browne that DPO lasts longer in vivo than rhEPO.[3] The dose of either 25 μg of DPO (0.049 μg/kg) or 16 000 IU (80 μg) of epoetin α per horse (34 IU/kg) was quite low, compared with the proposed dose of 300 IU/kg (1.5 μg/kg) of rhEPO for a visible hematological change in the horse[29] or the lowest dose of 6.25 μg of DPO per adult of 70 kg (0.089 μg/kg) in humans as suggested by the manufacturer or a dose of 50 IU/kg of rhEPO in human athletes.[47] Even at these doses that are lower than suggested, rhEPO and DPO were confirmed in post administration plasma samples, indicating that this LC−MS method is sufficiently sensitive for confirmation of DPO and rhEPO in the fight against abuse of these protein-based drugs in the horse racing industry.

It should be noted that it was after storage of the DPO administration samples at −20 °C for 6 months pending the development of this method that the DPO confirmation and detection results described above were obtained. After equine plasma samples from administration of rhEPO (60 000 IU intravenously per horse) were stored at −20 °C for 42 months, rhEPO was still confirmed up to 48 h post administration, which was consistent with the ELISA screening results obtained immediately after collection of the samples. These results indicated that rhEPO and DPO in equine plasma stored at −20 °C were stable during the storage period.

This method was also successfully used in the analysis of "real-world" plasma samples. In four out of seven racehorse serum samples submitted by a Racing Commission/jurisdiction in North America, the presence of DPO or/and rhEPO was confirmed, and the other three serum samples that were found to be negative were blank samples indistinguishably intermingled with the positive samples by the client. In the four positive serum samples, DPO/rhEPO concentration was approximately 0.9, 1.4, 2.0, and 6.3 ng/mL, respectively. Shown in Figure 5 are the LC−MS chromatograms and the product ions spectra for the positive

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serum sample with DPO/rhEPO concentration of ~0.9 ng/mL. The retention times of the two chromatographic peaks from the suspect sample in Figure 5 are close to those from DPO standard spiked into blank plasma (Figure 4). In Figure 5, all the major product ions of the two signature peptides were present for confirmation of DPO/rhEPO in the suspect sample. This method provided the correct confirmation results for all the seven samples, as later verified with the ELISA results from the Racing Commission from whom the samples were received for analysis.

CONCLUSION

A sensitive and selective LC−MS/MS method was developed for confirmation of the presence or absence of rhEPO and/or DPO in equine plasma. The confirmation was conducted using two unique and discriminating proteotypic peptides, ^{46}VNFYAWK^{52} (T_6) and ^{144}VYSNFLR^{150} (T_{17}), from rhEPO and DPO via LC retention times and major product ions. The limit of confirmation was 0.2 ng/mL for both rhEPO and DPO in equine plasma, and the LOD was 0.1 ng/mL. This method was successful in confirming the presence of rhEPO and DPO in plasma samples collected from research horses with rhEPO or DPO administration and from racehorses in competition in North America. To our knowledge, this is the first LC−MS method that is sufficiently sensitive for confirmation of the presence of rhEPO and/or DPO in "real-world" equine samples, resulting in the first confirmation reports for rhEPO/DPO in the racehorse anywhere in the world. Although the method does not distinguish between rhEPO and DPO, it does provide a powerful enforcement tool that was missing in the fight against abuse of rhEPO and DPO in the horse racing industry.

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SUPPORTING INFORMATION AVAILABLE

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

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