Detection and Confirmation of Oxyglobin and Hemopure in Equine Plasma and Urine by LC/Q-TOF-MS/MS

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ABSTRACT

Hemopure® and Oxyglobin® are hemoglobin-based oxygen carrier (HBOC) made from bovine hemoglobin (bHb). Besides the indicated use of HBOC as temporary blood substitutes in trauma and acute blood loss, they might be misused to enhance the performance of human and equine athletes. Since these agents increase the oxygen-carrying capacity of the circulatory system and oxygen delivery to tissues, they may enhance the performance of both equine and human athletes. For this reason, HBOC's are banned from use in athletic competition. Detection and confirmation of HBOC in equine plasma have been impossible so far, because of lack of an appropriate methodology. In this study, we report an LC-MS method for detection, quantification and confirmation of HBOC in equine plasma. For the specific identification of HBOC and distinguishing it from native Hb, digestion of Oxyglobin® or Hemopure by trypsin was necessary. Control equine plasma (1.0 mL) was supplemented with Oxyglobin® or Hemopure®, and extracted by solid-phase extraction (SPE). The extract was dried at 80 °C under a stream of air or nitrogen. The dried extract was dissolved in 0.5 mL NH_4HCO_3 (50 mM, pH 7.8), and 25 µL of trypsin (400 µg/mL in H_2O) was added. The mixture was incubated at 37 °C for 3 hr. An aliquot of 20 µL was injected into LC-MS for detection of tryptic peptides from digestion of HBOC. Analysis of the tryptic peptides resulted in the identification of a specific peptide with the sequence of 'AVEHLDDLPGALSELSDLHAHK' that is unique to the amino acid residues # 69-90 in bHb alpha chain. This amino acid sequence is very specific for bHb alpha chain and is not found in other proteins such as Hb of other species including the horse, as concluded from the Fasta search results against the current 'Swiss-prot' database. Thus, this peptide was targeted for detection and confirmation of Oxyglobin® and Hemopure in equine plasma by LC/Q-TOF-MS/MS. Oxyglobin® was quantified in the range of 100 to 5,000 µg/mL with external calibration. The limit of detection was 50 µg/mL, and the limit of confirmation was 250 µg/mL. The method is so selective that it works equally well in hemolyzed equine plasma and was able to distinguish hemolysed equine Hb from the bovine Hb of Oxyglobin® or Hemopure . The method is simple and inexpensive for the detection and confirmation of Oxyglobin® or Hemopure in equine plasma or human plasma.

Pharmacokinetics of Oxyglobin® in the Horse: Preliminary Results

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ABSTRACT

Oxyglobin® is a hemoglobin-based oxygen carrier (HBOC) made of glutaraldehyde-polymerized bovine hemoglobin (bHb). Products similar to Oxyglobin® are under development for use as temporary blood substitutes in trauma, shock and anemia. Since these agents increase the oxygen-carrying capacity of blood and thus, possibly tissue oxygenation, they may enhance the performance of both equine and human athletes. For this reason, HBOCs are banned from use in athletic competition. Quantification of Oxyglobin® in equine plasma and urine was developed using LC/Q-TOF-MS/MS. Level of Quantification (LOQ) was 50μg/mL. The purpose of this study was to determine the pharmacokinetics of Oxyglobin® following intravenous (iv) administration of 33.7 g of HBOC solution to horses.

The decline of the plasma concentration-time curve of HBOC was described by a bi-exponential equation. The alpha ($t_{1/2\alpha}$) and beta ($t_{1/2\beta}$) half-lives were 0.06 and 8.6 h, respectively. The derived maximal 0-time plasma concentration (C_{max}) was 2262.0 μg/mL and declined to 153.9 μg/mL at 24 h. The area below the plasma concentration time curve (AUC) was 11.1 h*μg/L, the steady state volume of distribution (Vss) was 38.4 L and clearance (CL) was 31.6 mL/h. The Vss was similar to the plasma volume of the horse. There were no changes in native Hb, total proteins (TP) or hematocrit (Hct). Following HBOC administration, plasma was pink and the plasma Hb concentration was 0.2 to 0.1 g/dL, as measured with standard colorimetric methods that cannot distinguish between native Hb and HBOC. No changes in urine color were noted over the 96-h collection period, which would suggest that renal excretion did not occur. Following the 5-min infusion of 33.7 g, early blood samples were collected from the contra-lateral vein at 2, 5, and 15 min. This allowed the description of the very rapid initial decline in HBOC. The bHb molecules in Oxyglobin® are not of uniform size and vary substantially in molecular weight (MW). Molecules of smaller MW (<60 kD) can rapidly pass across capillaries and enter the lymphatic system, thereby leaving the circulation. HBOC's, being large protein molecules compared to drugs, are not metabolized and are removed by the reticular endothelial system and eliminated by similar mechanisms that handle native Hb. This removal of the HBOC represents the slower β elimination phase.

This preliminary study shows the detection and quantification in equine plasma of a HBOC following an iv administration. The method can detect, quantify and distinguish native Hb from an administered HBOC.

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sent to Jan Wade 6/5/03

Proceedings of the 14th International Conference of Racing Analysts and Veterinarians, Florida, USA

PROPOSED METHODOLOGY FOR THE DETECTION OF OXYGLOBIN IN EQUINE PLASMA

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ABSTRACT

Oxyglobin, manufactured by Biopure Corporation, is a blood substitute used for transfusions in veterinary medicine. This substance is a mixture of bovine haemoglobin (Hb) polymers, the properties of which have reportedly attracted the interest of athletes wanting a temporary increase in the oxygen-carrying capacity of their circulatory systems. Our proposed methodologies aim to eliminate the possibility of abuse of this substance in Thoroughbred racing by providing methods for detection and confirmation of Oxyglobin in horse serum. Oxyglobin specifically comprises a mixture of <5% unstabilised Hb tetramers, ~50% Hb polymers between 65–30 kD, and ≤10% as >500 kD. Because equine Hb examined by size exclusion HPLC with 420 nm spectrophotometric detection and by electrospray ionisation (+) mass spectrometry, revealed its existence as 32 and 30 kD dimeric units ($\alpha_1\beta_2$ and $\alpha_2\beta_1$ dimers), identification of higher mw polymers based on mass selectivity should be possible. Unfortunately, native equine plasma was found to contain interfering high mw proteins absorbing at 420 nm, limiting size exclusion chromatography to a screening mechanism at best. Confirmation would then exploit evolved differences between bovine and equine Hb protein primary sequences. For example, trypsin digestion of Hbα (bovine) provides peptide fragments >500 daltons sized 2970.4, 2367.6, 1834.0, 1529.6, 1279.5, 1071.3, 817.9, 702.8, 672.8 and 531.6 daltons, whereas Hbα (equine) provides 3013.5, 2950.3, 1834.0, 1515.6, 1268.5, 1041.3, 817.9, 702.8 and 561.6. Note that there are both similarities and differences in these fragment arrays. ESI(+)MS studies of such fragments have revealed several candidates from trypsin-digested Oxyglobin as providing adequate response, lack of significant interference by miscuts, and ease of distinction from equine peptides, specifically the 2.970 kD T12α fragment, the 2.090 kD T6β fragment, the 2.368 kD T9α fragment, and the 1.530 kD T4α fragment. Ongoing studies will reveal the efficacy of measuring such fragments in plasma from Oxyglobin-dosed horses as evidence of recent Oxyglobin administration, as well as limitations of detectability post dose.

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INTRODUCTION

According to the manufacturers (Biopure Corporation), Oxyglobin, the market name for Bovine Hemoglobin Glutamer 200, consists of cross-linked Hb tetramers designed to replace temporarily the Hb lost by animals through anaemia or rapid blood loss. It is a dark purple, sterile, polyionic colloidal fluid, pH 7.8, with glutaraldehyde-polymerised, ultrapurified bovine Hb formulated in a modified lactated Ringer's solution (Meyer 2001). Bovine Hb polymers are present at 13 g/dl distributed as <5% unstabilised tetramers, approximately 50% with a molecular weight between 65 and 130 kD, and ≤10% with a molecular weight above 500 kD, with an estimated terminal elimination half-life of 18–43 h for dosages of 10–30 ml/kg in dogs (Driessen *et al.* 2001). A high molecular weight is important for this substance to prevent breakdown and excretion of Hb dimers with their subsequent renal toxicity (Meyer 2001). A major advantage of cell-free Hb solutions is their lack of the antigenic red cell stroma present in whole blood, thus avoiding the necessity for crossmatching prior to infusion (Kilbourn *et al.* 1994). Oxyglobin has a shelf life of 36 months at room temperature (2°–30°C).

The field of veterinary medicine has embraced Oxyglobin as an arterial oxygen carrier during

episodes of shock due to rapid blood loss, in stabilisation of anaemia, following carbon monoxide poisoning, during dialysis and possibly during other surgical emergencies, and human medicine may soon benefit similarly from an advanced preparation known as Hemopure. Normal Hb encapsulated within red blood cells is responsible for the transport of oxygen from the lungs to the tissue beds, but with anaemia, for example, oxygen transportation is compromised. Polymerised Hb molecules, like Hb within red blood cells, transport oxygen from the lungs to the tissues and compensate the decreased oxygen content of the blood due to low red cell count. According to the manufacturers, oxyglobin glutamers are 3 times more efficient in oxygenating tissues than natural blood cells, mainly by facilitation of oxygen loading to red blood cells present in the lungs, by improved oxygen off-loading from red blood cells to tissues and by effective distribution within the plasma space.

Meyer (2001) states that adequate transport of oxygen to tissues depends on oxygen content of the arterial blood and distribution of that blood to locations of oxygen consumption. That is where the efficacy of Hb-based solutions may be limited by their vasoactivity. Early cell-free Hb solutions were noted to cause vasoconstriction and increased systemic and pulmonary blood pressures. Nitric oxide (NO) is produced in vascular endothelial cells and is an important factor in the normal regulation of vascular smooth muscle tone, by relaxing the muscle in response to an increase in arterial pressure. Haemoglobin solutions reduce vascular NO activity by binding to and inactivating the NO molecule, rather than by inhibiting its production (Wennmalm *et al.* 1992; Kilbourn *et al.* 1994). Improved oxygen delivery to tissues may, therefore, not be realised when administering vasoactive Hb solutions such as Oxyglobin (Meyer 2001). These findings, however, lead to the speculation that the vascular reactivity of Hb solutions may prove useful in the treatment of septicaemic hypotension (Meyer 2001).

An interest in experimentation with oxygen carriers for enhancement of performance in stamina competitions such as cycling, marathon and distance running, rowing, cross-country skiing, etc. has led the athletic world to Oxyglobin (Grossekathoefer 2000) as well as Hemopure (Bovine Hemoglobin Glutamer 250) and Erythropoietin (EPO; Lasne and de Ceaurriz 2000), and the horse is no exception (Geor 2000). Early attempts at enhancement of oxygen transport involved months-long athletic training in high altitudes for development of polycythaemia, a natural phenomenon arising from diminished blood oxygen concentrations. Similar conditions arise from EPO because it increases the number of red cells with no regulation. A number of elite cyclists are believed to have died due to heart attacks (Mackay 2000) caused by EPO, and the International Cycling Union now uses a haematocrit level of 50% as the cut-off beyond which riders are deemed to have used EPO (see: www.times-olympics.co.uk/archive/d3news. html). The concern in the Sydney Olympic Games was whether athletes would be using Oxyglobin, since EPO could be detected to some extent (Hoberman 2001). Because of the advantage of making the blood more fluid without affecting haematocrit, and because there are not yet definitive tests to detect Oxyglobin, athletes are reported to have tried it.

Such use of Oxyglobin comes in spite of known side effects including kidney failure due to severe vasoconstriction, high blood pressure (Feuerstein 2001) of both the arterial and central venous types (Maxson *et al.* 1993; Giger *et al.* 1994), among others. In human use in South Africa, where there is major concern about prevalence of HIV in the blood supply, the plasma clearance of Hemopure has been shown to follow first order pharmacokinetics for either single or multiple dosage regimens; at a dose of 45 g Hb, the plasma elimination half-life of Hemopure is approximately 20 h with a clearance of approximately 0.12 l/h, and plasma concentrations of Hb are proportional to dose (Smalley 2001). We have resorted to technologies developed within the science of proteomics to design methods for the determination of Oxyglobin doping in performance horses, involving screening with simple chromatographic and spectrophotometric methods, and confirmation with more rigorous methods such as trypsin digestion followed by ESI MS identification of specific fragments. The following is an initial report of our efforts.

Materials and methods

Molecular weight standards, equine and bovine Hb standards were obtained from Sigma (Missouri, USA); equine Hb was also obtained readily from equine red blood cells lysed by exposure to hypotonic conditions.

Size exclusion chromatography

An HPLC system composed of a Beckman 114M pump, Agilent 1050 UV-Vis detector set at 420 nm, and H-P 3392A integrator enabled size exclusion chromatography (SEC) with a Waters YMC-Pack Diol-AMP-S-5 u, 20 nm, AMP-714-5, 300 x 6 mm id

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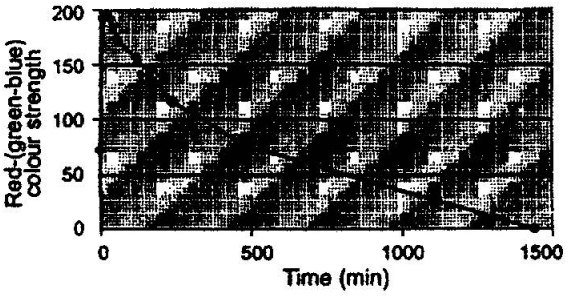

Fig 1: Serum samples from a 500 kg mare dosed with 125 ml Oxyglobin iv for a 16.25 g total dose of polymerised Hb. Samples displayed a significant colour change associated with this substance, and were photographed digitally, and sample pixel colour values tested with Microsoft Image Composer version 1.5 (Microsoft Corporation). The plot shows variation in red color strength based on a red-green-blue model ranging from 0 to 255. Time points include 0, 10, 20, 30 min, then 1, 2, 4, 8 and 24 h.

column. The pH 6 isocratic mobile phase was 0.1 M ammonium formate, 0.05 M NaCl, 5% methanol, in water with a flow rate of 0.6 ml/min. Standards for interpolation of molecular weights included thyroglobin, 670 kD; gamma-globulin, 158 kD; ovalbumin, 45 kD; myoglobin, 16 kD; and vitamin B-12, 1.3 kD.

Rockford, IL

Protein determinations

Trypsinisation of protein mixtures requires accurate knowledge of protein concentrations. The authors' laboratory compared 3 methods of constructing standard curves with a Coomassie reagent kit (Pierce #23236, Pierce Chemical Company) and found the microtitre plate direct microassay superior to standard and micro assays. Equal volumes (100 µl) of diluted protein sample (1:80 dilution) and Coomassie reagent were mixed for 1 min on a plate shaker. Diluted BSA stock standards 1–25 µg/ml were arranged alongside samples in 96-well microtitre plates, and absorbances were measured in a Bio-Tek (Vermont, USA) microtitre plate reader at 595 nm. The results were linear and reproducible, and reliably measured Oxyglobin concentrations, as determined by success at trypsin digestions.

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Trypsin digestion method

Pierce TPCK Trypsin is treated with L-(tosylamido-2-phenyl ethyl)chloromethyl ketone to inhibit chymotryptic activity and thereby prevent miscuts. Digestion requires a 1:50 trypsin:protein (w/w) ratio (Covey 1996). Digestion buffer is 0.1 M NH_4CO_3, pH 8.0, and the enzyme is prepared in digestion buffer. Example protocol: in a glass 1 ml

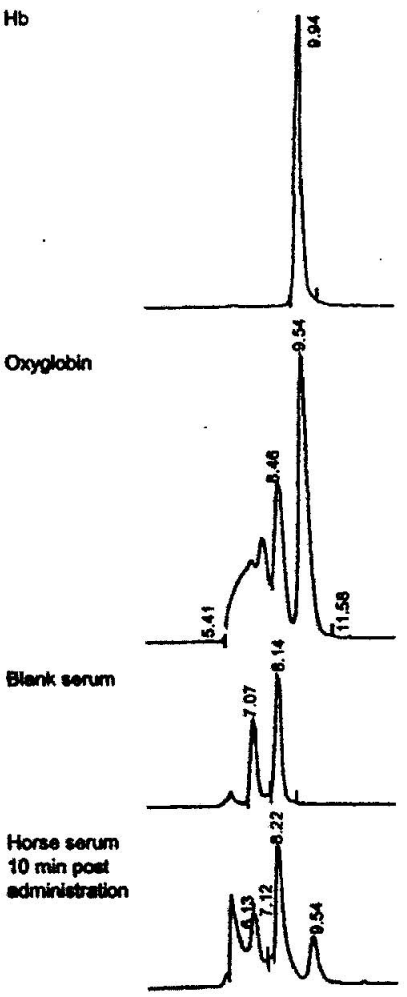

Fig 2: HPLC-SEC chromatography profiles of equine Hb standard from lysed RBC, Oxyglobin, blank horse serum, horse serum 10 min post Oxyglobin administration. The x-axis shows retention time increasing from left to right, while the y-axis shows visible light absorption at 420 nm. Molecular weight interpolation enabled assignment of Oxyglobin peaks as 64 kD (9.54' RT), 128 kD (8.46' RT), 192 kD (7.60' RT), 256 kD (6.85' RT), and larger concatemers between 5.4' and 6.8' RT, representing idealised concatemers of 64 kD Hb tetramers.

Reacti-vial (Pierce), 2 mg/ml bovine Hb (500 µl) was mixed with 20 µl of 1 mg/ml TPCK trypsin, both previously dissolved or diluted in digestion buffer. Reaction was allowed to proceed for 16 h at 37°C, with gentle mixing supplied by a small stir bar in a Pierce Reacti-Therm heating/stirring module.

ESI(+)-MS confirmation method

ESI(+)MS full scan analysis of tryptic digests was carried out on the Quattro II by direct infusion via a Harvard syringe pump and 500 µl syringe. Tuning parameters included: capillary: 2.90 kVolts: HV

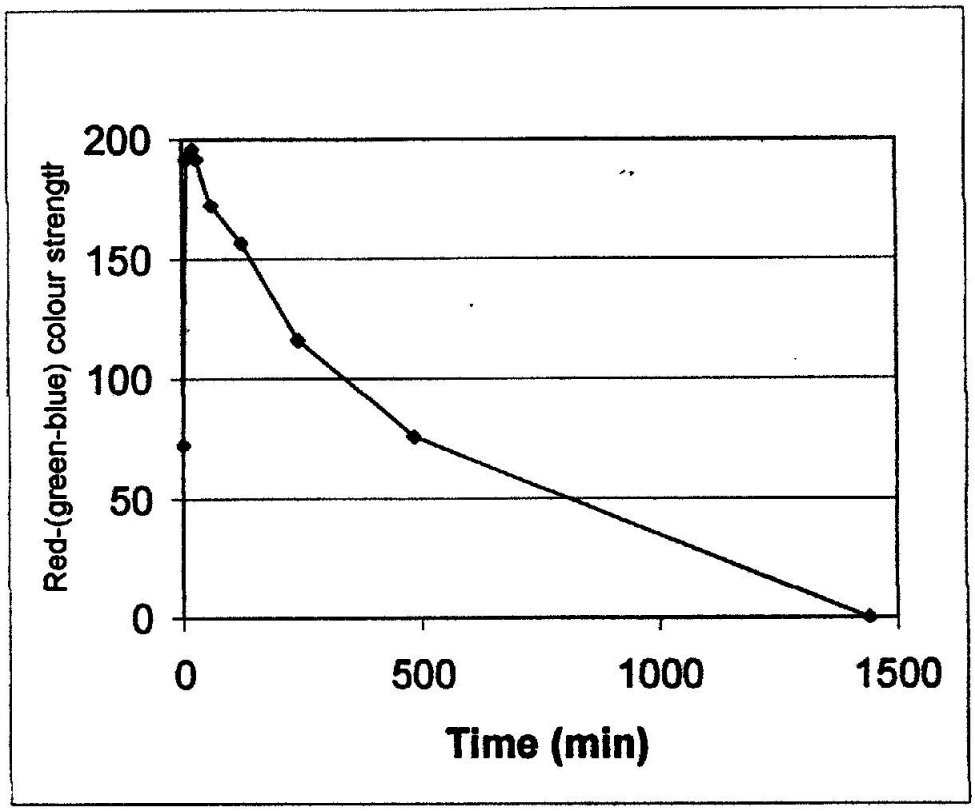

Fig. 1. Serum samples from a 500 kg mare dosed with 125 ml Oxyglobin® i.v. for a 16.25 g total dose of polymerized hemoglobin. Samples displayed a significant color change associated with this substance, and were photographed digitally, and sample pixel color values tested with Microsoft Image Composer version 1.5 (Microsoft Corporation). The plot shows variation in red color strength based on a red-green-blue model ranging from 0 to 255. Time points include 0, 10, 20, 30 min, then 1, 2, 4, 8 and 24 hr.

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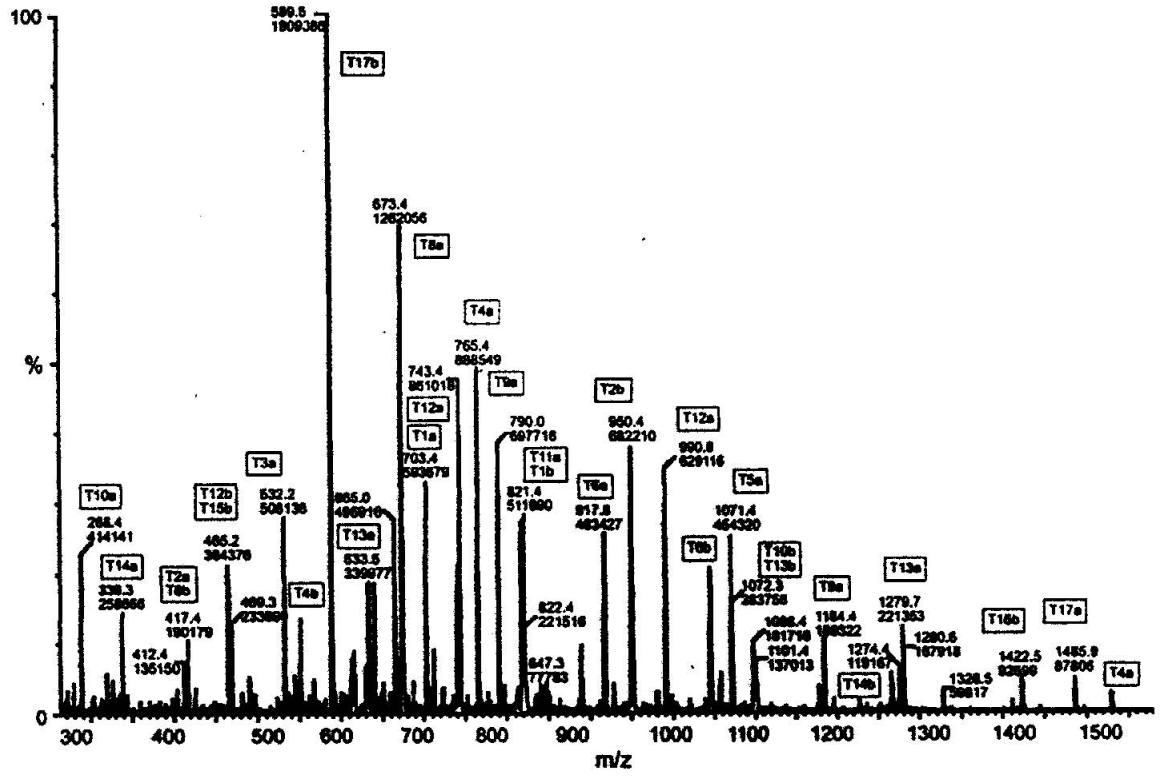

Fig 3: Bovine Hb standard (2 mg/ml) reacted with 20 µl dose of soluble trypsin endopeptidase, diluted to a protein concentration of 10 µg/ml in 0.05% formic acid: acetonitrile, 1:1, followed by direct infusion-ESI(+)MS analysis. Fragment labels indicate: T=trypsin, followed by fragment number (see Table 2), followed by peptide designation, a=alpha, b=beta.

Lens: 0.45 kVolts; Cone: 40 Volts; Skimmer Offset: 5 Volts; Skimmer: 0.6 Volts; RF Lens: 0.2 Volts; Source temp: 120°C; MS1 Ion Energy: 2.0 Volts; MS1 Ion Energy Ramp: 2.2 Volts; LM Resolution:14.3; HM Resolution: 14.3; Lens 5: 100 Volts; Lens 6: 5 Volts; Multiplier 1: 680 Volts.

RESULTS

Dosage of a 500 kg mare with one iv infusion container of Oxyglobin (125 ml bag containing 13 g/dl polymerised Hb) resulted in an obvious colour change in the animal's serum over time (Fig 1).

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Haemoglobin and Oxyglobin were compared spectrophotometrically in a Beckman UV-visible spectrometer and found to be basically indistinguishable in their absorption spectra in the visible region (data not shown). Absorption spectra disclosed maxima in the 420 and 544 nm regions, enabling selection of the 10-fold more sensitive 420 nm as an initial wavelength for direct SEC observation of standards. Chromatography of Hb standards was compared with that of Oxyglobin by using 420 nm detection and isocratic elution, resulting in chromatographic profiles such as those seen in Figure 2. Points of interest in this exercise include: 1) the single peak representing tetrameric Hb $\alpha_1\beta_2/\alpha_2\beta_1$ complexes from native Hb; 2) transformation of similar complexes into concatemers as seen in the Oxyglobin trace with over 3 additional higher mw peaks when haemolysis was absent; 3) presence of some interfering peaks in blank equine serum but with the lack of a Hb peak; and 4) the occurrence of

TABLE 1: Trypsin digestion of Hb alpha and beta chains, comparing equine and bovine types and showing fragments greater than 500 daltons, per Swiss Protein Database

Chain	Mw in daltons
Hbα (bovine)	2970.4, 2367.6, 1834.0, 1529.6, 1279.5, 1071.3, 817.9, 702.8, 672.8, 531.6
Hbα (equine)	3013.5, 2950.3, 1834.0, 1515.6, 1268.5, 1041.3, 817.9, 702.8, 561.6
Hbß (bovine)	2090.3, 1422.6, 1391.6, 1274.5, 1265.6, 1177.4, 1101.2, 1098.2, 1097.3, 950.1, 821.0, 739.8
Hbß (equine)	2000.2, 1802.0, 1426.6, 1391.6, 1358.4, 1274.5, 1265.6, 1149.4, 1126.2, 986.2, 889.0

TABLE 2a: Primary sequence of bovine Hb alpha chain (top), followed by listing of theoretical trypsin fragment sizes, their designations, sequences and +1, +2 and +3 projected m/z ion values

Fragment No.	Res No.	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]
T10	91–92	(K)LR(V)	287.36	288.37	144.69	96.80
T14	140–141	(K)YR(-)	337.38	338.39	169.70	113.47
T2	8–11	(K)GNVK(A)	416.48	417.49	209.25	139.83
T7	57–61	(K)GHGAK(V)	468.51	469.52	235.26	157.18
T3	12–16	(K)AAWGK(V)	531.61	532.62	266.81	178.21
T8	62–68	(K)VAAALTK(A)	672.82	673.83	337.42	225.28
T1	1–7	(-)VLSAADK(G)	702.81	703.81	352.41	235.28
T11	93–99	(R)VDPVNFK(L)	817.94	818.95	409.98	273.65
T5	32–40	(R)MFLSFPTTK(T)	1071.31	1072.31	536.66	358.11
T13	128–139	(K)FLANVSTVLTSK(Y)	1279.50	1280.51	640.76	427.51
T4	17–31	(K)VGGHAAEYGAEALER(M)	1529.63	1530.64	765.82	510.88
T6	41–56	(K)TYFPHFDLSHGSAQVK(G)	1834.02	1835.03	918.02	612.35
T9	69–90	(K)AVEHLDDLPGALSELSDLHAHK(L)	2367.60	2368.61	1184.81	790.21
T12	100–127	(K)LLSHSLLVTLASHLPSDFTPAVHASLDK(F)	2970.42	2971.43	1486.22	991.15

Hb alpha chain Bos taurus (bovine); Average mass = 15053.1949, monoisotopic mass = 15043.9042; N-terminus = H; C-terminus = OH

1 VLSAA DKGNV KAAWG KVGGH AAEYG AEALE RMFLS FPTTK TYFPH FDLSH GSAQV KGHGA KVAAA LTKAV
2 EHLDD LPGAL SELSD LHAHK LRVDP VNFKL LSHSL LVTLA SHLPS DFTPA VHASL DKFLA NVSTV LTSKY
141 R

Hb alpha chain Bos taurus (bovine); Trypsin:/K-\P /R-\P

TABLE 2b: Primary sequence of bovine Hb beta chain (top), followed by listing of theoretical trypsin fragment sizes, their designations, sequences and +1, +2 and +3 projected m/z ion values

Fragment No.	Res No.	Sequence	Theor (Bo)	[M+H]	[M+2H]	[M+3H]
T9	65–65	(K)K(V)	146.19	147.20	74.10	49.74
T7	59–60	(K)VK(A)	245.32	246.33	123.67	82.78
T3	17–18	(K)VK(V)	245.32	246.33	123.67	82.78
T18	144–145	(R)YH(-)	318.33	319.34	160.17	107.12
T8	61–64	(K)AHGK(K)	411.46	412.47	206.74	138.16
T15	116–119	(R)NFGK(E)	464.52	465.53	233.27	155.85
T11	76–81	(K)HLDDLK(G)	739.83	740.83	370.92	247.62
T1	1–7	(-)MLTAEEK(A)	820.96	821.97	411.49	274.66
T2	8–16	(K)AAVTAFWGK(V)	950.11	951.11	476.06	317.71
T10	66–75	(K)VLDSFSNGMK(H)	1097.26	1098.27	549.64	366.76
T13	95–103	(K)LHVDPENFK(L)	1098.22	1099.23	550.12	367.08
T4	19–29	(K)VDEVGGEALGR(L)	1101.18	1102.19	551.60	368.07
T17	132–143	(K)VVAGVANALAHR(Y)	1177.37	1178.38	589.69	393.47
T14	104–115	(K)LLGNVLVVLAR(N)	1265.61	1266.61	633.81	422.88
T5	30–39	(R)LLVVYPWTQR(F)	1274.53	1275.54	638.27	425.85
T12	82–94	(K)GTFAALSELHCDK(L)	1391.57	1392.58	696.79	464.86
T16	120–131	(K)EFTPVLQADFQK(V)	1422.60	1423.61	712.31	475.21
T6	40–58	(R)FFESFGDLSTADAVMNNK(V)	2090.30	2091.31	1046.16	697.77

Hb beta chain Bos taurus (bovine); Average mass = 15954.4238, monoisotopic mass = 15944.3102; N-terminus = H; C-terminus = OH

1 MLTAE EKAAV TAFWG KVKVD EVGGE ALFRL LVVYP WTQRF FESFG DLSTA DAVMN NPKVK AHGKK VLDSF
71 SNGMK HLDDL KGTFA ALSEL HCDKL HVDPE NFKLL GNVLV VVLAR NFGKE FTPVL QADFQ KVVAG VANAL
141 AHRYH

Hb beta chain Bos taurus (bovine); Trypsin:/K-\P /R-\P

TABLE 3: Candidate bovine Hb peaks to examine by ESI(+) MS/MS following tryptic digestion; middle and right sections indicate possible interferences by equine or bovine tryptic fragments, respectively, under worst-case conditions including incomplete digestion

Candidate confirmatory peptide	Measured		Equine	Equine		Bovine	Bovine	note
mw	m/z	ID, including charge	interferences seen on scan of list from +1 to +4 with 2 partials	m/z of possible interfering equine peak	see target or interference by MS?	interferences seen on scan of list from +1 to +4 with 2 partials	m/z of possible interfering bovine peak	
1177.4	589.3	T17ß+2	0		586	T4-5ß+4; T2-3ß+2	590.1; 589.3	**
1279.5	640.4	T13α+2	T6-7ß+1; T14-15ß+4	639.4; 640.1	638	T7-8ß+1	639.4	
2970.4	743.2	T12α+4	T6-7α+3; T5-6ß+3	743	744			
1529.6	765.4	T4α+2	T6-8ß+1	767.5	none	T7-9+1	767.5	*
2367.6	789.7	T9α+3	T11-12ß+3	791.8	791			*
2970.4	990.5	T12α+3	0		991.4	T1-3ß+2	990.5	**
2090.3	1045.5	T6ß+2	0		1044.3			*
1071.3	1071.6	T5α+1	T9-10α+3; T9-11ß+4	1073.6; 1071.9	none			
1279.5	1279.7	T13α+1	T14-15ß+2	1279.2	none			
1422.6	1422.7	T16ß+1	T11-13ß+2	1421.8	none			
2970.4	1485.3	T12α+2	0		1486.6			*
1529.6	1529.7	T4α+1	T12-14α+3	1528.7	none			

Note: no interferences with one another seen on examination of bovine tryptic fragments with charges of +1 to +3 and no partial fragments. * this fragment is probably a good candidate; **this fragment will probably work despite possible bovine interferences

Oxyglobin peaks in serum from an animal dosed with Oxyglobin. Both bovine and equine Hb standards made from dissolved crystalline material co-chromatographed with the native equine Hb, although tiny percentages of higher molecular weight inpurities were observed in these standards.

Confirmation of Oxyglobin dosing could take advantage of evolved differences between bovine and equine Hb protein primary sequences. As shown in Table 1, trypsin digestion gives both similar as well as distinguishable fragments when comparing bovine and equine proteins. Chances were considered good that the glutaraldehyde-cross-linked structure of Oxyglobin would be sufficiently random as to allow similar digestion with release of essentially intact bovine sequences distinguishable from native equine peptides that might occur in horse serum samples haemolysed during sample handling.

Figure 3 displays ESI(+) mass spectrometry of a bovine Hb standard reacted with soluble trypsin. Most of the fragments can be assigned as arising from the alpha (suffix 'a') or beta (suffix 'b') chain of Hb as shown. The expected fragments derive their name designations such as T14α from computer-simulated autodigestion via Biolynx software, a Micromass toolset used to analyse and manipulate protein or peptide sequences so as to facilitate the interpretation of data obtained from mass spectrometry. Table 2a lists the primary sequence of the bovine Hb alpha chain and its autodigest fragments, including predicted fragment m/z values assuming charges of +1, +2 or +3, whereas Table 2b does the same for the bovine Hb beta chain. If one takes into account that fragments may occur at several m/z values depending on relative charge states, that identity or close similarity of bovine and equine sequences could occur, and that trypsin may occasionally miscut, ie introduce too few cuts in a peptide sequence, then interference between fragments could confuse interpretation of full scan spectra of non-chromatographed material such as that seen in Figure 3. Based on comparison with trypsin digestion of equine Hb (data not shown), we are left with specific fragments that can be utilised for unambiguous confimation of Oxyglobin, and allowing some margin for error as summarised in Table 3. This lists bovine sequences in the left-most third and expected from Oxyglobin, along with measured values, their designations from Biolynx software, and potential interferences from one another; the middle third lists potential interferences from equine fragments under worst-case conditions including poor trypsin digestion; and the right third of the table lists similar

inteferences from other bovine sequences. As a result of this exhaustive analysis, the best candidates for Oxyglobin confirmation include the 2.970 kD T12α fragment, the 2.090 kD T6B fragment, the 2.368 kD T9α fragment, and the 1.530 kD T4α fragment. Tryptic digestion of Oxyglobin demonstrates that these fragments can be visualised by direct infusion ESI(+)MS/MS (data not shown), and experiments are under way to design HPLC chromatography for candidate fragments utilising microbore reverse phase columns.

Discussion

The prospects for visualisation of Oxyglobin concatemers by HPLC-SEC as a screening method are good, and depend upon careful analysis of chromatographic patterns as in Figure 2: however, owing to intense colorisation of dosed-horse serum, this may not be any better than direct examination and comparison to a colour chart, unless a more specific HPLC detector such as UV-diode array detector is introduced. Enhanced separation capabilities of capillary electrophoresis or capillary HPLC might alternatively enable differentiation by molecular weight.

It was our initial hope based on ESI(+)MS examination of bovine Hb Sigma standards at 10 µg/ml in 0.05% formic acid:acetonitrile 1:1, that the Quattro II MS could function as such a detector. Anticipation was heightened by transformation of full scan spectra, since calculated values of 15059.6 and 15960.1 mw (top of peak) compared well with anticipated sizes of 15,053 average mw (Hb alpha peptide) and 15,954 average mw (Hb beta peptide). However, full scan analysis of the randomly cross-linked Oxyglobin concatemers revealed a confusingly thick array of m/z fragments (data not shown). Protein digestion with endopeptidases could then be introduced as a means of simplifying the Oxyglobin-related peaks, and could be exploited as a confirmatory methodology after an initial colour-based or SEC-based screening method. Such endopeptidase digestion depends upon the derivation of Oxyglobin and related Hemopure from bovine Hb, and upon evolved sequence differences between bovine and equine alpha and beta Hb chains (Table 1).

Figure 3 demonstrates that endopeptidase digestion of bovine Hb provides interpretable fragments in the absence of chromatography, and separate digestion experiments have shown that similar fragments can be derived from Oxyglobin. In order to err on the side of worst-case digestion and protonation conditions, the authors considered situations in which multiple miscuts and charge states existed and could introduce fragments with m/z values corresponding exactly to ones considered specifically diagnostic for Oxyglobin, resulting in the information in Table 3. Although direct infusion experiments with analysis for specific m/z fragments and subsequent collision-induced dissociation for sequence information may eventually provide sufficient information for adequate Oxyglobin confirmation, we are currently studying the possibility of including a chromatographic component such as microbore HPLC with product ion monitoring by MS/MS (Covey 1996). Such techniques may not be as specific or sensitive as those utilising time-of-flight MS with accurate mass options, but should nonetheless be of sufficient sensitivity for a protein used in such high quantities.

Acknowledgements

This research was supported by the Kentucky Racing Commission, the National, Florida, Kentucky, Nebraska, Arkansas, Pennsylvania, Ohio, and Canadian Horsemen's Benevolent and Protective Associations, and Mrs John Hay Whitney. Thanks to Casey Nickerson for assistance in editing the manuscript.

Kentucky Agricultural Experiment Station Manuscript # 02-14-169 published with the approval of the Dean and Director, College of Agriculture and Kentucky Agricultural Experimental Station. Publication #300 from the Pharmacology and Experimental Therapeutics Laboratory, Department of Veterinary Science, University of Kentucky, Lexington, Kentucky.

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