

TIP Approved SOP:

Confirmation and Quantification of Oxyglobin[®] in Equine Plasma By Hyphenated Liquid Chromatography Quadrupole Time-Of-Flight Mass Spectrometry

DEVELOPED BY

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Confirmation and Quantification of Oxyglobin[®] in Equine Plasma by Liquid Chromatography Hyphenated with Tandem Mass Spectrometry

I. INTRODUCTION

Hemoglobin glutamer-200 (Oxyglobin[®], Biopure, Cambridge, MA) is a solution of glutaraldehyde-polymerized bovine hemoglobin (Hb). This hemoglobin-based oxygen carrier (HBOC) was developed for treatment of anemia in animals, regardless of the cause of anemia (hemolysis, blood loss, or ineffective erythropoiesis). Oxyglobin[®] is approved and marketed in the US and Europe for treatment of anemia in dogs. Though developed primarily as temporary blood substitutes, this agent is an excellent candidate for abuse in equine athletes, due to its potential to increase oxygen-carrying capacity of circulating blood and to improve tissue oxygenation.¹ Oxyglobin[®] has a higher oxygen partition pressure (P_{50}) than native Hb and thus, release oxygen easily to the tissues, particularly in situations of high tissue oxygen demand, such as during maximum athletic performance.^{2, 3, 4, 5} The results of a study of human volunteers during a submaximal exercise test and of exercising normovolemic pigs seems to support this concept.^{6, 7} To deter equine athletes from blood doping, the Association of Racing Commissioners International has banned Oxyglobin[®] and Hemopure[®] (Biopure, Cambridge, MA, USA) by including them in its list of “Prohibited Practices”. To date, there is no method available for the detection and confirmation of Oxyglobin[®] in equine athletes.

Techniques available for quantification of Oxyglobin[®] in blood are based on the appearance of free Hb in plasma and the application of readily available bed-side hemometers (HemoCue[®])⁸ or cooximeters to measure plasma Hb content. However these methods lack specificity in that they do not distinguish between native Hb (from hemolyzed red blood cells) and HBOCs.

This standard operating procedures (SOP) describes a reliable analytical method for screening, quantification and confirmation of Oxyglobin[®] in equine plasma by LC/Q-TOF-MS/MS (Micromass-Waters).

II. SCOPE

This SOP will be limited to screening, quantification and confirmation of Oxyglobin[®] in racehorse plasma samples. The scope of this work covers verifiable procedures to be used in quantifying and confirming the presence of Oxyglobin[®] in equine plasma using LC/Q-TOF-MS/MS.

III. PRINCIPLE OF THE METHOD

Oxyglobin[®] is glutaraldehyde-polymerized bovine hemoglobin. The analyte is separated from equine plasma by SPE, digested by an enzyme (trypsin), and then a tryptic peptide specific for bovine hemoglobin (bovine hemoglobin α chain residues #69-90) is targeted for detection, quantitation and confirmation of Oxyglobin[®].^{9, 10} The analyte is extracted from equine plasma by solid-phase extraction (SPE). The extracted analyte in elution solvent is dried, reconstituted in ammonium bicarbonate buffer (50 mM, pH 7.5 ~ 8.5), and digested by incubation with trypsin at 37 °C for 3 hours. The digests are analyzed by LC-MS/MS operated in electrospray ionization positive ion mode, and the specific tryptic peptide is

monitored for quantitation and confirmation of Oxyglobin[®]. The concentration of Oxyglobin[®] is determined by the external calibration method using the chromatographic peak area. The limit of quantitation (LOQ) for Oxyglobin[®] in equine plasma by this method is 50 µg/mL. The confirmable concentration of Oxyglobin[®] in equine plasma is 250 µg/mL.

Bovine Hb α chain (141 amino acids)

1 vlsaadkgnv kaawgkvvggh aeeygaeale rmflsfpttk tyfphfdlsh gsaqvkghga 61 kvaaaltkav
ehlddlpgal seldlhahk lrvdvvnfkl lshllvtla shlpsdftpa
121 vhasldkfla nvstvltsky r

Bovine Hb β chain (145 amino acids)

1 mltaeekaav tafwgkvkvd evggealgrl llvypwtqrf fesfgdlsta davmnnpkvk
61 ahgkklvdsf sngmkhlddl kgtfaasel hccklhvdpe nfkllgnlv vvlarnfgke
121 ftpvlqadfq kvvagvanal ahryh

Equine Hb α chain (142 amino acids)

1 mvlsaadktn vkaawskvgg hageygaeal ermflgfptt ktyfphfdls hgsaqvkahg
61 kkvgdaltla vghlddlpga lsnlhdlhah klrvdvnfk llshellstl avhlpndftp
121 avhasldkfl ssvstvltsk yr

Equine Hb β chain (146 amino acids)

1 vqlsgeekaa vlalwdkvne eevggealgr llvypwtqr ffdsfgdlsn pgavmgnpkv
61 kahgkklvhs fgegvhhdn lkgtfalse lhcklhvdpenfrllgnvl vvlarhfgk
121 dftpelqasy qkvvagvana lahkyh

Human Hb α chain (142 amino acids)

1 mvlspadktn vkaawgkvga hageygaeal ermflsfptt ktyfphfdls hgsaqvkghg
61 kkvadaltna vahvddmpna lsalsdlhah klrvdvnfk llshellvtl aahlpaeftp
121 avhasldkfl asvstvltsk yr

Human Hb β chain (147 amino acids)

1 mvhltpeeks avtalwgkvn vdevggealg rllvypwtq rffesfgdls tpdavmgnpk
61 vkahgkklvg afsdglahld nlkgtfatls elhcklhvdpenfrllgnvl lvcvlahhfg
121 keftppvqaa ykvvagvan alahkyh

Figure 1. Amino acid sequences of bovine, equine and human hemoglobins obtained from Protein Database of the National Center for Bioinformatics.

IV. REAGENTS

- A. Methanol, HPLC grade (Cat. No. A 452-4, Fisher Scientific.)
- B. Water, HPLC grade (Cat. No. W5-4, Fisher Scientific.)
- C. Acetonitrile, HPLC grade (Cat. No. A998-4, Fisher Scientific)
- D. Formic Acid, Suprapur (Super pure?) (EM Science)
- E. Ammonium bicarbonate, Certified (Cat. No. A643-500, Fisher Scientific)
- F. Trypsin, TPKC treated, from bovine pancreas (Cat. No. T1426, Sigma), stored at 0 °C (freezer)

V. SOLUTIONS (All labels must bear date and initials of the chemist who prepared the reagent)

A. Ammonium Bicarbonate Buffer Stock Solution (1.0 M, pH 7.8)

- 1. Reagents
 - a) Ammonium bicarbonate (NH₄HCO₃)
 - b) Water, HPLC grade
- 2. Procedure
 - a) Weigh 7.9 grams of ammonium bicarbonate and dissolve it in 100 mL water (HPLC grade) in a 200 mL beaker. The pH of the buffer is 7.8.
- 3. Storage Requirements
 - a) Store in a glass container at 4 °C (refrigerator).
 - b) Discard 6 months after preparation.

B. Ammonium Bicarbonate Buffer for Enzyme Digestion (50 mM, pH 7.8)

- 1. Reagents
 - a) Ammonium Bicarbonate Buffer Stock Solution (1.0 M, pH 7.8)
 - b) Water, HPLC grade
- 2. Procedure
 - a) Add 95 mL of water to a 200 mL reagent bottle.
 - b) Add 5 mL of ammonium bicarbonate buffer stock solution (1.0 M, pH 7.8). Mix.
- 3. Storage Requirements
 - a) Store in the reagent bottle at 4 °C (refrigerator).
 - b) Discard 30 days after preparation.

C. Trypsin Solution in H₂O (400 µg/mL)

- 1. Reagents

- a) Trypsin, TPCK treated, from bovine pancreas
 - b) Water, HPLC grade
2. Procedure
 - a) Weigh 1.xx grams of trypsin into a 7-mL vial.
 - b) Add yyyy μL of water ($\text{yyyy} = 1.\text{xx}/0.4 \times 1000$). Mix.
3. *Cautions*
 - a) Use only trypsin solution freshly prepared.
 - b) Do not store the trypsin solution for later use.

VI. OXYGLOBIN[®] REFERENCE MATERIAL

- A. Oxyglobin[®], 13 g/dL, manufactured from bovine hemoglobin (Biopure, Cambridge, MA), stored at 4 °C (refrigerator).

VII. PREPARATION OF WORKING OXYGLOBIN[®] REFERENCE SOLUTIONS

A. 10 mg/mL Oxyglobin[®] Solution in H₂O

1. Materials
 - a) Oxyglobin[®] stock solution (13 g/dL).
 - b) Water, HPLC grade.
2. Procedure
 - a) Add 1846 μL of water to a 4-mL vial.
 - b) Add 154 μL of Oxyglobin[®] stock solution to the vial. Mix.
3. *Cautions*
 - a) Use only working Oxyglobin[®] solution freshly prepared.
 - b) Do not store the working Oxyglobin[®] solution for later use.

B. 1.0 mg/mL Oxyglobin[®] Solution in H₂O

1. Materials
 - a) 10 mg/mL working Oxyglobin[®] solution.
 - b) Water, HPLC grade.
2. Procedure
 - a) Add 1800 μL of water to a 4-mL vial.
 - b) Add 200 μL of working Oxyglobin[®] solution (10 mg/mL) to the vial. Mix.
3. *Cautions*

- a) Use only working Oxyglobin[®] solution freshly prepared.
- b) Do not store the working Oxyglobin[®] solution for later use.

VIII. QC WORKING SOLUTIONS

A. 10 mg/mL Oxyglobin[®] Solution in H₂O

1. Materials
 - a) Oxyglobin[®] stock solution (13 g/dL).
 - b) Water, HPLC grade.
2. Procedure
 - a) Add 1846 μ L of water to a 4-mL vial.
 - b) Add 154 μ L of Oxyglobin[®] stock solution. Mix.
3. *Cautions*
 - a) Use only QC working Oxyglobin[®] solution freshly prepared.
 - b) Do not store the QC working solution for later use.

B. 1.0 mg/mL Oxyglobin[®] Solution in H₂O

1. Materials
 - a) Oxyglobin[®] stock solution (13 g/dL).
 - b) Water, HPLC grade.
2. Procedure
 - a) Add 1846 μ L of water to a 4-mL vial.
 - b) Add 154 μ L of Oxyglobin[®] stock solution. Mix.
3. *Cautions*
 - a) Use only QC working Oxyglobin[®] solution freshly prepared.
 - b) Do not store the QC working solution for later use.

IX. DEVICES FOR SOLID-PHASE EXTRACTION DEVICE AND ENZYME DIGESTION

- A. Speedisk[®] 48 Pressure Processor (J.T. Baker, Philipsburg, NJ) for solid-phase extraction.
- B. Sample Concentrator (Dri-Block DB-3, Techne) for drying extracts in elution solution.
- C. Water bath (Model 1245PC, VWR Scientific, Bridgeport, NJ).

X. MATERIALS

- A. Bond Elut ENV cartridges (100 mg/3mL, Varian, Part #: 12105014) for solid-phase extraction.
- B. Vortex mixer (Scientific Industries, Inc.).
- C. 16 × 100 mm test tubes.
- D. 12 × 75 mm test tubes.
- E. Test tube rack
- F. Pipettes and tips.
- G. 2 mL autosampler vials
- H. 250 uL Inert (Target PP Polyspring, National Scientific Company)
- I. Balance (Mettler AT 261 Delta range, Mettler-Toledo Inc.)
- J. Eye protection
- K. Gloves

XI. MATRIX

Equine Plasma.

XII. VOLUME OF MATRIX FOR ANALYSIS

1.0 mL of equine plasma.

XIII. CONTROL SAMPLES

A. Negative Control Sample

1. Equine plasma samples previously demonstrated by LC-MS to be negative for the presence of detectable Oxyglobin[®].
2. Store control samples at approximately -20 °C.

C. Positive Control Sample

1. Equine plasma samples supplemented with Oxyglobin[®] at concentrations of 50, 500 and 2500 µg/mL.

XIV. SAMPLE REQUIREMENTS FOR ANALYSIS

A. Calibrators

1. Prepare a set of calibrators for analysis of plasma samples.
2. Calibrator concentrations as designated in Table 1.
3. Prepare plasma calibrators using negative (control) plasma and working Oxyglobin[®] 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) plasma.

C. Positive control samples

1. Designate **plasma QC (or PC)1.....QC (or PC)n.**
2. Prepare positive control samples as described in Table 2.

D. Mobile phase blank

1. Designate **MB1.....MBn**

E. Test samples designated to use the date of which the sample is analyzed and raw data file designated to use sequence number.

XV. PLASMA CALIBRATOR AND SAMPLE PREPARATION

A. Calibrator and Sample Preparation for Oxyglobin[®] Quantitation and Confirmation

Table 1. Preparation of Plasma Calibrators for Oxyglobin[®] Quantitation

Target Conc. of Oxyglobin [®] (µg/mL)	Working Oxyglobin [®] Solution (mg/mL)	Vol. Of Spiked Working Solution (µL)	Volume of Plasma (mL)
50	1.0	50	1.0
100	1.0	100	1.0
250	10	25	1.0
500	10	50	1.0
1000	10	100	1.0
2500	130	19.2	1.0
5000	130	38.5	1.0

Table 2. Preparation of Plasma Positive Control (QC) Samples for Oxyglobin[®]

Target Conc. of Oxyglobin [®] (µg/mL)	QC Working Oxyglobin [®] Solution (mg/mL)	Vol. Of Spiked QC Working Solution (µL)	Volume of Plasma (mL)
100	1.0	100	1.0
100	1.0	100	1.0
500	10	50	1.0
500	10	50	1.0
2500	130	19.2	1.0
2500	130	19.2	1.0

1. Label 16 × 100 mm test tubes.
2. All samples are prepared in labeled tubes as per Table 3.
3. Mix by vortex the contents of each tube for 5 – 10 seconds.

Table 3. Preparation of Plasma Samples for Oxyglobin[®] Quantitation

Items	Blank	Sample
Blank Plasma (mL)	1.0	N/A
Plasma Samples (mL)	N/A	1.0

B. Sample Extraction by Solid-Phase Extraction

Safety Requirements: eye protection

1. Label solid-phase extraction cartridges (Bond Elut ENV, 100 mg/3mL).
2. Put the labeled cartridges in the Solid-phase extraction processor.
3. Add 1.0 mL of methanol to each cartridge, and allow methanol to pass through by gravity to waste.
4. Add 2.0 mL of H₂O to each cartridge, and allow H₂O to pass through by gravity to waste.
5. Load each equine plasma sample to the labeled cartridges. Allow the samples to pass through by gravity to waste.
6. Add 1.0 mL of H₂O to each cartridge. Allow H₂O to pass through by gravity to waste.
7. Add 1.0 mL of acetonitrile/H₂O (90/10, v/v) to each cartridge. Allow the mixed solvents to pass through by gravity to waste.
8. Add 1.0 mL of acetonitrile/H₂O/formic acid (80/20/0.2, v/v/v) to each cartridge to elute Oxyglobin[®] into a **labeled collection tube**. Allow the mixed solvents to pass through by gravity. Then by pressure, blow into the collection tube the solvent residues remaining in each cartridge bed.
9. In a fume hood, bring the extracts in collection tubes to dryness in a hot block at 80 °C under a stream of nitrogen.
10. Remove test tubes from the drying block, place in a rack, and allow to cool to room temperature.

C. Enzyme Digestion

1. Reconstitute each dried extract with 500 µL of 50 mM ammonium carbonate (pH 7.8).
2. Add 25 µL of 400 µg/mL trypsin (in H₂O) to each reconstituted extract. Mix by vortex.
3. Seal with Parafilm[®] the tubes containing the mixture of reconstituted extract and trypsin. Put the sealed tubes in a water bath at 37 °C. Incubate at 37 °C for 3 hours. Monitor with a thermometer the temperature of the water bath.
4. At the end of the 3 hour incubation, take the tubes out of water bath. Add 20 µL of 10% formic acid to each tube to stop digestion.
5. Transfer 100 µL of each digestion solution into a 200 µL insert in an autosampler vial. All the samples are now ready for LC/MS/MS analysis.

XVI. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION AND QUANTITATION OF OXYGLOBIN[®]

A. Instrumentation

1. Micromass Q-TOF mass spectrometer with MassLynx V3.5 for system control and data acquisition and processing.
2. Hewlett Packard 1100 binary HPLC pump, autosampler, column compartment and on-line degasser.

B. HPLC conditions

1. HPLC Column
 - a) Type: Zorbax 300 SB-C₈ Analytical Column (Part No. 865750-906, Agilent Technologies).
 - b) Dimension: 2.1 × 50 mm
 - c) Particle size: 3.5 Micron
 - d) Temperature: 27 °C
2. LC Guard Column
 - a) Type: Zorbax 300 SB-C₈ (Part No. 821125-918, Agilent Technologies).
 - b) Dimension: 2.1 × 12.5 mm
 - c) Particle size: 5 micron
 - d) Temperature: 27 °C
3. Mobile Phase
 - a) Mobile phase A: acetonitrile/H₂O/formic acid (5/95/0.2)
 - b) Mobile phase B: acetonitrile/H₂O/formic acid (95/5/0.2)
4. Mobile phase and flow rate gradient

Table 4. Gradients in LC mobile phase composition and flow rate

Time (min)	0	0.5	13.0	13.5	15.0	15.5	16.0	20.0	20.5	21.0
Mobile phase B%	15	15	40	80	80	15	15	15	15	15
Flow rate (ml/min)	0.2	0.2	0.2	0.2	0.2	0.2	0.4	0.4	0.2	0.2

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) Desolvation gas: nitrogen at 500 liter/h
- b) Nebulizer gas: nitrogen at 15 liter/h
- c) Collision gas: argon, Analyzer Vacuum reading 2.0e-5
- d) Capillary: 3000 (volts)
- e) Cone: 35 (volts)
- f) Extractor: 0
- g) RF Lens: 0.90
- h) Source Block Temp: 120
- i) Desolvation Temp: 400

3. Q-TOF settings

- a) LM Resolution: 0
- b) HM Resolution: 0
- c) Collision: 4.0
- d) Steering: 0
- e) Entrance: 47.0
- f) Multiplier: 550
- g) MCP: 2750
- h) Transport: 2.0
- i) Aperture 2: 20.0
- j) Acc. V: 200
- k) Focus: 130
- l) Guard: 47
- m) TOF: 7200
- n) Reflectron: 23
- o) Pre-Filter: 6.0

4. Acquisition parameters for Oxyglobin[®]

Parameters	Setting	
	TOF MSMS	<i>m/z</i> 738
Scan Range	<i>m/z</i> 100-1500	<i>m/z</i> 100-1500
Collision Energy (eV)	30	35
Cone (Volts)	35	35
Scan time (sec)	1.0	1.0
Inter Scan Time (sec)	0.1	0.1
Start Time (min)	5.0	5.0
End Time (min)	12.0	12.0

D. Sample list setup for Oxyglobin[®] analysis

1. Sample solvent blank
2. Blank plasma (QC negative control)
3. QC plasma sample (100 µg/mL for Oxyglobin[®])
4. QC plasma sample (500 µg/mL for Oxyglobin[®])
5. QC plasma sample (2500 µg/mL for Oxyglobin[®])
6. Sample solvent blank
7. Calibrator series 1 (50 µg/mL)
8. Calibrator series 2 (100 µg/mL)
9. Calibrator series 3 (250 µg/mL)
10. Calibrator series 4 (500 µg/mL)
11. Calibrator series 5 (1000 µg/mL)
12. Calibrator series 6 (2500 µg/mL)
13. Calibrator series 7 (5000 µg/mL)
14. Sample solvent blank
15. Sample 1, replicate 1
16. Sample 1, replicate 2
17. Sample 1, replicate 3
18. Sample solvent blank
19. Sample 2, replicate 1
20. Sample 2, replicate 2
21. Sample 2, replicate 3
22. Sample solvent blank
23. QC plasma sample (100 µg/mL for Oxyglobin[®])
24. QC plasma sample (500 µg/mL for Oxyglobin[®])
25. QC plasma sample (2500 µg/mL for Oxyglobin[®])

26. Sample solvent blank

There should be a mobile phase blank in between sample, calibrator and QC.

E. Criteria for Confirmation of Oxyglobin[®] from Equine Plasma Extracts

- a) The confirmation of Oxyglobin[®] is performed using the product ions of m/z 737.9 (a doubly charged y14 ion from the unique tryptic peptide derived from bovine Hb α chain residues # 69-90) and the daughter ions of m/z 893.5 (a singly charged b8 ion from the unique tryptic peptide), as shown in Figure 2. The product ion spectra should be obtained by averaging across the chromatographic peak at 20% peak height and background subtracting. The product ion spectra of m/z 737.9 and m/z 893.5 are interpreted in Table 5.
- b) For confirmation of Oxyglobin[®], all the b-series ions and y-series ions shown in Table 5 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 m/z 738 and m/z 893.5 must be recognized at retention time within ± 0.15 minute of that of an authentic standard run under identical conditions (Figure 3).

Table 5. Interpretation of the MS/MS spectra of the doubly charged y14 ion at m/z 737.9 and the singly charged b8 ion at m/z 893.5 from the specific tryptic peptide of bovine Hb α chain Residues # 69-90.

b-ion series and y-ion series of the doubly charged y14 ion at m/z 737.9														
b-ion series		b2	b3	b4	b5	b6								
Mass ^a		155	226	339	426	555								
sequence	P	G	A	L	S	E	L	S	D	L	H	A	H	K
Mass ^a					1136		920	807		605		355	284	
y-ion series					y10		y8	y7		y5		y3	y2	

b-ion series of the singly charged b8 ion at m/z 893.5								
b-ion series				b4	b5	b6	b7	b8
Mass ^a				437	550	665	780	893
sequence	A	V	E	H	L	D	D	L

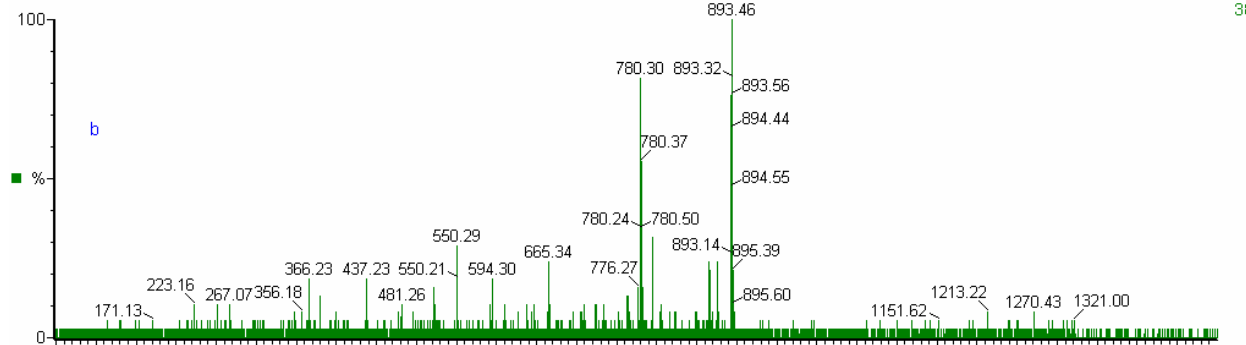
b-ion series			b3		b5
Mass ^a			366		594
sequence	H	L	D	D	L

^a Nominal mass for the b-ion series and y-ion series observed in the MS/MS spectra shown in Figure 2.

250 ug/ml oxyglobin/pl, digest

091703adm_pl11 99 (8.941) Cm (98:101-107:113)

3: TOF MSMS 893.50ES+
38



091703adm_pl11 99 (8.922) Cm (98:101-106:116)

2: TOF MSMS 738.00ES+
22

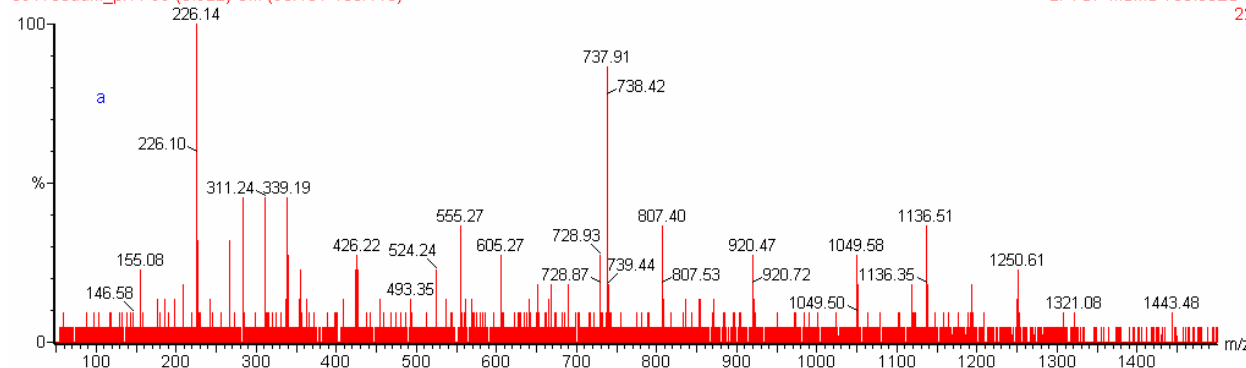


Figure 2. MS/MS spectra of the y14 ion at m/z 737.9 (a) and the b8 ion at m/z 893.5 (b) of the tryptic peptide from bovine Hb α chain residues # 69-90 showed that the presence of Oxyglobin[®] (250 $\mu\text{g}/\text{mL}$) spiked into blank equine plasma was confirmed. The confirmation was based on the b-ion series and y-ion series of the y14 ion and b8 ion. Interpretation of the spectra is presented in Table 5.

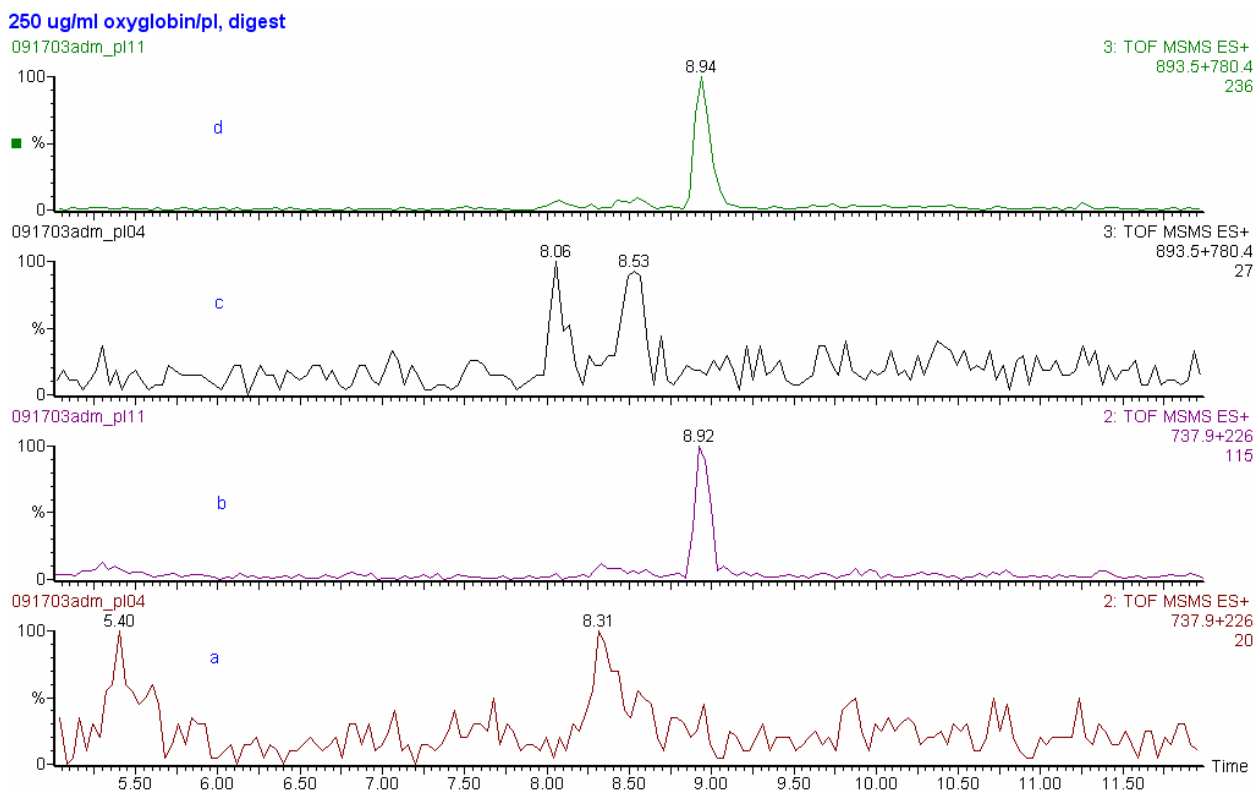


Figure 3. LC-MS/MS chromatograms of tryptic digests of blank equine plasma (a and c) and Oxyglobin[®] (250 µg/mL) spiked into blank equine plasma (b and d). Graphs (a) through (d) are from bottom to top. The chromatographic peaks at retention time of 8.92 min in graph (b) and of 8.94 min in graph (d) indicate the presence of Oxyglobin[®]. The chromatograms were reconstructed from the product ions of m/z 737.9 (a doubly charged y_{14} ion from the triply charged tryptic peptide from bovine Hb α chain residues # 69-90) and of m/z 893.5 (a b_8 ion from the same tryptic peptide). The ions used in reconstructing the chromatograms were ' m/z 737.9 + m/z 226.0' for graphs (a) and (b), and ' m/z 893.4 + m/z 780.3' for graphs (c) and (d). The collision energy was 30×2 eV for CID of m/z 737.9 and 35×1 eV for CID of m/z 893.5.

Compound 1 name: Oxyglobin738 Method File: Oxyglobin738
Coefficient of Determination: 0.994629
Calibration curve: $0.0422493 * x + -0.844068$
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

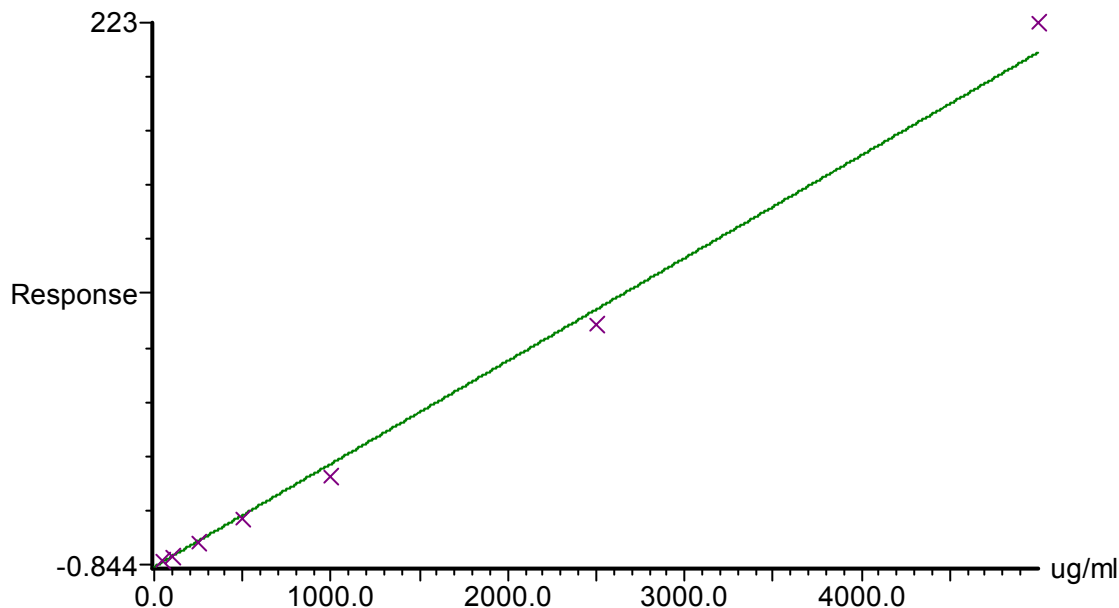


Figure 4. Calibration curve for quantitation of Oxyglobin[®] in equine plasma

F. Criteria for Oxyglobin[®] Quantitation from Equine Plasma

1. Determination of Oxyglobin[®]

- The product ions used for quantification of Oxyglobin[®] are “ m/z 737.9 + m/z 226”.
- Plot the peak area of the quantifying ions for each calibrator versus the concentration of Oxyglobin[®] in the calibrator (external calibration). Use the Masslynx software’s Quantification function to perform calibration and data analysis. Print the compound summary quantification report and calibration curve (Figure 4). The coefficient of determination should be greater than 0.98.
- Examine the reported concentration for all samples. The accuracy of concentration of QC samples should be between 80% and 120 %.

XVII. CRITERIA FOR REPORTING A SAMPLE AS A POSITIVE FOR OXYGLOBIN[®]

Report a test sample as a positive per this standard operating procedure for Oxyglobin[®] if the sample contains Oxyglobin[®] at concentration greater than the limit of confirmation (250 μ g/mL), and all of the following criteria are met:

- The test sample contains Oxyglobin[®] according to the chromatographic and product ion criteria described in XVI (E).
- The limit of quantitation of Oxyglobin[®] in the test sample is better than 100 µg/mL plasma.
- The signal-to-noise ratio of the chromatographic peak reconstructed using ‘*m/z* 737.9 + *m/z* 226’ for Oxyglobin[®] in each replicate of the test sample is greater than 5.
- The concentration for confirmation of Oxyglobin[®] in the test sample is greater than the confirmable concentration (250 µg/mL).

Table 6. Accuracy ^a and precision (C.V.)^b for quantification of Oxyglobin[®] in equine plasma (n=6)

Added (µg/mL)	Intra-day			Inter-day		
	Detected (± SD, µg/mL)	Accuracy (%)	C.V. (%)	Detected (± SD, µg/mL)	Accuracy (%)	C.V. (%)
100	97±16	97	17	116±14	116	12
500	489±27	98	5.6	433±43	87	10
2500	2573±70	103	2.7	2547±322	102	13

^a Accuracy = quantified / added × 100.

^b Coefficient of variation (CV; %) = standard deviation of the conc. quantified / mean of the conc. quantified × 100.

Table 7. Stability of Oxyglobin[®] under different storage conditions

Added (µg/mL)	4 °C		-20 °C		-70 °C	
	Days of storage	Detected (µg/mL)	Days of storage	Detected (µg/mL)	Days of storage	Detected (µg/mL)
100	1	111	7	110	8	55
100	4	101	15	101	15	106
100	5	91	21	101	21	142
100	6	108	28	142	28	109
100	7	117	35	99	38	94
100	8	84				
100	11	105				
500	1	464	7	491	8	466
500	4	438	15	614	15	432
500	5	393	21	503	21	445
500	6	525	28	520	28	452
500	7	433	35	455	38	421
500	8	522				
500	11	453				
2500	1	2282	7	2651	8	2934
2500	4	2120	15	2956	15	2542
2500	5	2188	21	2453	21	2518
2500	6	2585	28	2255	28	2606
2500	7	2335	35	2295	38	2551
2500	8	2627				
2500	11	2571				

XVIII. MEASUREMENT UNCERTAINTY

Without inter laboratory assessment of a developed method, the measurement uncertainty is assessed according to the following statements.

1. Method measurement uncertainty is established based on method validation.
2. The 95% confidence interval is expressed as “± Standard Deviation × coverage factor (k)” (SD×k) for both unknown determinations as well as threshold control values.
3. The measurement uncertainty coverage factor (k, for 95% confidence level) is expressed as the following values depending on the number of data points.

k= 2.0 for 20-50 data points

k= 2.3 for 10-19 data points

k= 2.5 for 4-9 data points

4. The 95% confidence level of a measurement of concentration should be expressed as “mean value \pm (k \times SD)”.

Examples of calculating measurement uncertainty are shown below.

Measurement uncertainty for Oxyglobin[®] in plasma samples

Symbol	Source of Uncertainty	Value of Units	Distribution	Divisor	Standard Uncertainty	Degree of Freedom (n-1)	Other
U1	Intermediate precision	12	N	1	12	9	Oxyglobin [®] 100 μ g/mL
U2	Intermediate precision	7.2	N	1	7.2	9	Oxyglobin [®] 500 μ g/mL
U3	Intermediate precision	8.9	N	1	8.9	9	Oxyglobin [®] 2500 μ g/mL
Combined Uncertainty		1: $(U_1^2)^{1/2} = 12$; 2: $(U_2^2)^{1/2} = 7.2$; 3: $(U_3^2)^{1/2} = 8.9$					
Expanded Uncertainty (k= 2.5, 4-9 data points)		1: $(12 \times 2.5) = 30\%$ 2: $(7.2 \times 2.5) = 18\%$ 3: $(8.9 \times 2.5) = 22\%$, Oxyglobin [®] = concentration \pm 30%					

XIX. POSITIVE SAMPLE DATA PACKET ASSEMBLY ORDER

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

XX. INTERFERING SUBSTANCES

No known substances have been found to interfere with the determination of Oxyglobin[®] by this procedure.

XXI. REFERENCES:

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- (9) Guan, F.; Uboh, C.; Soma, L.; Luo, Y.; Driessen, B. *Analytical Chemistry*. 2003, submitted.
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APPENDIX 1:

Physical appearance of plasma samples before and after administration of Oxyglobin to horses.

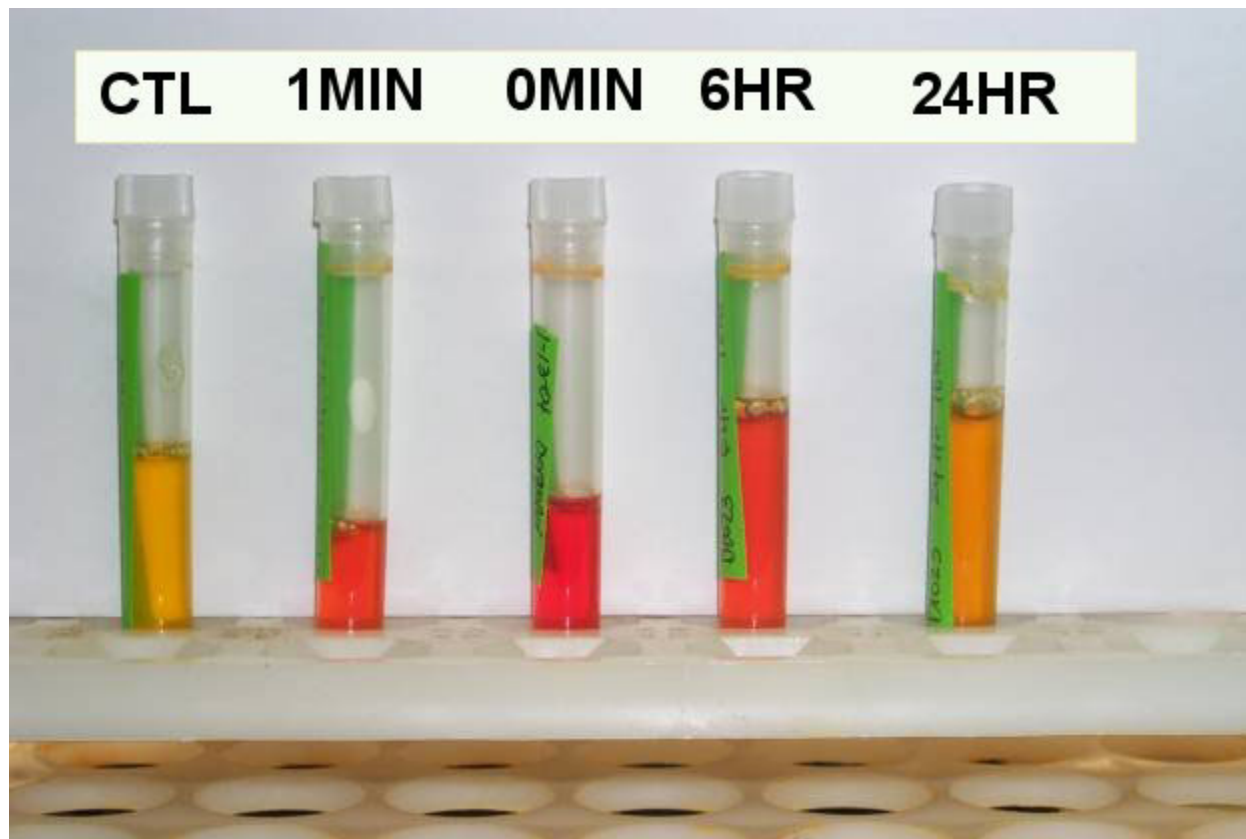


Figure 1. Color change in plasma following intravenous (iv) administration of 32.5 grams/horse Oxyglobin®. CTL = plasma before Oxyglobin® infusion and 1 MIN = plasma collected during Oxyglobin® infusion. Time 0 = plasma collected immediately following completion of the iv administration of Oxyglobin®. 24 hr = plasma collected 24 hr post treatment and shows minimal discoloration of plasma.

APPENDIX II

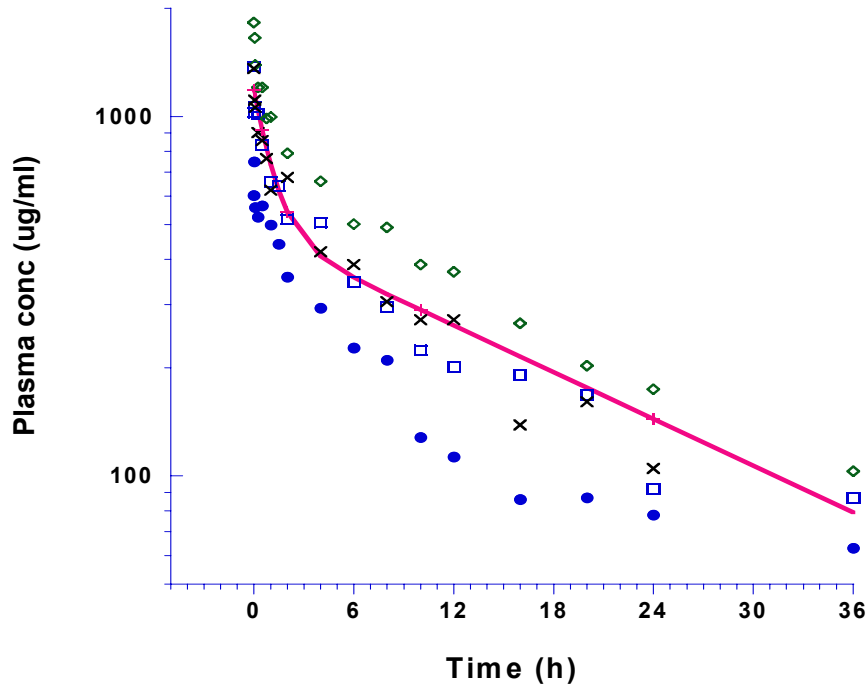


Figure 2. Plasma concentration vs time curve for Hemoglobin Based Oxygen Carrier (HBOC), Oxyglobin®, following iv administration of 32.5 g/horse (n = 4 horses).