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**CONFIRMATION OF RECOMBINANT HUMAN ERYTHROPOIETIN
AND DARBEPOETIN-ALFA IN EQUINE PLASMA BY LIQUID
CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY**

DEVELOPED FOR TESTING INTEGRITY PROGRAM (TIP)

BY

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Confirmation of Recombinant Human Erythropoietin and Darbepoetin Alpha in Equine Plasma by LC-MS/MS

I. INTRODUCTION

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). Genetically engineered recombinant human EPO (rhEPO) is indicated for the treatment of anemia in humans. Darbepoetin alpha (DPO) (NESP, or Aranesp®) is a second generation rhEPO with five of its 165 amino acids intentionally modified to increase duration of action while retaining the pharmacological effect of rhEPO. The ability of these agents to stimulate red blood cell production has led to their abuse as blood doping agents in human endurance sports and horse racing. 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. Deaths in endurance athletes were attributed to rhEPO-induced erythrocytosis. 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 erythropoietin causing inhibition of erythropoiesis and death of some horses.

Concerns of the international sports community over the abuse of rhEPO and DPO in human athletes have led to the evaluation of various methods for the detection and confirmation of these protein-based drugs. Initially, methods by enzyme-linked immunosorbent assay (ELISA) were developed for detection of rhEPO. 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.

Electrophoretic method combining Western blotting was developed for the detection of rhEPO and DPO in human urine, based on the principle that rhEPO molecule is less negatively charged than endogenous human EPO molecule. 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. 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. However, the method occasionally produced false positive results. 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 and the intact proteins. Capillary electrophoresis-mass spectrometry (CE-MS) methods were also reported for separation of

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glycoforms of rhEPO and DPO standards, and for quality control analysis of rhEPO in biotech products. 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. 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 samples because the concentration of rhEPO or DPO in plasma was higher than that in urine. Despite the inherent difficulties, a sensitive and reliable LC-MS/MS method has been developed 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 the presence of rhEPO/DPO in plasma samples from racehorses in North America.

II. SCOPE

This SOP is limited to confirmation of rhEPO/DPO in racehorse plasma samples that have tested positive for rhEPO/DPO by immunoassay (ELISA). The scope of this work covers verifiable procedures to be used in confirming the presence of rhEPO/DPO in equine plasma. This method is not for screening purpose. The method does not differentiate between rhEPO and DPO. The limitation does not exclude this SOP from its usefulness in confirmation of rhEPO/DPO in equine plasma for anti-doping purpose because neither rhEPO nor DPO is naturally present in the horse. In other words, confirmation, by this method, of the presence of rhEPO/DPO in an equine plasma sample from a racehorse certainly reveals illegal abuse of rhEPO or DPO.

III. PRINCIPLE OF THE METHOD

rhEPO and DPO are protein-based drugs. The analytes are digested by a proteolytic enzyme (trypsin), and two resulting tryptic peptides specific for rhEPO/DPO, $^{46}\text{VNFYAWK}^{52}$ (T₆) and $^{144}\text{VYSNFLR}^{150}$ (T₁₇), are targeted for confirmation of rhEPO/DPO. The analytes are extracted from equine plasma by immunoaffinity separation with anti-rhEPO antibody linked to magnetic beads. The extracted analytes in elution buffer are subjected to buffer exchange to ammonium bicarbonate buffer (50 mM, pH 7.8), and digested with trypsin by incubation at 37 °C for 3 hours. The digests are analyzed by LC-MS/MS operated in electrospray ionization positive ion mode, and the two signature tryptic peptides are monitored for confirmation of rhEPO/DPO. The criteria for confirmation include chromatographic retention time of the two signature peptides and their major product ions in MS/MS. The concentration of rhEPO/DPO is semi-quantified by the external calibration method using the chromatographic peak area. The limit of detection (LOD) for rhEPO/DPO in equine plasma by this method is 0.2 ng/2 mL. The lowest confirmable concentration of rhEPO/DPO in equine plasma is 0.4 ng/2 mL.

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Human EPO

1 APPRLICDSR VLERYLLEAK EAENITTTGCA EHCSLNENIT
41 VPDTKVNIFYA WKRMEVGQQA VEVWQGLALL SEAVLRGQAL
81 LVNSSQPWEP LQLHVVDKAVS GLRSLTTLLR ALGAQKEAIS
121 PPDAASAAPL RTITADTFRK LFRVYSNFLR GKLKLYTGEA
161 CRTGD

Glycosylation site: ^{24}N , ^{38}N , ^{83}N , and ^{126}S . Disulphide bonds: $^7\text{C} - ^{161}\text{C}$, $^{29}\text{C} - ^{33}\text{C}$

DPO

Amino acid sequence similar to that of human EPO except for change of five amino acids at the following positions: A30N, H32T, P87V, W88N, P90T

Equine EPO

1 APPRLICDSR VLERYILEAR EAENVMTMGCA EGCSFGENVVT
41 VPDTKVNIFYS WKRMEVEQQA VEVWQGLALL SEAI LQGQAL
81 LANSSQPSET LRLHVVDKAVS SLRSLTSLLR ALGAQKEAIS
121 PPDAASAAPL RTFAVDTLCK LFRI YSNFLR GKLKLYTGEA
161 CRTGD

Figure 1. Amino acid sequence of human and equine EPOs and DPO.

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Table 1. Tryptic peptides predicted from simulated digestion of human and equine EPOs and actually detected following digestion of DPO

Fragment	Human EPO			Note	Equine EPO		
	Start-end	Sequence	[M+2H] ²⁺ (calc.) ^a		Start-end	Sequence	[M+2H] ²⁺ (calc.)
T ₁	1-4	APPR			1-4	APPR	
T ₂	5-10	LICDSR	353.9	T2-T20	5-10	LICDSR	353.9
T ₃	11-14	VLER			11-14	VLER	
T ₄	15-20	YLLEAK	368.9	Detected	15-20	YILEAR	382.9
T ₅	21-45	EAENITTGC AEHCSLNE NITVPDTK	1346.0	Glycopeptide ^b	21-45	EAENVTMG CAEGCSFG ENVTVPDTK	1295.4
T ₆	46-52	VNFYAWK ^c	464.5	Detected	46-52	VNFYSWK	472.5
T ₇	53-53	R			53	R	
T ₈	54-76	MEVGQQAV EVWQGLALL SEAVLR	1264.5	Detected	54-92	MEVEQQAV EVWQGLAL LSEAILQGQ ALLANSSQP SETLR	2120.4
T ₉	77-97	GQALLVNSS QPWEPLQLH VDK	1180.8	Glycopeptide	93-97	LHVDK	
T ₁₀	98-103	AVSGLR	301.9		98-103	AVSSLR	316.9
T ₁₁	104-110	SLTTLLR	402.5	Detected	104-110	SLTSLLR	395.5
T ₁₂	111-116	ALGAQK	294.3		111-116	ALGAQK	294.3
T ₁₃	117-131	EAISPPDA ASAAPLR	733.8	Glycopeptide	117-131	EAISPPDAA SAAPLR	733.8
T ₁₄	132-139	TITADTFR	463.0	Detected	132-140	TFAVDTLCK	499.26
T ₁₅	140-140	K					
T ₁₆	141-143	LFR			141-143	LFR	
T ₁₇	144-150	VYSNFLR ^c	450.0	Detected	144-150	IYSNFLR	457.0
T ₁₈	151-152	GK			151-152	GK	
T ₁₉	153-154	LK			153-154	LK	
T ₂₀	155-162	LYTGEACR	457.0		155-162	LYTGEACR	457.0
T ₂₁	163-165	TGD			163-165	TGD	
T _{2-T20}	5-10 155-162	LICDSR LYTGEACR	539.6 (triply charged)	Detected			

^a The *m/z* value for the [M+2H]²⁺ is the calculated average (not the mono-isotopic value).

^b The glycopeptides were not observed under the LC-MS conditions used in this SOP.

^c The unique tryptic peptides, ⁴⁶VNFYAWK⁵² (T₆) and ¹⁴⁴VYSNFLR¹⁵⁰ (T₁₇), were used for confirmation of rhEPO and DPO in equine plasma.

IV. REAGENTS

A. Water, HPLC grade (Cat. No. W5-4, Fisher Scientific.)

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- B. Boric acid, for Molecular Biology (Cat. # B-6768-500G, Sigma)
- C. Phosphate buffered saline powder (Cat. # P-5368, Sigma).
- D. Bovine serum albumin (BSA), (Cat. # A2153-10G, Sigma)
- E. Na₂EDTA, for Molecular Biology, (Cat. # E5134, Sigma)
- F. Sodium azide, (Cat. # S-8032, Sigma)
- G. Tris(hydroxymethyl)aminomethane (Tris base), Biotechnology Performance Certified (Cat. # T-6066, Sigma)
- H. Igepal CA-630, (Cat. # I8896-50ML, Sigma)
- I. PEG 6,000, (Cat. # 81255, Fluka)
- J. Acetonitrile, HPLC grade (Cat. No. A998-4 , Fisher Scientific)
- K. Formic Acid, Suprapur (Super pure) (EM Science)
- L. Ammonium bicarbonate, Certified (Cat. No. A643-500, Fisher Scientific)
- M. Anti-rhEPO antibody, polyclonal, from rabbit IgG (Cat. No. AB-286-NA, R&D Systems), stored at – 20 °C.
- N. Trypsin, of porcine origin, Sequencing Grade Modified, (Cat. No. V511A, Promega), stored at -70 °C.
- O. Bradykini Fragment 2-9, Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (Cat. No. B1901-1MG, Sigma)

V. SOLUTIONS

A. Borate buffer (0.1 M, pH 9.5)

1. Reagents
 - a) Boric acid powder
 - b) Water, HPLC grade
 - c) NaOH solution, 10 M.
2. Procedure
 - a) Weigh 0.62 grams of boric acid.
 - b) Dissolve it in 80 mL of water.
 - c) Adjust pH to 9.5 with 10 M NaOH.
 - d) Make up to 100 mL with water, and label.
3. Storage Requirements
 - a) Store the buffer at 4 °C (refrigerator).
 - b) Discard 12 months after the date of preparation.

B. Phosphate Buffered Saline (PBS, pH 7.4)

1. Reagents
 - a) Phosphate buffered saline powder
 - b) Water, HPLC grade
2. Procedure

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- a) Transfer the content of a pack of phosphate buffered saline powder to 1-L reagent bottle.
- b) Add 1000 mL of water.
- c) Gently shake the bottle to dissolve the powder and then label

3. Storage Requirements

- a) Store the buffer at 4 °C (refrigerator).
- b) Discard 3 months after the date of preparation.

C. PBS (pH 7.4) with 0.1% (w/v) BSA, 2 mM EDTA, and 0.02% (w/v) NaN₃

1. Reagents

- a) PBS solution
- b) Bovine serum albumin (BSA), Na₂EDTA dihydrate, NaN₃

2. Procedure

- a) Weigh 1.0 g of BSA and add to 1000 mL of PBS (pH 7.4).
- b) Weigh 0.744 g of Na₂EDTA dihydrate and add to 1000 mL of the PBS.
- c) Weigh 0.2 g of NaN₃ and add to 1000 mL of the PBS.
- c) Gently shake the bottle to dissolve BSA, Na₂EDTA, and NaN₃ and label.

3. Storage Requirements

- a) Store the buffer at 4 °C (refrigerator).
- b) Discard 12 months after the date of preparation.

D. Tris buffer (0.2 M, pH 8.5) with 0.1% (w/v) BSA

1. Reagents

- a) Tris base
- b) BSA
- c) HCl, concentrated

2. Procedure

- a) Weigh 2.42 g of Tris base and add to 80 mL of water.
- b) Gently shake to make sure that the powder is completely dissolved.
- c) Adjust to pH 8.5 using concentrated HCl.
- c) Weigh 10 mg of BSA and add to the buffer.
- d) Make up to 100 mL, and label.

3. Storage Requirements

- a) Store the buffer at 4 °C (refrigerator).
- b) Discard 12 months after the date of preparation.

E. One percent (w/v) Igepal CA-630 in PBS (pH 7.4)

1. Reagents

- a) PBS solution (pH 7.4)
- b) Igepal CA-630

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2. Procedure

- a) Add 10 mL of Igepal CA-630 to 1000 mL of PBS
- b) Sonicate in a sonication bath to make sure that Igepal CA-630 is completely dissolved and then label.

3. Storage Requirements

- a) Store the buffer at 4 °C (refrigerator).
- b) Discard 12 months after the date of preparation.

F. 0.1% (w/v) PEG 6000 in PBS (pH 2.0)

1. Reagents

- a) PBS solution (pH 7.4)
- b) Concentrated HCl
- c) PEG 6000

2. Procedure

- a) Transfer 200 mL of PBS (pH 7.4) to a clean/fresh beaker
- b) Adjust pH to 2.0 using concentrated HCl.
- c) Weigh 0.20 g of PEG 6000 and add to the PBS (pH 2.0).
- d) Gently shake to dissolve PEG 6000, and label.

3. Storage Requirements

- a) Store the buffer at 4 °C (refrigerator).
- b) Discard 12 months after the date of preparation.

G. Ammonium Bicarbonate Buffer (50 mM, pH 7.8)

1. Reagents

- a) Ammonium bicarbonate powder, Certified
- b) Water, HPLC grade

2. Procedure

- a) Weigh 0.79 grams of ammonium bicarbonate.
- b) Dissolve it in 200 mL of water.
- c) The pH of this buffer is 7.8 and then label.

3. Storage Requirements

- a) Store the buffer at 4 °C (refrigerator).
- b) Discard 14 days after the date of preparation.

H. Trypsin Solution in Bicarbonate Buffer (20 µg/100 µL)

1. Reagents

- a) Trypsin, Sequencing Grade Modified, of porcine origin
- b) Bicarbonate buffer (50 mM, pH 7.8)

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2. Procedure

- a) Add 100 μ L of the bicarbonate buffer to trypsin (20 μ g) in the original vial.
- b) Mix by brief vortex and then label.

3. **Caution!**

- a) Use only trypsin solution freshly prepared.
- b) Do not store the trypsin solution for later use.

I. 10% Formic Acid

1. Reagents

- a) Formic acid
- b) Water, HPLC grade

2. Procedure

- a) Add 1.0 mL of formic acid to 9.0 mL of water.
- b) Mix by brief vortex and label.

3. Storage Requirements

- a) Store at 4 °C.

J. Bradykinin Fragment 2-9

1. Reagents

- a) bradykinin fragment 2-9
- b) Formic acid
- c) Water, HPLC grade
- d) Acetonitrile, HPLC grade

2. Procedure

- a) Add 50 mL of acetonitrile to 50 mL of water.
- b) Add 0.1 mL of formic acid
- c) Mix by brief vortex.
- d) Transfer 1.0 mL of the mixture of MeCN/H₂O/formic acid (50/50/0.1, v/v/v) and add to 1.0 mg of bradykinin fragment 2-9 in the original vial. Mix to dissolve. The concentration of bradykinin fragment 2-9 in the stock solution is 1.0 mg/mL.
- e) Transfer 10 μ L of bradykinin fragment 2-9 stock solution (1.0 mg/mL) and add to 10 mL of the mixture of MeCN/H₂O/formic acid (50/50/0.1, v/v/v). Mix. The concentration of the working solution is 1.0 μ g/mL, and label.

3. Storage Requirements

- a) Store the stock solution (1 mg/mL) at -20 °C.
- b) Store the working solution (1 μ g/mL) at 4 °C.

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VI. rhEPO AND DPO REFERENCE MATERIALS

- A. rhEPO; HSA-free EPOGEN (Epoetin alpha), 3.19 mg/mL containing carbohydrates, 1.91 mg/mL protein only, in 100 mM NaCl / 20 mM Na Citrate, pH 7.0; from Amgen Inc. (CA); stored at -70 °C.
- B. Darbepoetin alpha (NESP, or Aranesp) in polysorbate solution, 500 µg/mL, from Amgen Inc. (CA), stored at 4 °C.

VII. PREPARATION OF WORKING rhEPO and DPO REFERENCE SOLUTIONS

A. 100 µg/mL rhEPO Solution in H₂O

1. Materials
 - a) rhEPO stock solution (1.91 mg/mL).
 - b) Water, HPLC grade.
2. Procedure
 - a) Add 94.8 µL of water to a plastic microcentrifuge vial (1.5 mL).
 - b) Add 5.2 µL of rhEPO stock solution to the vial in (a).
 - c) Mix and label.
3. Storage requirements
 - a) Store at - 70 °C.
 - b) Discard 12 months after the date of preparation.

B. 10 µg/mL rhEPO Solution in H₂O

1. Materials
 - a) 100 µg/mL working rhEPO solution.
 - b) Water, HPLC grade.
2. Procedure
 - a) Add 90 µL of water to a plastic microcentrifuge vial (1.5 mL).
 - b) Add 10 µL of working rhEPO solution (100 µg/mL) to the vial in (a).
 - c) Mix and label.
3. Storage requirements
 - a) Store at - 70 °C.
 - b) Discard 30 days after the date of preparation.

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C. 1.0 µg/mL rhEPO Solution in H₂O

1. Materials
 - a) 10 µg/mL working rhEPO solution.
 - b) Water, HPLC grade.
2. Procedure
 - a) Add 90 µL of water to a plastic microcentrifuge vial (1.5 mL).
 - b) Add 10 µL of working rhEPO solution (10 µg/mL) to the vial in (a).
 - c) Mix and label.
3. **Caution!**
 - a) Use only 1.0 µg/mL working rhEPO solution freshly prepared.
 - b) Do NOT store this working solution for later use.

D. 0.1 µg/mL rhEPO Solution in H₂O

1. Materials
 - a) 1.0 µg/mL working rhEPO solution.
 - b) Water, HPLC grade.
2. Procedure
 - a) Add 90 µL of water to a plastic microcentrifuge vial (1.5 mL).
 - b) Add 10 µL of working rhEPO solution (1.0 µg/mL) to the vial in (a).
 - c) Mix and label.
3. **Caution!**
 - a) Use only 0.1 µg/mL working rhEPO solution freshly prepared.
 - b) Do NOT store this working solution for later use.

E. 0.01 µg/mL rhEPO Solution in H₂O

1. Materials
 - a) 0.1 µg/mL working rhEPO solution.
 - b) Water, HPLC grade.
2. Procedure
 - a) Add 90 µL of water to a plastic microcentrifuge vial (1.5 mL).
 - b) Add 10 µL of working rhEPO solution (0.10 µg/mL) to the vial in (a).
 - c) Mix and label.
3. **Caution!**
 - a) Use only 0.1 µg/mL working rhEPO solution freshly prepared.
 - b) Do NOT store this working solution for later use.

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F. 20 µg/mL DPO Solution in H₂O

1. Materials
 - a) DPO stock solution (500 µg/mL).
 - b) Water, HPLC grade.
2. Procedure
 - a) Add 96 µL of water to a plastic microcentrifuge vial (1.5 mL).
 - b) Add 4.0 µL of DPO stock solution (500 µg/mL) to the vial in (a).
 - c) Mix and label.
3. Storage requirements
 - a) Store at – 70 °C.
 - b) Discard 6 months after the date of preparation.

G. 1.0 µg/mL DPO Solution in H₂O

1. Materials
 - a) 20 µg/mL working DPO solution.
 - b) Water, HPLC grade.
2. Procedure
 - d) Add 95 µL of water to a plastic microcentrifuge vial (1.5 mL).
 - e) Add 5 µL of working DPO solution (20 µg/mL) to the vial in (p).
 - f) Mix and label.
3. **Caution!**
 - a) Use only 1.0 µg/mL working rhEPO solution freshly prepared.
 - b) Do NOT store this working solution for later use.

H. 0.1 µg/mL DPO Solution in H₂O

1. Materials
 - a) 1.0 µg/mL working DPO solution.
 - b) Water, HPLC grade.
2. Procedure
 - a) Add 90 µL of water to a plastic microcentrifuge vial (1.5 mL).
 - b) Add 10 µL of working DPO solution (1.0 µg/mL) to the vial in (a).
 - c) Mix and label.
3. **Caution!**
 - a) Use only 0.1 µg/mL working rhEPO solution freshly prepared.
 - b) Do NOT store this working solution for later use.

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I. 0.01 µg/mL DPO Solution in H₂O

1. Materials
 - a) 0.1 µg/mL working DPO solution.
 - b) Water, HPLC grade.
2. Procedure
 - a) Add 90 µL of water to a plastic microcentrifuge vial (1.5 mL).
 - b) Add 10 µL of working DPO solution (0.10 µg/mL) to the vial in (a).
 - c) Mix and label.
3. **Caution!**
 - a) Use only 0.1 µg/mL working rhEPO solution freshly prepared.
 - b) Do NOT store this working solution for later use.

VIII. DEVICES FOR IMMUNOAFFINITY SEPARATION, BUFFER EXCHANGE AND ENZYME DIGESTION (Please see Appendix 1 for phone numbers)

- A. Dynal MPC-L magnetic particle separator (Cat. # 120-21D, Invitrogen) for immunoaffinity separation.
- B. Millipore Centrifugal Filter Devices (Ultrafree-CL), 0.22 micrometer pore (Cat. # UFC4 0GV 00, Fisher Scientific).
- C. Millipore Centricon YM-30 Centrifugal Concentrators (30 KDa) (Cat. # 4209, Fisher Scientific).
- D. Isotemp Standard Laboratory Incubator (Model No. 637 D) (Cat. # 11-690-637D, Fisher Scientific)
- E. Tube Shaker/Rotator, small shaker (14 x 10-19 mm tubes) (Cat. # 13-687-10, Fisher Scientific)
- F. Centrifuge, Sorvall Legend Mach 1.6R Tabletop (Cat. # 75004337, Kendro), with Fixed-Angle Rotor (holding twelve 16-mL tubes) (Cat. # 75002006, Kendro)
- G. Water bath (Model 1245PC, VWR Scientific, Bridgeport, NJ).

IX. MATERIALS

- A. Vortex mixer (Scientific Industries, Inc.).
- B. 1.5 mL plastic microcentrifuge vials (Cat. No. 05-408-137, Fisher Scientific).
- C. Pipettes and tips.
- D. 2 mL autosampler vials
- E. 250 uL Insert (Target PP Polyspring, National Scientific Company)
- F. Balance (Mettler AT 261 Delta range, Mettler-Toledo Inc.)
- G. Eye protection
- H. Gloves

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X. MATRIX

Equine Plasma.

XI. VOLUME OF MATRIX FOR ANALYSIS

2.0 mL of equine plasma.

XII. CONTROL SAMPLES

A. Negative Control Sample

1. Equine plasma samples previously demonstrated by LC-MS to be negative for the presence of detectable rhEPO and DPO.
2. Store control samples at approximately -20°C .

B. Column Test Sample

Tryptic digest of rhEPO or DPO standard at concentration of 10 ng/100 μL .

XIII. SAMPLE REQUIREMENTS FOR ANALYSIS

A. Calibrators

1. Prepare a set of calibrators for analysis of plasma samples.
2. Calibrator concentrations are designated in Table 2.
3. Prepare plasma calibrators using negative (control) equine plasma and working rhEPO or DPO standard solutions as described in Section VII.

B. Negative (control) sample

1. Designate **plasma NC or plasma blank**.
2. Prepare negative (control) sample from negative (control) equine plasma.

C. Solvent blank

1. Designate **solvent blank**
2. Use aqueous 0.1% formic acid as solvent blank

- D. Test samples are designated to use the date of which the sample is analyzed and raw data files are designated to use sequential numbers.

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Table 2. Preparation of Plasma Calibrators for rhEPO/DPO confirmation

Target Conc. of rhEPO/DPO (ng/mL)	Working rhEPO/DPO Solution (µg/mL)	Vol. of Spiked Working Solution (µL)	Volume of Plasma (mL)
0	N/A	N/A	2.0
0.1	0.01	20	2.0
0.2	0.01	40	2.0
0.5	0.1	10	2.0
1.0	0.1	20	2.0
2.5	1.0	5.0	2.0

XIV. IMMUNOAFFINITY SEPARATION OF rhEPO AND DPO FROM PLASMA SAMPLES

A. Linkage of anti-rhEPO antibody to magnetic beads in preparation for immunoaffinity separation

1. Shake magnetic beads in the original bottle (Dynabeads M-280, Cat. # 142.03, Invitrogen) for 1 minute to suspend them uniformly, and immediately transfer 2.0 mL of the uniformly suspended beads into a sterile plastic 5-mL tube (Cat. # T2076S, Argos Technologies).
2. Place the tube in a magnetic particle separator (DynaL MPC-L, Cat.# 120-21D, Invitrogen) for 1 minute to separate the magnetic beads from solution. Discard the supernatant, and save the magnetic beads.
3. Remove the tube from MPC-L. Add 2.0 mL of 0.1 M borate buffer (pH 9.5) to the tube to wash the beads, and mix on a tube shaker/rotator (Cat. 13-687-10, Fisher Scientific) for 2 min.
4. Remove the tube from the tube shaker, and place it in MPC-L to separate the magnetic beads from solution. Discard the supernatant, and add 2.0 mL of the borate buffer to the beads to re-suspend them.
5. Dissolve 1.0 mg of anti-rhEPO polyclonal antibody (Cat. # AB-286-NA, R&D Systems) in 1.0 mL of 0.1 M borate buffer (pH 9.5).
6. Add 1.0 mL of the anti-rhEPO antibody solution (1.0 mg/mL) to the magnetic beads in 2.0 mL of the borate buffer. Place the tube containing the anti-rhEPO antibody and magnetic beads in the tube shaker. Then place the tube and the shaker in an incubator at 37 °C, and incubate overnight (for 16-24 h) while the tube is continually shaken to avoid precipitation of magnetic beads.
7. After incubation, separate the coated magnetic beads, discard the supernatant, and *save* the beads as are described above.
8. Wash the coated beads twice with the following buffers, 2 mL of PBS (pH 7.4) with 0.1% BSA plus 2 mM EDTA and 0.02% (w/v) sodium azide (*Buffer C*), for 5 minutes each time.

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9. Separate the coated magnetic beads from washing buffer using MPC-L, discard the supernatant, and add 2 mL of 0.2 M TRIS (pH 8.5) plus 0.1% BSA to the beads. Incubate at 37 °C for 4 h.
10. Separate the coated beads from TRIS buffer using MPC-L. Wash the beads with 2 mL of Buffer C, for 5 minutes.
11. Separate the magnetic beads from the washing buffer. Add 2 mL of Buffer C, and store the magnetic beads coated with anti-rhEPO antibody in 2.0 mL (the original volume of magnetic beads) of Buffer C at 4°C. Now the anti-rhEPO antibody is ready for use in immunoaffinity separation.

B. Incubation of Anti-rhEPO Antibody with Plasma Containing rhEPO or DPO

Requirements for SAFETY: wear gloves, lab coat and eye goggles

1. Transfer 400 µL of the magnetic beads coated with anti-rhEPO antibody into each sterile plastic 5-mL tube (Cat. # T2076S, Argos Technologies).
2. Place the tubes in an MPC-L (Cat.# 120-21D, Invitrogen) for 1 minute to separate the magnetic beads from solution. Discard the supernatant, and leave the magnetic beads in the tubes.
3. Remove the tubes from the MPC-L. Add 2.0 mL of PBS (pH 7.4) with 0.1% BSA plus 2 mM EDTA and 0.02% (w/v) sodium azide (Buffer C) to each tube to wash the magnetic beads, and mix on a tube shaker/rotator (Cat. 13-687-10, Fisher Scientific) for 5 minutes.
4. Separate the magnetic beads and discard the supernatant as are described in Step 3 above. Add 0.4 mL of Buffer C to the magnetic beads in each tube, and mix briefly by vortex.
5. Prepare rhEPO (or DPO) calibrators in plasma as described in Table 2.
6. To the magnetic beads with anti-rhEPO antibody in each tube, add a plasma sample (2.0 mL). Mix briefly by vortex.
7. Place the tubes containing the magnetic beads and plasma sample in a shaker/rotator (Cat. 13-687-10, Fisher Scientific). Then place the tubes and the shaker in an incubator at 37 °C, and incubate overnight (for 16-24 h) while the tubes are continually shaking.

C. Elution of rhEPO or DPO from anti-rhEPO antibody

1. After the incubation for 16-24 h, take the tubes out of the incubator, and centrifuge the tubes at 100 x g for 1 minute to get sample droplets off tube lids. Separate the magnetic beads from plasma in each tube, and discard plasma as are described above. Leave the magnetic beads in the tubes.
2. To the magnetic beads in each tube, add 2.0 mL of 1% Igepal Ca-630 in PBS (pH 7.4) (washing buffer) to wash the beads. Place the tubes in the tube shaker/rotator, and shake for 5 minutes. Centrifuge the tubes at 100 x g for 1 minute. Separate the magnetic beads from the washing buffer, discard it, and leave the beads in the tubes, as are described above.
3. Repeat washing of the magnetic beads (Step 2) 3 more times.
4. To the washed magnetic beads in each tube, add 1.0 mL of 0.1% PEG 6,000 in PBS (pH 2.0) (elution buffer) to elute rhEPO (or DPO) from anti-rhEPO antibody on the beads.

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Place the tubes in the tube shaker, and shake at ambient temperature for 30 minutes. Then place the tubes in the MPC-L for 1 minute, and collect the supernatant into a Millipore Ultrafree-CL HV filter (0.22 μm) (Cat. # UFC4 OGV 00, Fisher Scientific).

D. Buffer Exchange

1. Centrifuge the eluate in each Millipore Ultrafree-CL HV filter (0.22 μm) at 2500 x g for 5 minutes at 20 °C.
2. Transfer the filtrate into a Centricon Centrifugal Filter Device (molecular weight cutoff = 30 KDa) (Cat. # 4209, Fisher Scientific), put on the cap, and centrifuge at 3500 x g for 35 minutes at 20 °C. Please note that the centrifugation time may be extended to ensure that the volume of the retentate on the filter membrane is less than 100 μL (or the retentate is almost invisible).
3. Add 0.4 mL of NH_4CO_3 buffer (50 mM, pH 7.8) to each Filter Device, and centrifuge at 3500 x g for 15 minutes at 20 °C. Discard the filtrate.
4. Repeat Step 3 five more times.
5. Reverse each Filter Device with the cap **ON**, centrifuge it at 500 x g for 2 minutes at 20 °C to spin down the retentate into the cap. Transfer the retentate (40 -80 μL) to a fresh and labeled 1.5-mL plastic microcentrifuge vial (Cat. # 05-408-137, Fisher Scientific). Make up to a final volume of 86 μL .

E. Enzyme Digestion of rhEPO or DPO extract

1. Place the vials containing rhEPO or DPO extract in a water bath at 80 °C for 10 minutes to denature proteins. Remove them from the water bath, and allow them to cool down to ambient temperature (wait for at least 10 minutes).
2. Add 100 μL of NH_4CO_3 buffer (50 mM, pH 7.8) to 20 μg of trypsin in the original vial (Sequencing Grade Modified, Cat. # V5111 , Promega). Please NOTE that the trypsin vial taken out from a - 80°C freezer should be allowed to warm up to ambient temperature (wait for at least 15 minutes).
3. Transfer 10 μL of the trypsin solution (20 $\mu\text{g}/100 \mu\text{L}$) to each vial containing denatured rhEPO or DPO extract, and mix briefly by vortex.
4. Place each vial containing the analyte and enzyme in a water bath at 37 °C, and incubate for 3 h.
5. After 3 h incubation, add 4 μL of 10% formic acid to each vial to quench the digestion/reaction. Mix briefly by vortex. Now the digest is ready for LC-MS/MS analysis.

XV. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL CONFIRMATION OF rhEPO/DPO

A. Instrumentation

1. Finnigan LTQ linear ion trap mass spectrometer with Xcalibur v 1.4 for system control and data acquisition and processing (Thermo Fisher Scientific).

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2. Surveyor Plus LC pump, autosampler, column compartment and on-line degasser (Thermo Fisher Scientific).

B. HPLC conditions

1. HPLC Column
 - a) Type: Zorbax 300 SB-C₁₈ Analytical Column (Part No. 865630-902, Agilent Technologies).
 - b) Dimension: 1.0× 50 mm
 - c) Particle size: 3.5 Micron
 - d) Temperature: 26 °C
2. LC Guard Column
 - a) Type: Zorbax StableBond Guard (Part No. 5185-5920, Agilent Technologies).
 - b) Dimension: 1.0 × 17 mm
 - c) Particle size: 5 micron
 - d) Temperature: 26 °C
3. Mobile Phase
 - a) Mobile phase A: acetonitrile/H₂O/formic acid (5/95/0.1)
 - b) Mobile phase B: acetonitrile/H₂O/formic acid (95/5/0.1)
4. Mobile phase gradient

Table 3. LC mobile phase gradient

Time (min)	0	1.0	19.0	19.5	20.0	26.0	26.5	31.0	31.5	32.0
Mobile phase A%	100	10 0	73	20	20	20	100	100	100	100
Mobile phase B%	0	0	27	80	80	80	0	0	0	0
Flow rate (µl/min)	50	50	50	50	100	100	100	100	50	50

5. Injection Volume: 20 µL.

C. Mass Spectrometric Conditions

1. Ionization mode
 - a) Electrospray ionization (ESI)
 - b) Positive ion mode
2. ESI source settings
 - a) Spray Voltage (kV): 4.5
 - b) Sheath Gas Flow Rate: 20

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- c) Auxiliary Gas Flow Rate: 3.2
 - d) Sweep Gas Flow Rate: 2.3
 - e) Capillary Voltage (V): 25.9 (volts)
 - f) Capillary Temperature (°C): 325
 - g) Tube Lens (V): 80
3. LTQ calibration and tune
- a) The mass spectrometer is calibrated monthly, according to the User’s Manual.
 - b) The sheath gas flow rate, auxiliary gas flow rate, and sweep gas flow rate are semi-automatically tuned by syringe infusion of bradykinin fragment 2-9 (1 µg/mL in MeCN/H²O/formic acid, 50/50/0.1, v/v/v) at 5 µL/min into LC flow of 45 µL/min (80% mobile phase A + 20 mobile phase B). The doubly charged peptide (Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) at *m/z* 453 is monitored for optimization of the gas flows.
 - c) Other ESI source parameters except capillary temperature, and LTQ electron- optical parameters are automatically tuned by infusion of bradykinin fragment 2-9 into LC flow as described above (b).

Table 4. Acquisition parameters for confirmation of rhEPO/DPO

Parameters	Setting	
	0-16 min	16-21 min
LC run time	0-16 min	16-21 min
Precursor ion	<i>m/z</i> 450.0	<i>m/z</i> 464.5
Scan Range	<i>m/z</i> 200-850	<i>m/z</i> 200-850
Normalized Collision Energy (%)	25	25
Isolation Width (<i>m/z</i>)	1.5	1.5
Activation Q	0.25	0.25
Activation Time (ms)	30	30

D. Sample list setup for rhEPO/DPO analysis

1. Blank solvent (0.1% formic acid in H₂O)
2. Column Test (tryptic digest of 10 ng of rhEPO/DPO standard in 100 µL of digestion buffer plus 4 µL of 10% formic acid)
3. Column Test (tryptic digest of 10 ng of rhEPO/DPO standard in 100 µL of digestion buffer plus 4 µL of 10% formic acid)
4. Blank solvent (0.1% formic acid in H₂O)
5. Blank solvent (0.1% formic acid in H₂O)
6. Blank plasma (negative control)
7. Calibrator series 1 (0.2 ng/2 mL)
8. Calibrator series 2 (0.4 ng/2 mL)
9. Calibrator series 3 (1.0 ng/2 mL)

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10. Calibrator series 4 (2.0 ng/2 mL)
11. Calibrator series 5 (5.0 ng/2 mL)
12. Blank solvent (0.1% formic acid in H₂O)
13. Sample 1
14. Blank solvent (0.1% formic acid in H₂O)
15. Sample 2
16. Blank solvent (0.1% formic acid in H₂O)
17. Sample 3
18. Blank solvent (0.1% formic acid in H₂O)
19. Column Test (tryptic digest of 10 ng of rhEPO/DPO standard in 100 µL of digestion buffer plus 4 µL of 10% formic acid)
20. Blank solvent (MeCN/H₂O/formic acid, 50/50/0.1, v/v/v)
21. Blank solvent (MeCN/H₂O/formic acid, 50/50/0.1, v/v/v)

E. Criteria for Confirmation of rhEPO/DPO from Equine Plasma Extracts

- a) The confirmation of rhEPO/DPO is performed using the two signature tryptic peptides, ⁴⁶VNFYAWK⁵² (T₆) and ¹⁴⁴VYSNFLR¹⁵⁰ (T₁₇). The product ion spectra of the doubly charged T₁₇ (*m/z* 450.0) and T₆ (*m/z* 464.5) are shown in Figure 2. The product ion spectra should be obtained by averaging across the chromatographic peak at 15% peak height and background subtracting.
- b) For confirmation of rhEPO/DPO, the minimum requirement is that the a₂, b₂, and y₅ product ions for T₁₇, and b₂ and y₅ product ions for T₆ (Figure 2) must be present at intensity of signal/background noise > 2:1 in the product ion spectra.
- c) Under LC-MS/MS analytical conditions, all of the above product ions of T₁₇ and T₆ must be recognized at retention times within ±0.5 minute of those from an authentic rhEPO/DPO standard spiked into plasma, extracted, digested and run under identical conditions (Figure 3).

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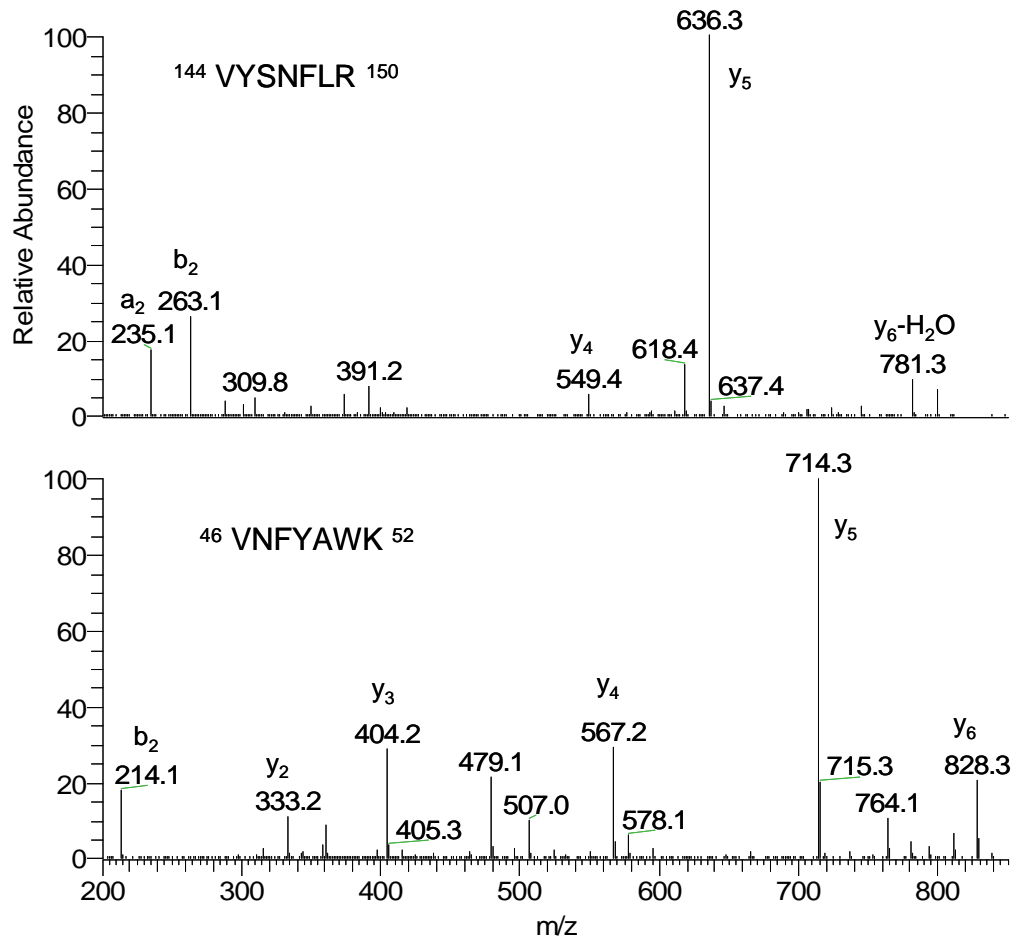


Figure 2. Product ion spectra of the doubly charged tryptic peptides T₁₇ (¹⁴⁴VYSNFLR¹⁵⁰) and T₆ (⁴⁶VNFYAWK⁵²) specific for confirmation of rhEPO and DPO. Un-annotated are the y₂ at m/z 283.3, b₃ at m/z 373.3, b₆ at m/z 724.3, and y₆ at m/z 799.4 from signature peptide T₁₇ (top panel); and b₃ at m/z 361.2, and y₆ - NH₃ at m/z 811.4 from signature peptide T₆ (bottom panel).

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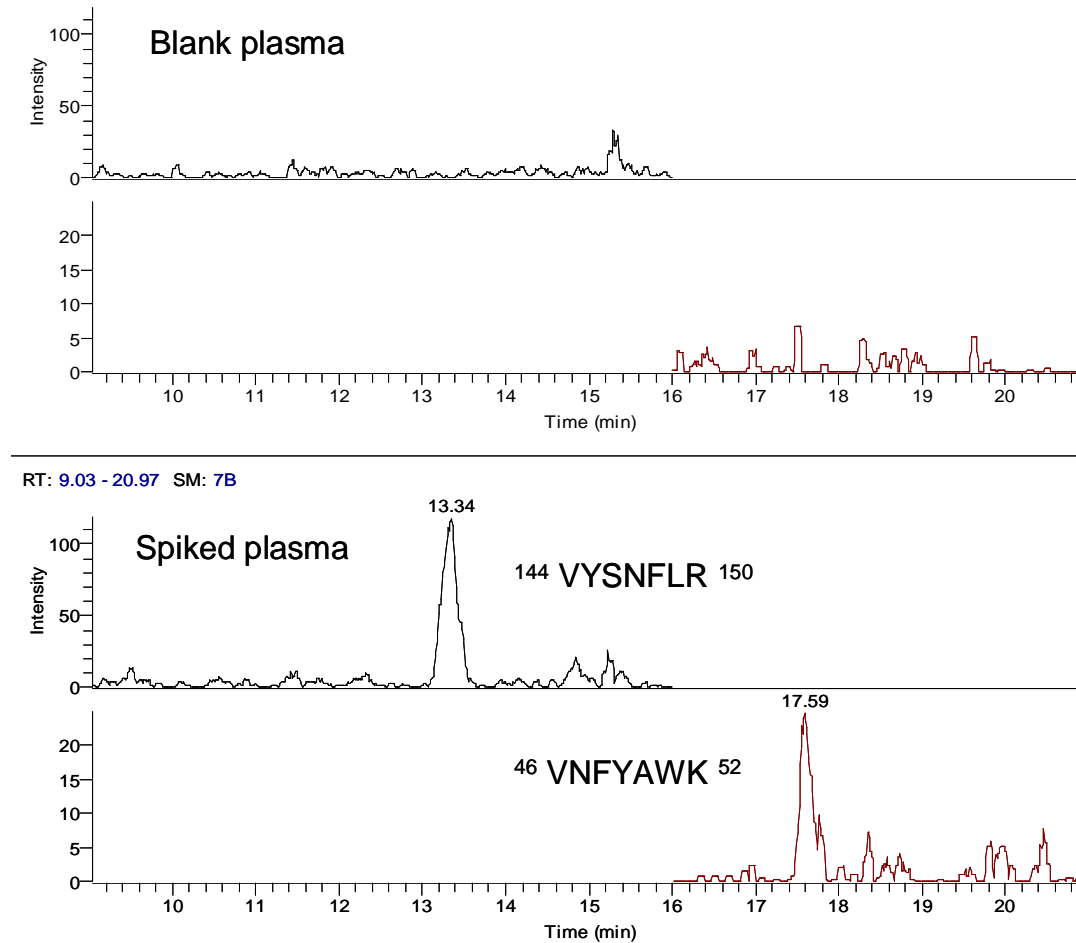


Figure 3. LC-MS/MS chromatograms of blank equine plasma (top two panels) and those of DPO spiked at 0.5 ng/mL into blank plasma (bottom two panels) showing the two chromatographic peaks of the two signature proteotypic peptides and the absence of interferences from blank plasma. The chromatograms were 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.

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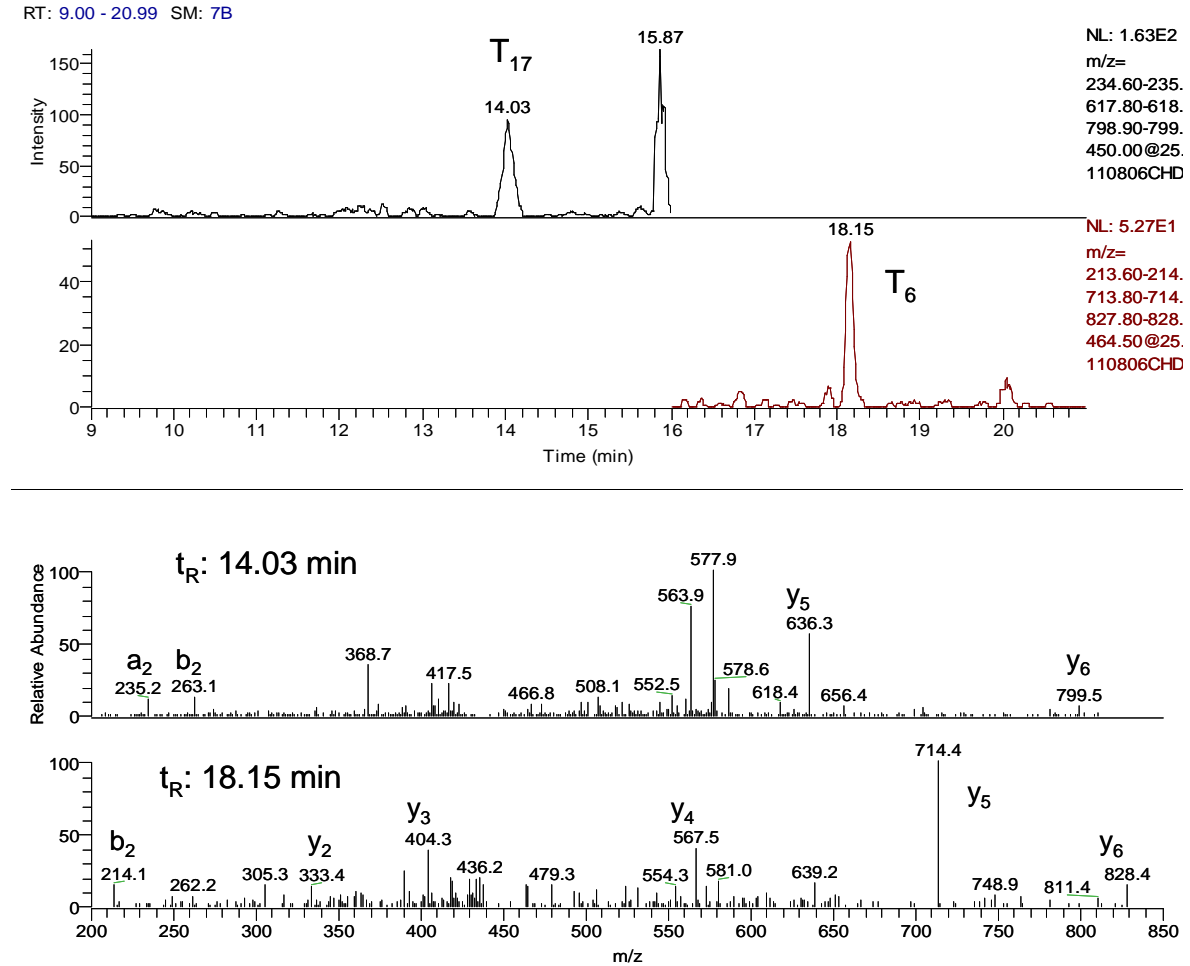


Figure 4. LC-MS/MS chromatograms and product ions spectra showing confirmation of DPO at 0.2 ng/mL in equine plasma.

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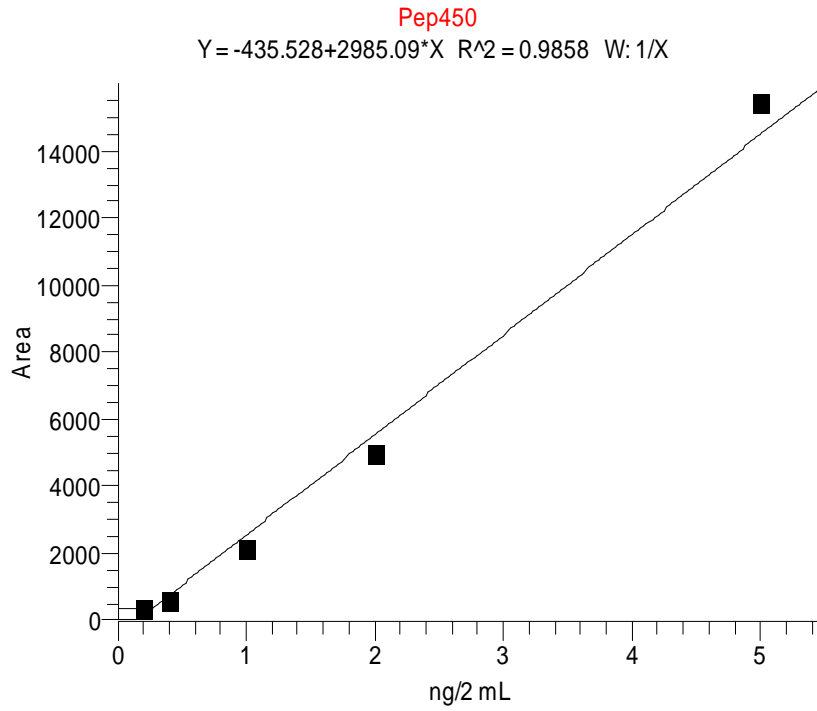


Figure 5. External calibration curve for rhEPO in equine plasma using the doubly charged signature peptide T₁₇ (¹⁴⁴VYSNFLR¹⁵⁰) at *m/z* 450 by LC-MS/MS.

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F. Criteria for Semi-quantification of rhEPO/DPO from Equine Plasma

- a) The signature peptide T₁₇ is used for quantification of rhEPO/DPO. Its product ions used for quantification are *m/z* 235.1 (a₂), 263.1 (b₂), 636.3 (y₅) and 799.5 (y₆). In some cases, the T₆ may be used for quantification.
- b) Plot the peak area of the quantifying ions for each calibrator versus the concentration of rhEPO/DPO in the calibrator (external calibration). Use the Xcalibur software's quantification function to perform calibration and data analysis. Print the summary quantification report and calibration curve (Figure 5). The coefficient of determination should be greater than 0.96.
- c) Examine the reported concentration for all samples.

XVI. CRITERIA FOR REPORTING A SAMPLE AS A POSITIVE FOR rhEPO/DPO

Report a test sample as positive per this standard operating procedure for rhEPO/DPO if the sample contains rhEPO/DPO at concentration greater than the limit of confirmation (0.2 ng/mL), and all of the following criteria are met:

- The test sample contains rhEPO/DPO according to the chromatographic and product ion criteria described in XV (E).
- The limit of detection of rhEPO/DPO in the calibrators is 0.1 ng/mL plasma.
- The signal-to-noise ratio of the chromatographic peaks reconstructed using “*m/z* 235.1 (a₂), 263.1 (b₂), 636.3 (y₅) and 799.5 (y₆)” from the T₁₇, and “*m/z* 214.1 (b₂), 714.3 (y₅), and 828.4 (y₆)” from the T₆ from the test sample is greater than 3.
- The concentration for confirmation of rhEPO/DPO in the test sample is greater than the confirmable concentration (0.2 ng/mL).

XVII. POSITIVE SAMPLE DATA PACKET ASSEMBLY ORDER

1. Sample Transfer Sheet (WS # 32)
2. Sample Usage Sheet (Form #7)
3. Sample List
4. Tune Page Settings
5. LC Method
6. MS Method
7. Semi-quantification Report
8. Quantification Calibration Curve
9. Reconstructed-Chromatogram Comparison for confirmation

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10. Spectrum Comparison for confirmation

Other Required Documentation

In addition to the positive data packet, the following documentation is required:

Sample list print-out that is maintained in the three-ring binder
Routine usage checklist completion (and maintenance log if needed)
Sample Analysis logbook, indicating date, project, operator initials, and listing of official samples.

Data packets for samples determined to be negative will contain the following elements:

1. Sample Transfer Sheet (WS # 32)
2. Sample Usage Sheet (Form #7)
3. Confidence Determination Report
4. Detection Report

XVIII. INTERFERING SUBSTANCES

No known substances have been found to interfere with the determination of rhEPO/DPO by this procedure.

XIX. STORAGE AND STABILITY OF rhEPO AND DPO

A. Storage and stability of rhEPO and DPO in equine plasma

1. rhEPO or DPO in equine plasma should be stored at $-20\text{ }^{\circ}\text{C}$ or $-70\text{ }^{\circ}\text{C}$.
2. rhEPO/DPO in equine plasma is stable for at least 3 years at $-20\text{ }^{\circ}\text{C}$ or $-70\text{ }^{\circ}\text{C}$.

B. Storage of rhEPO and DPO extracted from equine plasma

rhEPO/DPO extract in ammonium bicarbonate buffer (50 mM, pH 7.8) should be digested immediately after immunoaffinity separation, or stored at $-20\text{ }^{\circ}\text{C}$ or $-70\text{ }^{\circ}\text{C}$ until digested by trypsin.

C. Storage and stability of rhEPO/DPO digest

1. rhEPO/DPO digest from tryptic digestion should be analyzed immediately after completion of digestion, or stored at $-70\text{ }^{\circ}\text{C}$ until analyzed by LC-MS/MS.
2. rhEPO/DPO digest is stable for 36 h in autosampler tray at $23\text{ }^{\circ}\text{C}$.

XX. REFERENCES:

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Appendix 1 **Materials and devices for EPO method**

Product	Vendor	Telephone #	Catalog #	Quantity	Unit	Price (Unit)
Sequencing Grade Modified Trypsin	Promega	1-800-356-9526	V5111	1	Pack/100 µg	\$60
Formic acid, Suprapur grade	EMD Chemicals	1-800-222-0342	11670-1	1	Bottle	\$80
5-mL Sterile Microtubes	Argos Technologies	1-800-886-8675	T2076S	1	Pack/25	\$75
Dynal MPC-L magnetic particle separator	Invitrogen	1-800-955-6288	120-21D	1	Each	\$300
1.5 mL microcentrifuge tubes	Fisher Scientific	1-800-766-7000	05-408-137	1	Pack/500	\$400
Millipore Centrifugal Filter Devices (Ultrafree-CL), 0.22 micrometer pore	Fisher Scientific	1-800-766-7000	UFC4 0GV 00	1	Pack/100	\$200
Millipore Centricon YM-30 Centrifugal Concentrators (30 KDa)	Fisher Scientific	1-800-766-7000	4209	1	Pack/100	\$300
Fisher Isotemp Standard Lab Incubator (medium Model)	Fisher Scientific	1-800-766-7000	11-690- 637D	1	Each	\$200
Tube Shaker/Rotator, small shaker (14 x 10-19 mm tubes) <i>Centrifuge</i>	Fisher Scientific	1-800-766-7000	13-687-10	1	Each	\$200
Sorvall Legend Mach 1.6R Tabletop Centrifuge	Kendro	1-800-522-7746	75004337	1	Each	\$700
Fixed-Angle Rotor (holding twelve 16-mL tubes) for Legend Mach 1.6R	Kendro	1-800-522-7746	75002006	1	Each	\$200
Multiple Bucket (holding five 50-mL tubes) for TTH-440 Swinging Bucket Rotor	Kendro	1-800-522-7746	75002028	4	Each	4 x
Multiple Bucket (holding fourtenn 15-mL tubes) for TTH-440 Swinging Bucket Rotor <i>LC Column</i>	Kendro	1-800-522-7746	75002027	4	Each	4 x
Zorbax 300SB-C18 (1.0 x 50 mm, 3.5 micron)	Agilent	1-800-227-9770	865630-902	1	Each	\$300
Microbore Guard (1.0 x 17 mm), Zorbax 300 StableBond	Agilent	1-800-227-9770	5185-5920	1	Pack/3	\$200
ColumnSavers	Mac-Mod	1-800-441-7508	MMCS210	1	Pack/10	\$100
PEEK tubing, 0.0025" (65 micrometer) ID	Upchurch Scientific	1-800-426-0191	1560	3	Pack	3 x