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The Detection of Haemoglobin Based Oxygen Carriers

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Introduction

Blood substitutes are being developed by several companies as an alternative to blood transfusions because of the problems in obtaining sufficient whole blood for medical demands coupled with the difficulty in ensuring that the blood is not contaminated (Scott et al 1997). The contamination problem is particularly severe in those countries with a high incidence of HIV. The products being developed are designed to be used in operations and emergencies in place of donated blood. They do not and are not intended to carry out all the functions of whole blood but only make up volume expansion after blood loss and deliver oxygen to the tissues. There are two main types of blood substitute under development, those based on fluorocarbons and those based on some form of modified haemoglobin. The detection of perfluorocarbons has already been described (Mathurin et al 2001). It is clear from the abuse of erythropoietin (EPO) in endurance sports that some athletes will seek a performance advantage by using products which enhance the oxygen carrying capacity of their blood (Birkeland and Hemmersbach 1999). The addition of a blood substitute to a subject's normal blood would have just this effect. In addition the enhancement of oxygen carrying capacity would be immediate whereas with EPO a course of injections lasting at least two weeks is needed before the effect is obtained.

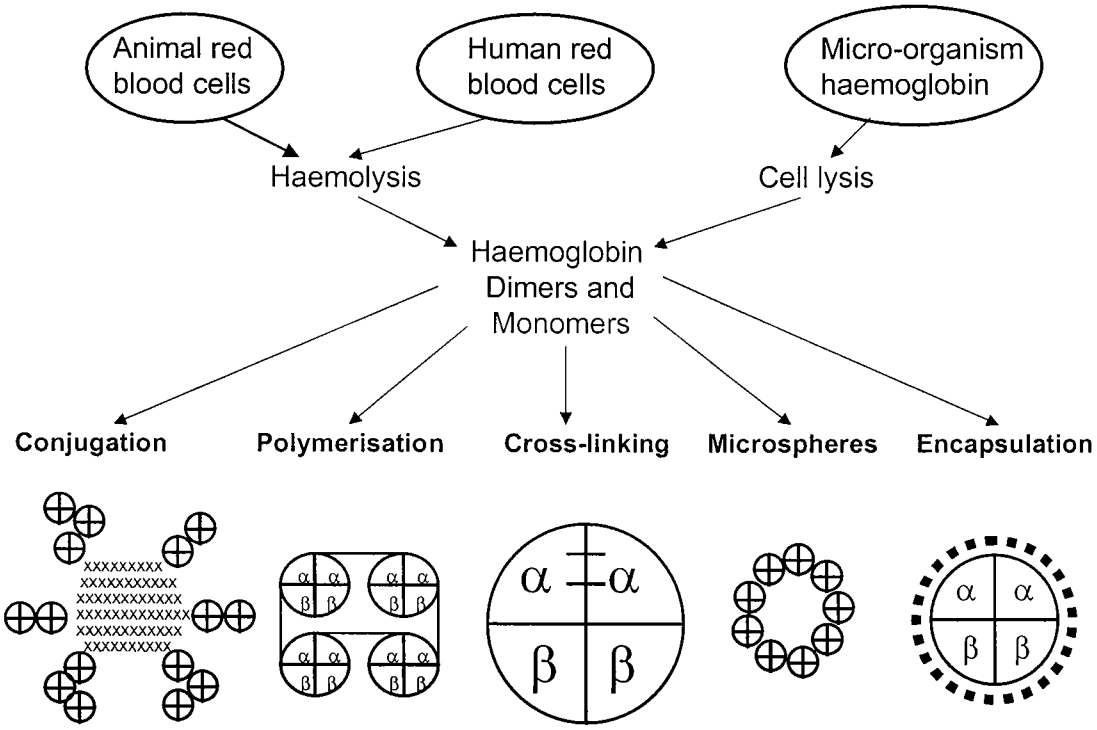
The most promising of the blood substitutes are those based on haemoglobin (Hb); the so called haemoglobin based oxygen carriers (HBOCs). Haemoglobin has a molecular weight of 64kDa with a tetrameric structure comprising two alpha and two beta polypeptide chains (Alayash 1999) and in normal blood is virtually all within the red blood cells. It has been known for some time that haemoglobin can also transport oxygen outside the red blood cell however there are three main reasons why unmodified haemoglobin cannot be used. These are:

1. Haemoglobin outside the red blood cell tends to break up into α/β heterodimers. These dimers are rapidly filtered by the kidney and are nephrotoxic in high concentration.
2. The oxygen affinity of free human haemoglobin is so high as to prevent it releasing oxygen to the tissues.
3. Haemoglobin infusions increase blood pressure as a result of vasoconstriction. This is due to binding of the haemoglobin to nitric oxide, which is important in controlling the vascular tone of blood vessels.

For these reasons various attempts have been made to modify haemoglobin so that it can safely and effectively transport oxygen in the plasma outside a red blood cell. The approaches used include various chemical modifications of human haemoglobin and animal haemoglobins, as well as the production of modified haemoglobins using

recombinant technology. Figure 1 shows some of the approaches that have been taken.

Figure 1
Haemoglobin based blood substitutes



It should be possible to overcome all three problems associated with free haemoglobin by the use of appropriately modified recombinant haemoglobin. However this has not yet been achieved. Most of the research effort has been expended in chemical modification of human and bovine haemoglobin to overcome the problems associated with free haemoglobin in the plasma. Table 1 lists some of the products under commercial development and their stage of development in 2001.

Table 1 Development Stages of some HBOCs

Product	*Manufacturer*	*Modifications*	*Class*	*Clinical Trial Phase*
PHP	Apex Biocience	Pyridoxylated human Hb conjugated to polyoxyethylene	Conjugated Hb tetramers	1-2
PEG-Haemoglobin	Enzon	Bovine Hb conjugated to polyethylene glycol (PEG)	Conjugated Hb tetramers	2
Polyheme	Northfield Laboratories	Gluteraldehyde-polymerised pyridoxylated human Hb	Polymerised Hb	3

Hemopure	Biopure	Gluteraldehyde-polymerised bovine Hb	Polymerised Hb	3
Hemolink	Hemosol	α-Raffinose polymerised human Hb	Polymerised Hb	3
Optro	Somatogen	Recombinant di-alpha human Hb	Recombinant / intra cross-linked Hb	Stopped clinical trials in 1998 due to high blood pressure concerns
HemAssist	Baxter	Human Hb internally cross-linked with bis(3,5-dibromosalicyl) fumarate (DBBF)	Intra cross-linked Hb	Stopped clinical trials in 1998 due to high blood pressure concerns

It can be seen that the products closest to human application are those based on polymerised haemoglobin. Polymerisation overcomes both the toxicity due to dissociation and the vasoconstriction as the higher molecular weight species are stable and also cannot cross the endothelial cell barrier to react with nitric oxide. The problem of high oxygen affinity with free human haemoglobin can be overcome by modification using pyridoxal phosphate (Bunn 1995). However if bovine rather than human haemoglobin is used as the source then the oxygen affinity is suitable. Thus by polymerising bovine haemoglobin the potential exists for a safe and effective oxygen carrier. This is the approach used by the Biopure corporation in the preparation of Hemopure. The properties of Hemopure compared to whole blood are shown in Table 2 where the obvious advantages regarding useful life and storage conditions can be seen.

Table 2 Comparison of Hemopure and red blood cells

Characteristics	Hemopure	Transfused RBCs
Haemoglobin (g/L)	120-140	100-150
Half-life in humans	1 day	> 1 month
Storage	Room temperature	Refrigerated
Shelf life	At least one year	35-42 days
Preparation	Ready to use	Requires testing, typing and cross-matching
Compatibility	Universal	Type specific
Effectiveness	Immediate oxygen delivery	Dependent on length of storage
Purity	Processed to remove infectious agents	Tested and screened for infectious agents
Raw material	Bovine Hb – abundant, controlled source	Human blood – limited availability

The aim of this research was to develop screening and confirmatory methods which would be applicable to any HBOC based on polymerised haemoglobin.

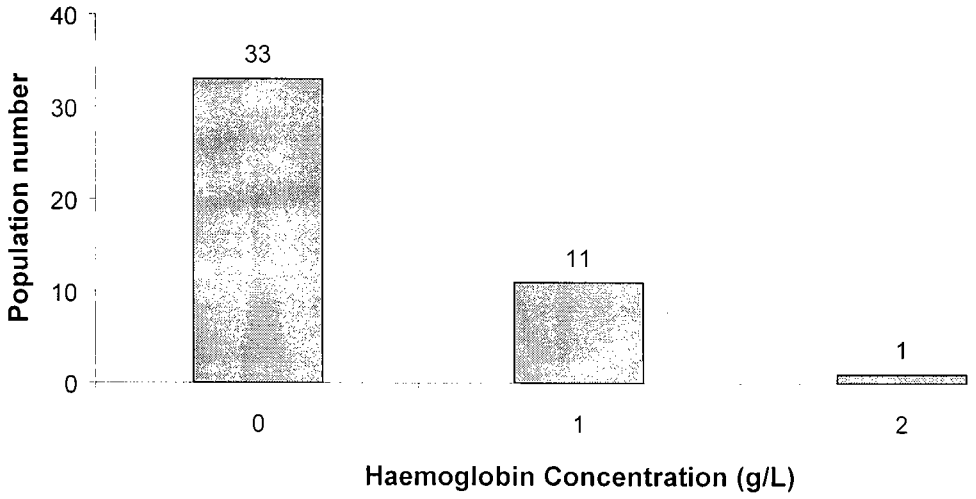
Experimental

The HBOCs Oxyglobin and Hemopure were kindly supplied by the Biopure Corporation USA. Oxyglobin is a veterinary product approved for use in dogs in the USA whilst the human product Hemopure is approved for use in South Africa. The development of the test for HBOCs in blood was based around these products. The blood measurements were made using an Advia 120 (Bayer Diagnostics USA). The HPLC used was an Alliance 2690 with 996 diode array detector using Millenium software (Waters USA). The LC-MS used was a Finnigan MAT 900 with API III ESI interface (Thermo Finnigan, Bremen)..

Results and Discussion

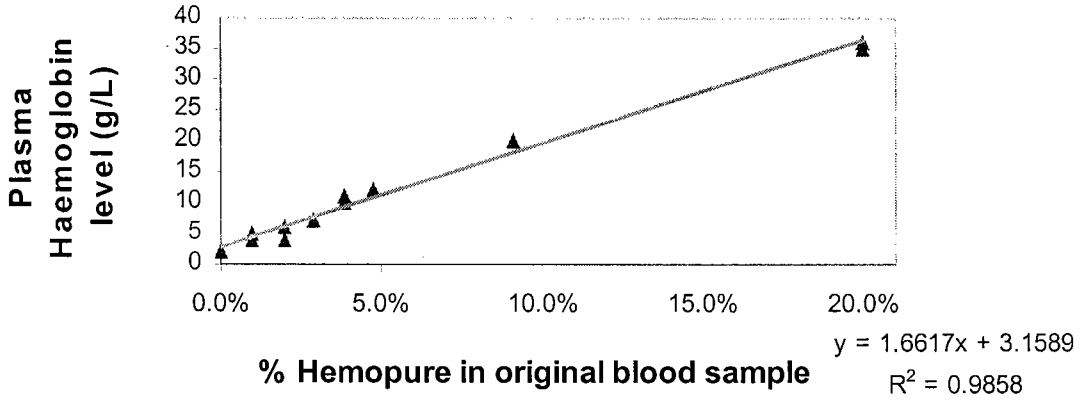
The screening method was based on the detection of extracellular haemoglobin. The concentration of haemoglobin in plasma is normally low and hence the detection of significant amounts of haemoglobin in plasma could either come from haemolysis of the red cells or from the presence of an HBOC. In order to obtain an estimate of the extent and frequency of haemolysis in normal blood collections, plasma was prepared from the blood of 45 athletes. The samples were collected as part of the Australian Sports Drug Agency's routine anti-doping program. The results are shown in Figure 3. It can be seen that in 73% of the subjects no haemoglobin was detected by the Advia 120. Only one subject had a plasma haemoglobin concentration above 1 g/L.

Figure 2 Population Distribution in Athletes of Plasma Hb Concentrations



In order to estimate what level of Hemopure administration could be detected by measuring plasma haemoglobin fresh whole blood was spiked with varying concentrations of Hemopure and the plasma haemoglobin levels measured. The results are shown in Figure 3.

Figure 3 Spiking of hemopure into whole blood

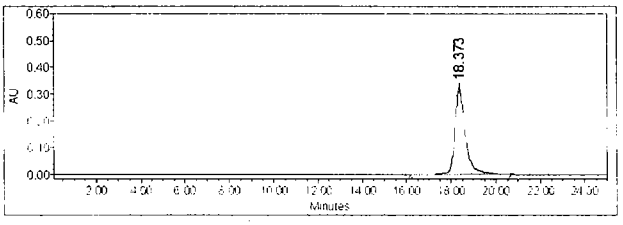


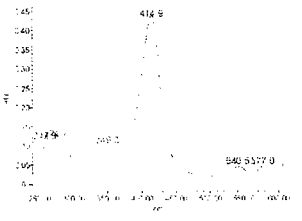
The infusion of one unit (500mL) of Hemopure by an individual would correspond to approximately 10% Hemopure and thus give a plasma haemoglobin level of nearly 20 g/L. A screen based on investigating samples with a plasma haemoglobin concentration greater than 1 g/L would have a false positive rate of approximately 2% based on the data in Figure 2. However such a screen would clearly detect all users of HBOCs for some days after a normal infusion, based on the half-life of Hemopure.

To confirm that the plasma haemoglobin detected in the screen is from an HBOC and not from the haemolysis of red blood cells it is necessary to show that the haemoglobin detected has been cross-linked and/or polymerised. In the case of Hemopure and the other two HBOCs which are in stage 3 clinical trials the haemoglobin is both cross-linked and polymerised. Thus natural haemoglobin would have a molecular weight of 64kDa or 32kDa (due to the dissociation of the tetramer to dimers) whilst a polymerised haemoglobin would have an average molecular weight above 128kDa. Size exclusion chromatography (SEC) with diode array detection was used to carry out the separation (Hughes et al 1996). The column used was a Biosil SEC 250 (Bio-Rad) with a mobile phase of water containing 0.75M magnesium chloride, 0.05M bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane, and 0.1mM EDTA. The flow rate was 0.5mL/min and the column effluent was monitored at 424nm. The results obtained when a sample of human haemoglobin and a sample of Hemopure were analysed using SEC are shown in Figure 4. Here it can be seen that Hemopure contains very little material with a molecular weight corresponding to that of haemoglobin with most of it eluting earlier and hence of higher molecular weight. However the absorbance spectra of the material detected at 424nm was very similar for both samples indicating that the material in Hemopure was of haemoglobin origin. In order to determine whether SEC was capable of detecting Hemopure in plasma, samples of whole blood were spiked with varying levels of Hemopure. Figure 5 shows the results obtained at a spiking level of 10% in plasma. The peaks corresponding to polymerised haemoglobin and their confirming absorbance spectra were still evident at a spiking level of 1%.

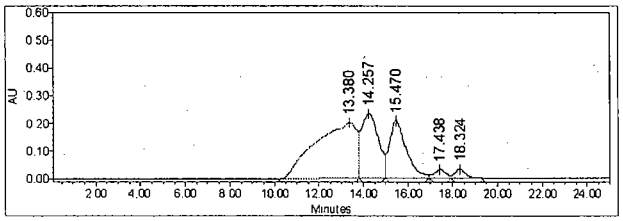
Figure 4 Size-exclusion Chromatograms and Spectra

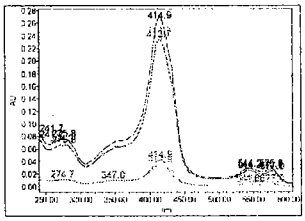
Human Hb Standard





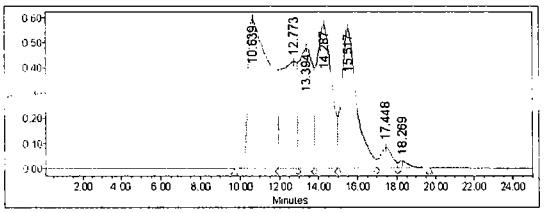
Hemopure Standard





- Each peak seen in Hemopure has an identical emission spectra to both each other and the Hb standard.

Figure 5 HemopureSpiked in Plasma



10% Hemopure (13 g/L) in plasma

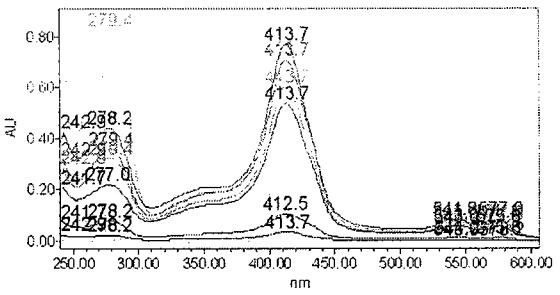


	Image	Retention Time	Lambda Max.	Max. Absorbance	Source Name
1		10.630	413.7	0.76907	10% Hemopure in Plasma
2		12.780	413.7	0.53563	10% Hemopure in Plasma
3		13.397	413.7	0.60244	10% Hemopure in Plasma
4		14.280	279.4	0.86685	10% Hemopure in Plasma
5		15.513	413.7	0.70563	10% Hemopure in Plasma
6		17.447	412.5	0.10257	10% Hemopure in Plasma
7		18.263	413.7	0.03405	10% Hemopure in Plasma

Overlay of spectra exhibited by the peaks observed in the 10% Hemopure in plasma

- **It can be seen that Hemopure spiked in plasma still displays the characteristic peaks**
- **The overlays of the spectra from these peaks demonstrate that they have almost identical spectra to the human Hb standard. This was still evident in Hemopure spiked in plasma at a concentration of 1g/L**

Whilst the confirmation by SEC with the detection of material having an absorbance spectrum virtually identical to that of haemoglobin and a molecular weight significantly greater than 64k is sufficient to prove the presence of a polymerised haemoglobin, it does not enable the identification of which particular form of polymerised haemoglobin is present. To do this one needs mass spectral analysis to distinguish between the different forms of haemoglobin and the different cross linking agents. The experiments carried out in this study were preliminary investigations into distinguishing cross-linked Hb from non cross-linked Hb using enzymatic digestion, with trypsin, of the Hb and determination of the molecular masses of the resulting peptides using ESI/MS. The trypsin digests were directly infused without

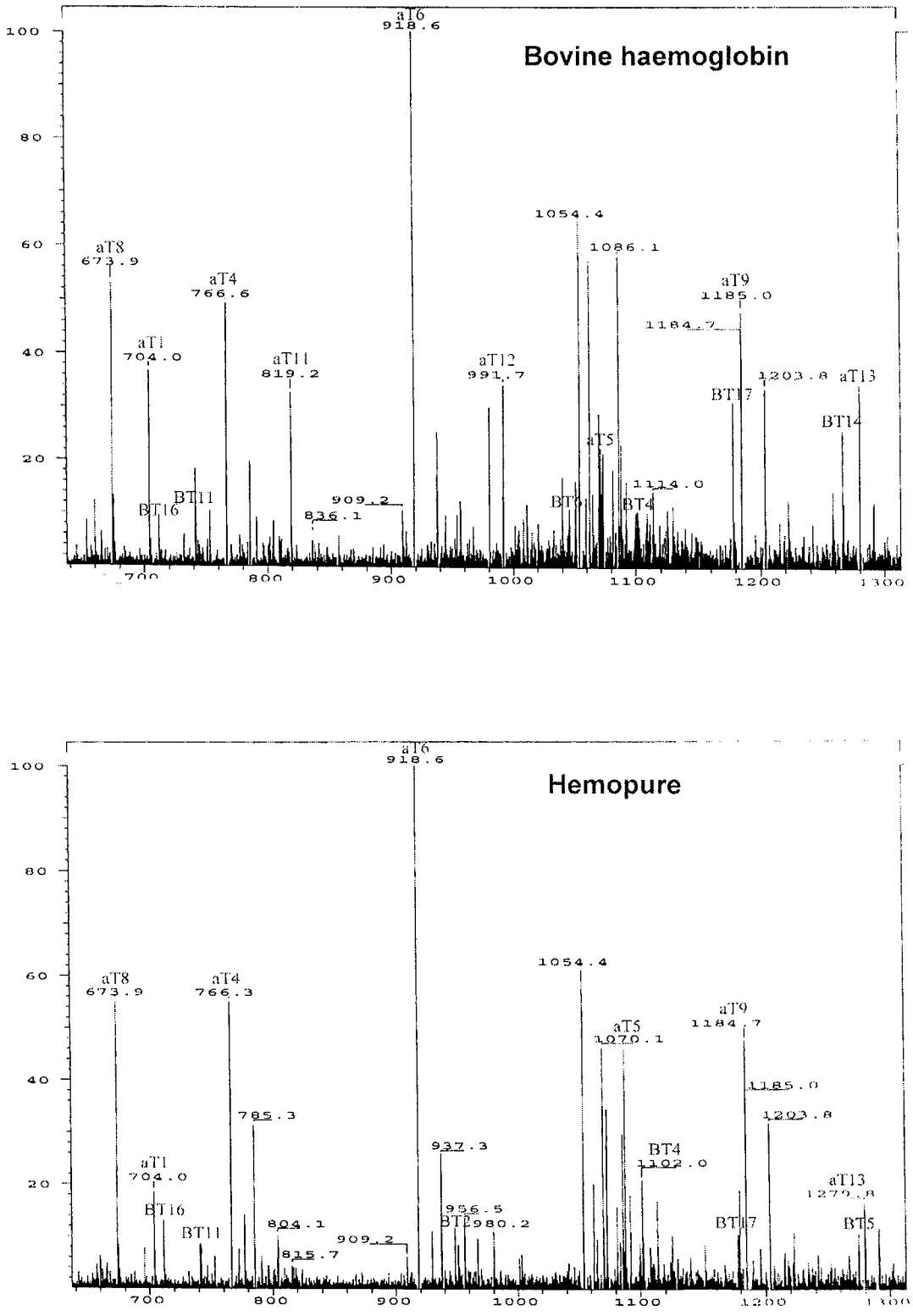
chromatographic separation. The experiments were performed with Hemopure and with bovine haemoglobin. The aim was to detect differences confirming cross-linking of the haemoglobin present. Whilst their quaternary structure is very similar, human and bovine Hb are only 85% homologous in amino acid sequence and hence the peptide fragments are very different. Thus the detection of peptides from bovine haemoglobin would be proof of the presence of an HBOC based on bovine haemoglobin such as Hemopure. However the intention was to detect differences that could be used to confirm cross-linking so that an HBOC based on human haemoglobin could also be detected. Trypsin specifically hydrolyses at the carboxylic sides of lysine and arginine. Glutaraldehyde, the cross-linking agent of Hemopure, reacts primarily with the amino group of lysine and hence trypsin would not cleave at a site containing this cross-link

Table 3 shows the peptide fragments expected from a trypsin digest of the alpha chain of bovine haemoglobin. The actual mass spectra obtained from bovine haemoglobin and Hemopure digests are shown in Figure 6. The spectra are similar confirming that Hemopure is made from bovine haemoglobin. However there are two significant differences namely the absence of the masses 819 and 992 in the Hemopure spectrum. The loss of these two peptide fragments means that cleavage did not occur at the lysine at position 99 in the alpha chain of bovine haemoglobin. The reason for this is that the glutaraldehyde used to cross-link the bovine haemoglobin does so by reacting with the lysine at position 99 on the alpha chain. Hence the trypsin can no longer cleave at this point. The spectrum of Hemopure confirms that whilst the material originates from bovine haemoglobin it is not natural having been chemically modified at position 99 on the alpha chain. This technique should be able to detect cross-linking in any haemoglobin based material.

Table 3 Predicted peptide sequences and the corresponding ions (ESI/MS) expected from a trypsin digest of the alpha chain of bovine haemoglobin.

Mass $[M+H]^+$	*Mass* $[M+2H]^{2+}$	*Mass* $[M+3H]^{3+}$	*Mass* $[M+4H]^{4+}$	*Position*	*Code*	*Peptide sequence*
703.398	-	-	-	1-7	aT1	VLSAADK
417.246	-	-	-	8-11	aT2	GNVK
532.288	-	-	-	12-16	aT3	AAWGK
1529.734	765.371	510.583	-	17-31	aT4	VGGHAAEYGAEALER
1071.554	536.281	-	-	32-40	aT5	MFLSFPTTK
1833.892	917.450	611.969	-	41-56	aT6	TYFPHFDLSHGSAQVK
469.252	-	-	-	57-61	aT7	GHGAK
673.424	-	-	-	62-68	aT8	VAAALTK
2367.194	1184.101	789.737	592.555	69-90	aT9	AVEHLDDLPGALSELSDLHA HK
288.203	-	-	-	91-92	aT10	LR
818.441	-	-	-	93-99	aT11	VDPVNFK
2969.609	1485.309	990.542	743.158	100-127	aT12	LLSHSLLVTLASHLPSDFTP AVHASLDK
1279.726	640.367	-	-	128-139	aT13	FLANVSTVLTSK
338.182	-	-	-	140-141	aT14	YR

Figure 6 ESI Mass Spectra of Bovine Haemoglobin and Hemopure following Tryptic Digestion.



Conclusions

The measurement of the haemoglobin content of plasma is a simple method for the detection of HBOCs in blood. From our studies on blood collected from elite athletes plasma haemoglobin concentrations above 1 g/L are infrequent. Where the plasma haemoglobin concentration is above 1 g/L a method is needed to distinguish haemolysis from the presence of an HBOC. Spiking trials with Hemopure in plasma have shown that size exclusion chromatography with diode array detection can readily distinguish free haemoglobin from the polymerised material present in Hemopure. ESI/MS of trypsin digests of Hemopure has shown that it is possible both to identify the source of the haemoglobin used and to show that it has been chemically modified. Future investigations will focus on identifying Hemopure in plasma both from spiking trials and from subjects who have been administered Hemopure. The methods developed using Hemopure will then be applied to other HBOCs based on polymerised human haemoglobin.

References

Alayash, A.I. (1999) Nature Biotechnology 17: 545-549.

Birkeland, K.I. and Hemmersbach, P. (1999) Sports Medicine 28: 25-33.

Bunn, H.F. (1995) Transfusion Clinique et Biologique 6: 433-439.

Hughes Jr, G.S., Antal, E.J., Locker, P.J., Francom, S.F., Adams, W.J. and Jacobs Jr, E.E. (1996) Critical Care Medicine 27: 756-764.

Mathurin, J.C., de Ceaurriz, J., Audran, M. and Krafft, M.P. (2001) Biomed. Chromatogr. 15(7): 443-451.

Scott, M.G., Kucik, D.F., Goodnough, L.T. and Monk, T.G. (1997) Clinical Chemistry 43: 1724-1731.