Methodologies for Detection of Hemoglobin-Based Oxygen Carriers

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Abstract

Blood substitutes based on hemoglobin or hemoglobin-based oxygen carriers (HBOCs) are oxygen-carrying therapeutic agents developed for use in operations and emergencies in place of donated blood. Increased oxygen-carrying capacity through the use of blood substitutes could help elite athletes to lengthen endurance capacity and improve their performance. As blood substitutes become more readily available, it is essential that a qualitative detection method for their abuse in sport is available. Ideally, such a method would be simple and inexpensive. This study investigates methods that could be used as screening procedures to easily detect HBOCs in plasma and develops tests that can unequivocally confirm their presence. The investigation into the screening method indicates that the direct visual screening of plasma discoloration is the most appropriate with detection limits of less than 1% HBOC in plasma. Two methods are shown to confirm the presence of exogenous hemoglobin in plasma samples, size-exclusion chromatography with photodiode array detection and high-performance liquid chromatography analysis of enzymatic digests with detection by electrospray mass spectrometry. This work emphasizes the need for cooperation between drug developers and sports testing laboratories to ensure that methods for the detection of putative doping agents are available prior to product release.

Introduction

The desire for athletes to excel has led some to misuse drugs that have been developed to alleviate suffering and disease. A recent example is the use of recombinant human erythropoietin (EPO) by endurance athletes to enhance their aerobic performance (1). Although there is now a method to detect doping with recombinant EPO (2), the method was not applied widely until more than 10 years had elapsed since recombinant EPO was commercially available. It is obviously desirable to have methods available to detect drugs that may be abused in sport as soon as possible, after they are approved for therapeutic use.

Blood substitutes are products that have the potential to be abused in sport and have been prohibited by the International Olympic Committee since January 2000. They are being devel-

oped by several companies as an alternative to blood transfusions because of the difficulty of obtaining adequate supplies of uncontaminated whole blood (3). They do not carry out all the functions of whole blood, but they do deliver oxygen to the tissues and make up plasma volume after blood loss (4). In fact, one product has been shown to be more efficient than blood in oxygen delivery and thus has a real potential for doping in sport (5).

The blood substitutes that are closest to commercial availability are those based on hemoglobin, either human or animal, that has been chemically treated to induce cross-linking or polymerization (or both). This exogenous hemoglobin is not contained within red blood cells but circulates in the plasma. The chemical modification is essential to prevent the toxic effects associated with high levels of extracellular hemoglobin in the blood (6). Table I gives some details of the three hemoglobin-based oxgen carriers (HBOCs) that are currently in Phase 3 clinical trials in the United States. Hemopure, which is based on bovine hemoglobin, is the only product that has been approved for human use and then only in South Africa in limited circumstances (7). We have used this product as a model for the development of methods to detect HBOCs in blood.

In normal blood, oxygen is transported by the hemoglobin that is held within red blood cells. The hemoglobin is maintained in its native tetrameric structure (two alpha and two beta subunits) within the erythrocytes (8), and there is very little free hemoglobin in the plasma. The HBOCs function by transporting oxygen using modified hemoglobins that are circulating in the plasma. Thus, the presence of significant concentrations of hemoglobin, determined by the standard cyanmethemoglobin method, would always be observed in the plasma of any athlete who had infused an HBOC. Although this observation is a necessary condition for deciding if doping has occurred, it is not a sufficient condition. This is attributable to other possible causes for elevated plasma hemoglobin, particularly red cell hemolysis, which can result from extreme exercise or during sample collection. A confirmation method is needed that can distinguish free hemoglobin derived from the subject's own red blood cells to that which is caused by an HBOC. The common difference between native hemoglobin and the hemoglobin present in the HBOCs (listed in Table I) is that the hemoglobin in HBOCs has been chemically modified

and polymerized, with the result that the molecular weight has risen from approximately 64 kDa in native tetrameric human hemoglobin to values well in excess of 100 kDa for the HBOCs.

Size-exclusion chromatography (SEC) is a technique that separates compounds of large molecular weight, such as polymers and proteins, primarily on the basis of size, with larger compounds eluting earlier than small ones. The technique has been used for investigations into chemically modified hemoglobins (9). As hemoglobin has an intense red color, it is possible by using photodiode array (PDA) detection to both selectively detect hemoglobins in the presence of other plasma proteins and to obtain their characteristic (UV–vis) spectra. Retention times from liquid chromatography coupled with matching (UV–vis) spectra are accepted as proof of compound identity in the detection of residues in food, live animals, and animal products (10).

A second confirmation method is based on measuring the chemical differences that exist between native hemoglobins and polymerized hemoglobins using electrospray ionization (ESI) mass spectrometry (MS). The identification and characterization of proteins by the analysis of enzymatic digests using ESI–MS is a well-established technique. It has already been shown that Hemopure, which is a polymerized bovine hemoglobin, can be readily distinguished from native bovine hemoglobin using direct infusion ESI–MS of a tryptic digest. (11). It should be possible to apply similar techniques to detect the differences between polymerized and native human hemoglobins to confirm the presence of the HBOCs Polyheme and Hemolink. Because Hemopure is bovine in origin, and there are significant differences in the amino acid sequences between bovine and human hemoglobin, the detection of bovine sequences in human plasma would be proof of doping. A method for doing so using ESI-MS–MS has recently been published (12).

Experimental

Materials and chemicals

All reagents were of analytical-reagent or high-performance liquid chromatography (HPLC) grade or better. Water was from a Milli-Q water purification system (Millipore, Sydney, Australia). Magnesium chloride hexahydrate certified American Chemical Society grade, and bis tris enzyme grade were from Research Organics (Cleveland, OH). Ethylenediaminetetraacetic acid disodium salt (EDTA), dihydrate was from Merck (Kilsyth, Australia). Human hemoglobin standard and bovine hemoglobin standard were acquired from Sigma-Aldrich (St. Louis, MO). Hemopure and Oxyglobin was from Biopure Corp. (Cambridge, MA). Hemopure contains 130 g/L of polymerized hemoglobin. Formic acid was 98%. Trypsin TPCK was from Sigma-Aldrich. Human plasma from subjects administered Hemopure was provided by the Biopure Corp. from studies performed. Blank plasma was obtained from local volunteers.

HPLC analysis

The HPLC–PDA system used was a Waters Alliance 2695 HPLC system coupled to a 996 PDA detector (Waters, Milford, MA). Samples were analyzed using Waters Millennium Chromatography Manager software.

The HPLC column was a Bio-Sil SEC-250 Gel Filtration 300 × 7.8 mm (Bio-Rad, Hercules, CA) protected by a Security-Guard GFC-3000 4- × 3-mm guard column (Phenomenex, Sydney Australia). The injection volume was 20 µL. The mobile phase consisted of 152.5 g/L magnesium chloride hexahydrate, 10.45 g/L bis-tris, and 0.037 g/L EDTA dihydrate in water. The pH was adjusted to 6.5 with hydrochloric acid (1M) and the mobile phase was filtered each day before use. The flow rate was 0.5 mL/min with detection at 280 nm to 610 nm.

LC–MS analysis

The LC–MS system was a Waters Alliance 2795 separations module coupled to a Micromass Quattro micro triple stage quadrupole MS with a Z-spray electrospray interface. The HPLC column was a Phenomenex Jupiter 4-µm Proteo 90 A (150 × 2.1 mm) protected by a Phenomenex Security Guard Max-RP 4- × 2-mm guard column. The injection volume was 10 µL. The mobile phase was acetonitrile (ACN) in water containing a constant 0.2% formic acid. Initially, the composition was 5% ACN, which was maintained for 5 min before increasing linearly to 40% ACN at 60 min, and then to 90% ACN at 80 min. The equilibration time between runs was 10 min. The flow rate was 0.2 mL/min.

The MS parameters were as follows: the ESI capillary was at 3.5 kV, the cone voltage at 30 V, the source temperature at 115°C, the desolvation temperature was 180°C, the cone gas flow was 60 L/h, and the desolvation gas flow 560 L/h. The scan range was from 400 to 1,500 amu with a cycle time of 2.1 s. In MS–MS mode the collision energy was 30 eV using argon with a pressure of 3×10^{-3} mbar.

Sample preparation

Plasma was prepared from whole blood by centrifugation at 3,500 rpm for 15 min using a Heraeus Sepatech Labofuge 200 centrifuge. The blood was collected by venepuncture into EDTA K3 anticoagulant and stored at 4°C until analysis.

Tryptic digests were prepared by taking 0.1 mL of sample (Hemopure or plasma) and denaturing by boiling at 100°C for 5 min. Ten microliters of 0.5M NH_4HCO_3 was added to give a concentration of circa 50mM NH_4HCO_3. Twenty micrograms of trypsin was added to give a substrate-to-enzyme ratio of approximately 50:1 (w/w).

Table I. Polymerized Hemoglobins Currently in Phase 3 Clinical Trials in the United States.

Product	Manufacturer	Modifications
Hemopure	Biopure	Glutaraldehyde-polymerized bovine Hb
Hemolink	Hemosol	O-Raffinose polymerized human Hb
PolyHeme	Northfield Laboratories	Glutaraldehyde-polymerized, pyridoxylated human Hb

The digestion was carried out at 37°C for 4.5 h and halted by adding 20 µL of 0.5M HCl.

Results and Discussion

It is relatively simple to determine whether there are significant concentrations of extracellular hemoglobin in blood. The blood is centrifuged and the resulting plasma is examined. The concentration of hemoglobin may be measured by any hematology analyzer, typically using the cyanmethemoglobin method (13). Using such methodology, it has been found that approximately 98% of the 300 blood samples arriving at our laboratory have plasma hemoglobin concentrations of 1 g/L or below, with most having no measurable hemoglobin. It was also shown by spiking Hemopure into plasma that the measured plasma hemoglobin concentrations increased linearly with added Hemopure with a limit of detection of less than 1 g/L (11).

During this work it was noted that merely observing the color of the separated plasma was a good guide to the presence of and concentration of hemoglobin. Plasma is normally a pale yellow color and the presence of hemoglobin from hemolysis or the presence of an HBOC such as Hemopure causes the color to change to red/burgundy. A black and white rendition of the color chart prepared from plasma with no hemoglobin, plasma from lysed blood, and plasma prepared from blood spiked with varying levels of Hemopure is shown in Figure 1.

The intensity of the shading in Figure 1 shows that plasma prepared from blood spiked with 0.6% Hemopure can be readily detected. This equates to a concentration of approximately 1.6 g/L of hemoglobin in plasma (the plasma volume is typically close to half that of the total blood volume).

Administration of Hemopure at its lowest recommended dose of 250 mL would result in a plasma hemoglobin concentration of approximately 13 g/L in an adult male. As the half life of Hemopure in humans is approximately 24 h, this means that a screening test with a cut-off of 2 g/L would detect Hemopure doping for up to 3 days after its use. Because its use would be at competition with infusion just prior to the event, this detection period is reasonable for doping detection purposes. We have found that concentrations of plasma hemoglobin greater than 2 g/L are infrequent, and a simple visual inspection of the plasma is a sensitive test for the presence of HBOCs. This screening procedure involving the visual inspection of plasma samples prepared from whole blood has been in use in our laboratory for over 1 year. Of the more than 500 samples collected in this period, only one has had a plasma hemoglobin concentration greater than 2 g/L because of hemolysis.

In the event that a sample is observed with a plasma hemoglobin concentration greater than 2 g/L, a definitive test is needed to distinguish between hemolysis and HBOC abuse. The first confirmation procedure developed was based on the use of SEC to separate native hemoglobin from the polymerized hemoglobin that would be present in an HBOC. The method used was based on a published method (14). As can be seen in Figure 2, plasma from lysed blood and Hemopure spiked into plasma are clearly resolved by the column enabling hemolyzed blood to be readily distinguished from a sample containing a polymerized hemoglobin. The presence of many other proteins in the plasma can be seen from the 280-nm trace in Figure 2. However, by monitoring at 410 nm, predominantly hemoglobin-containing peaks are observed.

Under the conditions used for this analysis, native hemoglobin breaks down to its dimeric form with a molecular weight of 32 kDa and elutes at 18.7 min. There is no difference in the retention times of human and bovine hemoglobins. Cross-linked bovine hemoglobin (64 kDa) elutes at 17.8 min and the polymeric dimer (128 kDa) elutes at 15.7 min. The UV–vis spectrum can be obtained from the relevant peaks to confirm that the material being detected at the high molecular weights is derived from hemoglobin. Figure 3 shows the chromatogram obtained from a plasma sample taken from a human subject given Hemopure. Also shown is the close match between the spectra taken from one of the Hemopure peaks (15.7 min) and the peak caused by native human hemoglobin (18.7 min).

Spiking trials have shown that Hemopure is detectable in plasma at concentrations down to 2 g/L, although at this level the spectra do not match those of native hemoglobin because of interference below 310 nm from other plasma proteins. The lowest concentration of Hemopure that can be expected to
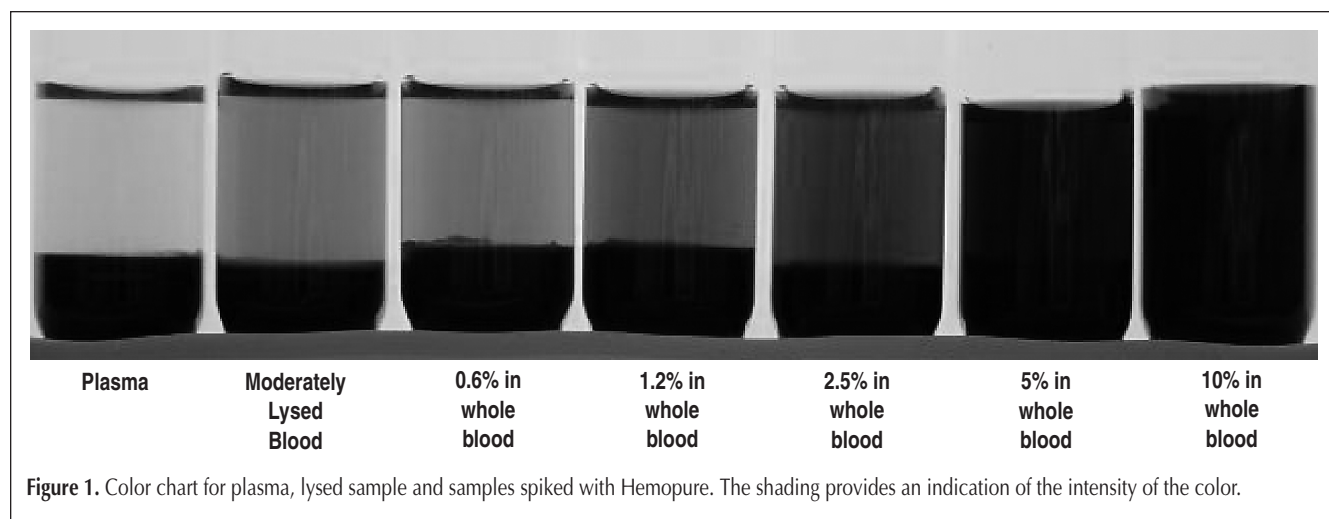

| Plasma | Moderately Lysed Blood | 0.6% in whole blood | 1.2% in whole blood | 2.5% in whole blood | 5% in whole blood | 10% in whole blood |

Figure 1. Color chart for plasma, lysed sample and samples spiked with Hemopure. The shading provides an indication of the intensity of the color.

give acceptable library matches is approximately 5 g/L. It was for this reason that an additional confirmatory method was evaluated.

In sports drug testing, mass spectral confirmation is required for all banned substances other than large biomolecules such as the peptide hormones. As Hemopure is derived from bovine hemoglobin, which has significant differences in the peptide sequences of the alpha and beta subunits compared to human hemoglobin, the detection of nonhuman hemoglobin sequences in human plasma would be proof of doping. Such an approach using digestion with trypsin and analysis of the resulting peptide sequences using LC–MS–MS has recently been published (12). Our initial approach was to look for differences between hemoglobin and polymerized hemoglobin using LC–MS of tryptic digests so that the method could be applied to any HBOC based on polymerized hemoglobin, not just HBOCs such as Hemopure, which use bovine hemoglobin.

We have also developed an LC–MS–MS method to detect the bovine specific sequences found in Hemopure and shown that the method works not only in spiked plasma samples but also in plasma samples from subjects who have been infused with Hemopure. The method is based on the differences in the peptide sequences expected from tryptic digestion of hemoglobin. The 141 amino acid alpha chains of human and bovine hemoglobin are shown in Figure 4. Also shown are the peptides that would result from tryptic digestion. Trypsin cleaves the C-terminal peptide bonds of arginine and lysine except those followed by proline. It can be seen that there are large regions of identical amino acid sequences with some small but significant differences in other regions.

The peptides aT3 (amino acids 12 to 16) and aT5 (32 to 40) are common to most hemoglobins, including human and bovine; but aT4, aT8, and aT9 are different in human and bovine hemoglobin. The aT8 and aT9 segments are unusual peptide sequences and so far have only been reported in bovine and yak hemoglobins (15). With ESI, these peptides acquire up to three positive charges (aT3$^+$, aT5^{2+}, aT4^{2+}, aT8$^+$, and aT9^{3+}). The base peaks are used as precursor ions for MS–MS studies. The extracted ion chromatograms found from the LC–MS–MS analysis of the tryptic digest of normal human plasma sample and a plasma sample taken from a subject infused with Hemopure are shown in Figures 5A and 5B. The top row of each contains the aT3 and aT5 common segment windows that are used to confirm that the material under investigation contains hemoglobin peaks. These hemoglobin peaks are small in the normal plasma sample (Figure 5A) used for this experiment because it was a typical one with low hemoglobin concentration. The low intensity of the human hemoglobin peaks coupled with the absence of peaks in the bovine windows (aT4, aT8, and aT9) demonstrates the specificity of the method and shows that the other plasma proteins present do not interfere. The plasma sample from an administration study in Figure 5B has peaks in all windows confirming the presence of bovine hemoglobin, as well as a small but significant amount of human hemoglobin. This was to be expected because previous analysis of the sample by SEC–PDA had shown it contained hemoglobin corresponding to that from lysed blood as well as Hemopure. Thus, the LC–MS–MS method can confirm

the presence of bovine based hemoglobin in plasma taken from a subject given Hemopure even in the presence of significant hemolysis.

Confirmation of the presence of bovine hemoglobin in plasma at the levels of 1 to 2 g/L needed for doping control will be readily achievable, as the plasma sample from the mentioned administration study was diluted 10 times prior to analysis. Estimated detection limits of 0.25 g/L have been reported (12).

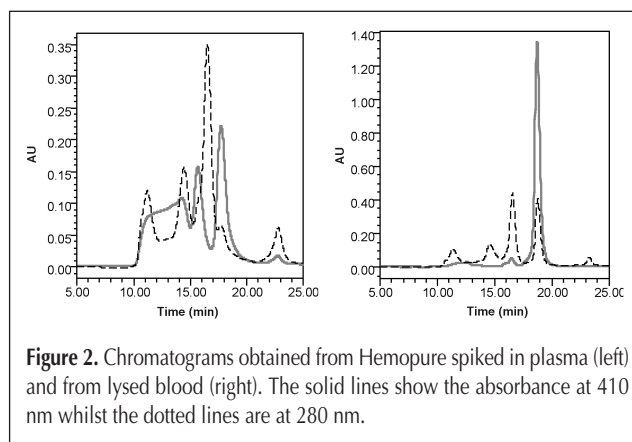The SEC–PDA method, when used in conjunction with the

Figure 2. Chromatograms obtained from Hemopure spiked in plasma (left) and from lysed blood (right). The solid lines show the absorbance at 410 nm whilst the dotted lines are at 280 nm.
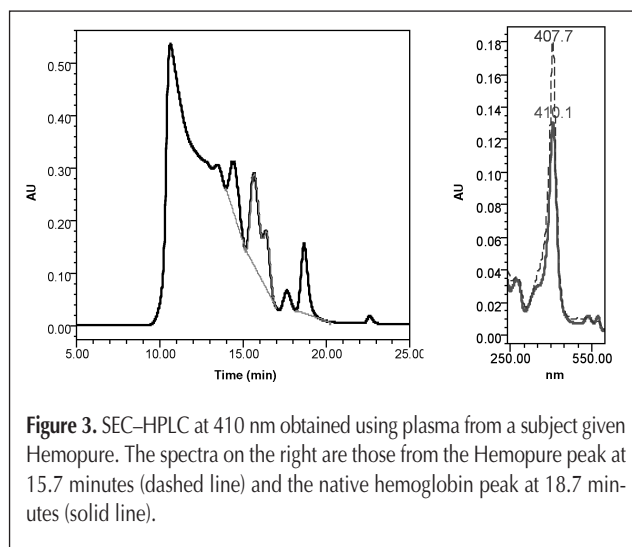
Figure 3. SEC–HPLC at 410 nm obtained using plasma from a subject given Hemopure. The spectra on the right are those from the Hemopure peak at 15.7 minutes (dashed line) and the native hemoglobin peak at 18.7 minutes (solid line).

Figure 4. Amino acid sequences of the alpha chains of human and bovine hemoglobins. The segments produced by tryptic digestion are also shown. The cleavage occurs at the positions with underlined lighter text (K, R).
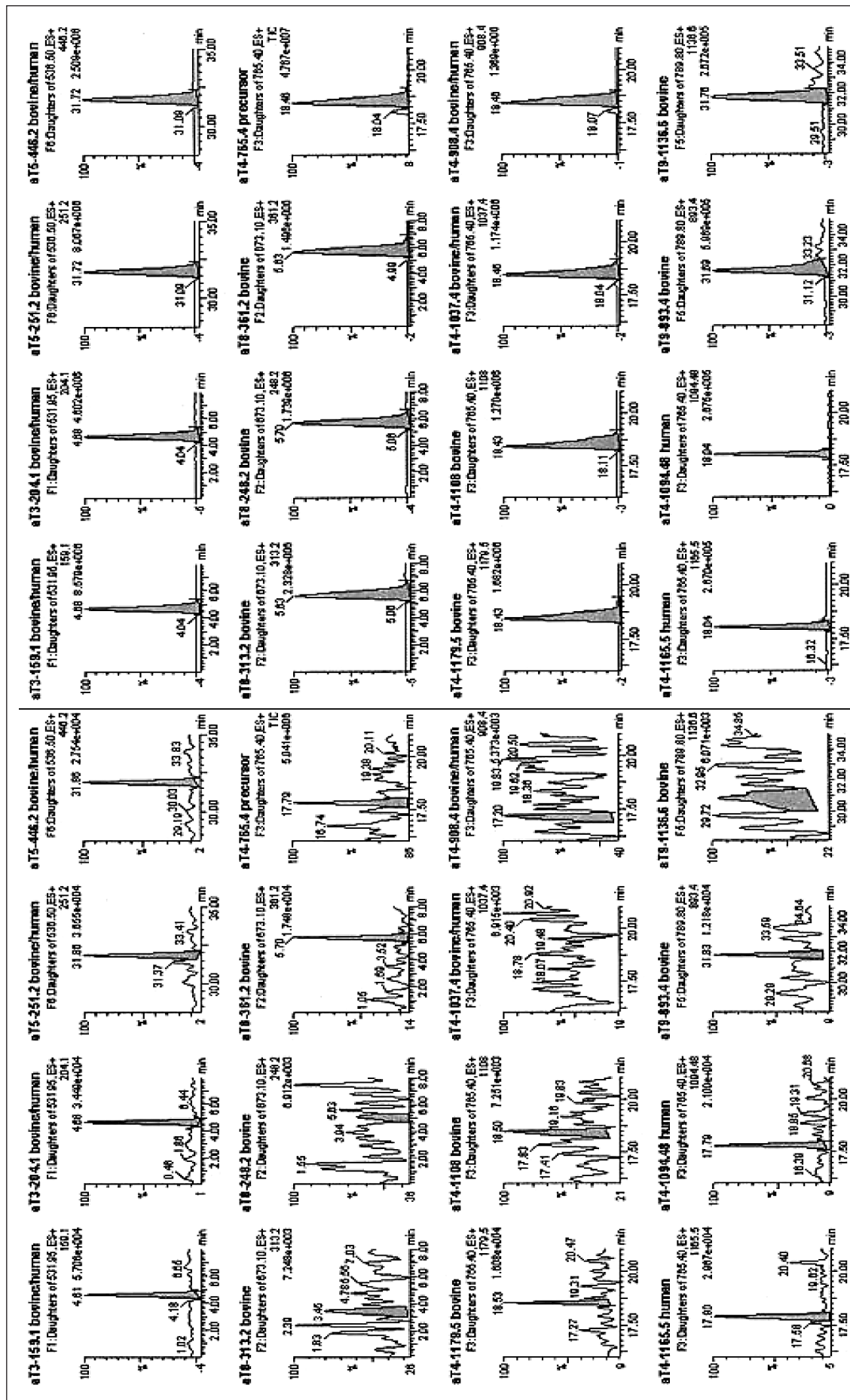
Figure 5. LC–MS–MS data from tryptic digests of human plasma. The left box (A) includes plasma from normal human subject. The right box (B) includes plasma from subject infused with Hemopure. The windows from left to right in each box are notated as: Segment (m/z parent → m/z daughter); aT3 common sequence (532.0 → 159.1); aT3 common sequence (532.0 → 204.1); aT5 common sequence (536.5 → 251.2); aT5 common sequence (536.5 → 446.2); aT8 bovine only (673.1 → 313.2); aT8 (673.1 → 248.2); aT8 (673.1 → 361.2); aT4 both human and bovine, different transitions due to variation in sequence between bovine (retention time 18.46mins) and human (retention time 18.04) and some ions are common and others are not, aT4 precursor ions (765.4); aT4 bovine (765.4 → 1179.5); aT4 common (765.4 → 1037.4); aT4 common (765.4 → 908.4); aT4 human (765.4 → 1165.5); aT4 human (765.4 → 1094.5); aT9 bovine (789.8 → 893.4); and aT9 bovine (789.8 → 1136.6).
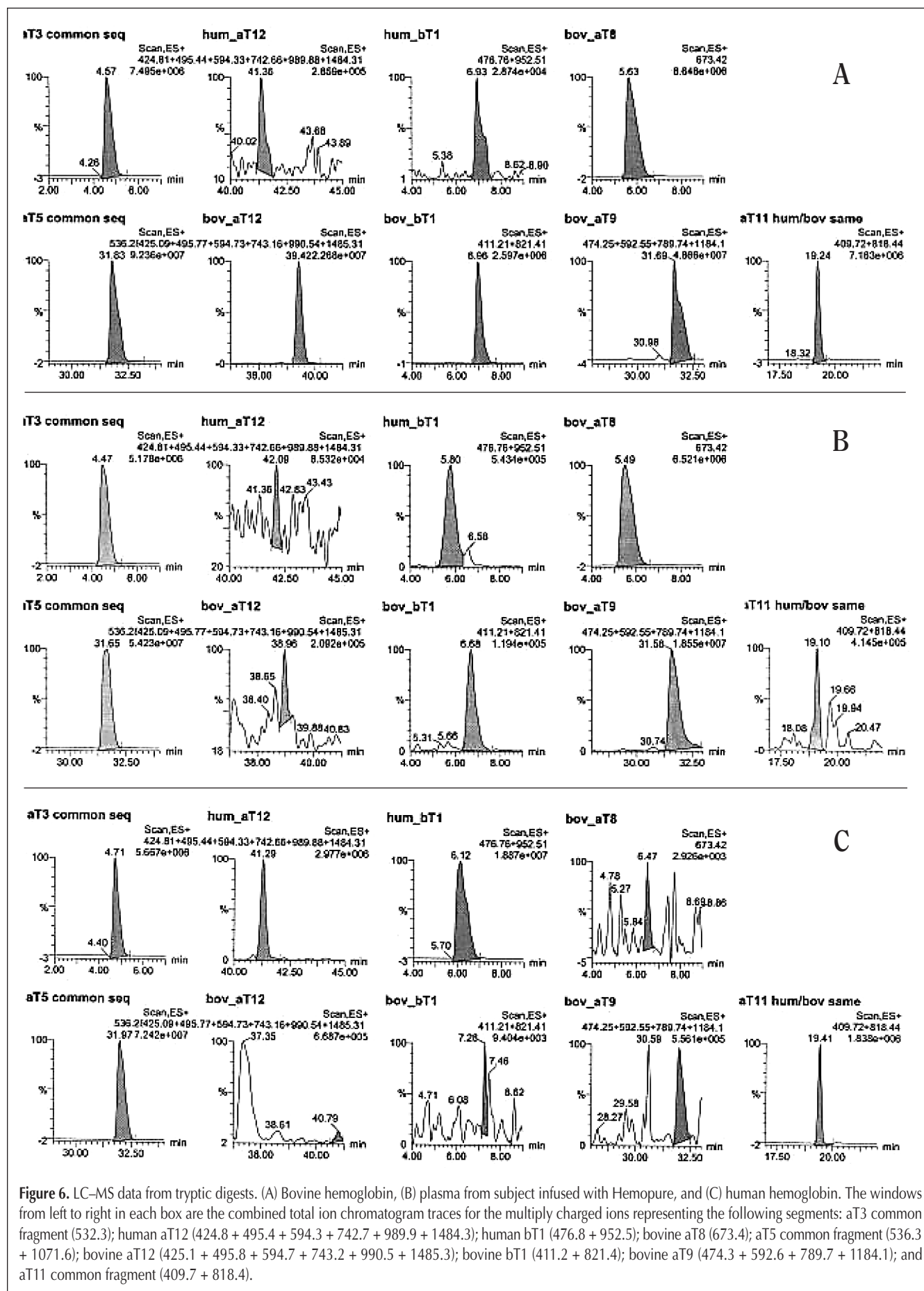
Figure 6. LC–MS data from tryptic digests. (A) Bovine hemoglobin, (B) plasma from subject infused with Hemopure, and (C) human hemoglobin. The windows from left to right in each box are the combined total ion chromatogram traces for the multiply charged ions representing the following segments: aT3 common fragment (532.3); human aT12 (424.8 + 495.4 + 594.3 + 742.7 + 989.9 + 1484.3); human bT1 (476.8 + 952.5); bovine aT8 (673.4); aT5 common fragment (536.3 + 1071.6); bovine aT12 (425.1 + 495.8 + 594.7 + 743.2 + 990.5 + 1485.3); bovine bT1 (411.2 + 821.4); bovine aT9 (474.3 + 592.6 + 789.7 + 1184.1); and aT11 common fragment (409.7 + 818.4).

LC–MS–MS methodology described previously, is capable of proving that high plasma hemoglobin concentrations are (*a*) because of high molecular weight hemoglobins and (*b*) that the hemoglobin is of bovine origin. The presence of either is proof of doping, but the LC–MS–MS method in its current form does not prove that the bovine hemoglobin is from a polymerized hemoglobin or HBOC and will not detect other HBOCs based on human hemoglobin.

It should be possible using similar methodology to detect the chemical cross linking that is used in all advanced HBOCs, and, thus, have a mass spectral method that can be used to confirm that a chemically modified hemoglobin is present and identify which HBOC has been used. It has already been shown by using the direct infusion of tryptic digests into ESI-MS that Hemopure contains hemoglobin of bovine origin that has been chemically modified (11). The method is based on the fact that the chemical cross linking agents used to prepare HBOCs (glutaraldehyde, in the case of Hemopure) change the peptide sequences that are obtained after tryptic digestion. These experiments have been continued to allow us to determine whether similar results can be obtained from plasma samples containing Hemopure.

Although direct infusion has been widely used for the rapid characterization of hemoglobins (16), it is of limited use in the presence of high concentrations of other proteins, those that occur in plasma. LC–MS analysis of tryptic digests has confirmed that the lysine at position 99 in both alpha chains reacts with glutaraldyde to stabilize the tetrameric structure. This stabilized tetramer is a significant component of the veterinary HBOC Oxyglobin. The LC–MS experiments have established other reaction sites such as the *N*-terminal methionine of the beta chain. When cross-linking with glutaraldehyde occurs, the reaction is between the carbonyl of the glutaraldehyde and the free amino groups of *N*-terminal amino acids or amino acids with a free amino group on a side chain such as lysine or arginine. As the latter are also the amino acids where trypsin cleaves the chain, the reaction with glutaraldehyde will prevent this cleavage and result in different peptide segments. The reaction of the lysine at position 99 of the alpha chain in Hemopure and Oxyglobin results in much lower concentrations of segments aT11 and aT12. Similar reductions are found for bT1, the first segment of the beta chain, which is also involved in the polymerization. Figure 6 shows the extracted ion chromatograms from the LC–MS analysis of bovine hemoglobin (Figure 6A), and a plasma sample from a subject given Hemopure (Figure 6B). As seen previously from the LC–MS–MS results, the aT3 and aT5 are common peaks for most hemoglobins, but aT8 and aT9 are specific for bovine. Thus, bovine hemoglobin is present. However, aT11, bovine aT12, and bT1 peaks are of much lower intensity in the sample containing Hemopure, indicating that a considerable amount of the material has cross-linking present. The output obtained from human hemoglobin is shown in Figure 6C, with significant peaks in the human aT12 and bT1 windows but negligible peaks in the bovine specific windows. The relative areas of the peaks obtained are set out in Table II along with the relative areas from a digest of Hemopure. The ratios have been calculated relative to the area of the common aT3 peak. Repeat analyses have shown the reproducibility to be good with less than 5% variation.

The predicted ratios given in Table II for glutaraldehyde polymerized pyridoxylated human hemoglobin are based on published data relating to the reaction between pyridoxyl 5'-phosphate and human hemoglobin. Anywhere from two to six sites on the two alpha and two beta chains in the native tetramer can react (17). The reaction sites are the *N*-terminal valine of the beta chain, the lysine at position 82 on the beta chain, and the *N*-terminal valine on the alpha chain. In all cases, both *N*-terminal valine on the beta chain has been reacted with pyridoxyl phosphate. The pyridoxylation is needed to modify the oxygen affinity of extra cellular human hemoglobin (18). The reaction of human hemoglobin with glutaraldehyde should result in linkage of the alpha chains at lysine 99 as in bovine hemoglobin. Based on the mentioned reactions, the tryptic digestion of such an HBOC would be expected to result in normal concentrations of aT3 and aT5, low concentrations of aT11 and aT12, and low concentrations of bT1 compared the values found from a similar concentration of native human hemoglobin.

Work is proceeding on using MS–MS to confirm that the peaks identified in the LC–MS analysis have the expected peptide sequences. It is anticipated that the use of LC–MS–MS will improve the reproducibility and selectivity of the method.

Table II. Ratios Found for the Characteristic Fragments of Hemoglobins and Hemopure Relative to the Common aT3 Sequence.*

Sample digested	aT3 Area	Ratios of segment areas to aT3							
		aT5	aT11	hum aT12	bov aT12	bov bT1	hum bT1	bov aT8	bov aT9
Bovine hemoglobin	2.9×10^6	13.2	0.6	0.0	2.0	0.3	0.0	1.6	7.4
Hemopure	2.5×10^6	14.7	0.1	0.0	0.4	0.0	0.0	2.0	11.1
Hemopure administration	2.3×10^6	11.1	0.0	0.0	0.0	0.0	0.1	1.8	4.3
Human hemoglobin	1.8×10^6	15.6	0.2	0.5	0.0	0.0	5.9	0.0	0.1
Predicted gluteraldehyde polymerized pyridoxylated human hemoglobin	1.5 to 3.0 $\times 10^6$	8 to 18	< 0.1	< 0.1	0.0	0.0	< 1	0.0	< 0.2

* The table also gives a prediction for polymerized human (hum) hemoglobin based on the published structures and observations for bovine (bov) material and is expected to have a greatly reduced aT11, aT12, and bT1 sequence.

Conclusion

We have demonstrated that the qualitative detection of HBOCs in blood samples is readily achievable by a simple visual examination of plasma. This screening protocol has a low false-positive rate, with less than 1% of samples requiring confirmation using a threshold of 2 g/L. We have evaluated three confirmation methods: one using SEC–PDA and two using LC–MS with ESI. The SEC method is based on the detection of polymeric hemoglobins in plasma using their UV–vis spectra to confirm identity. The method has been validated using Hemopure and should be applicable to any other HBOCs based on polymeric hemoglobins such as Polyheme and Hemolink. The confirmation level for this method is approximately 5 g/L for Hemopure in plasma.

The first LC–MS confirmation method uses MS–MS to detect peptide sequences, which are different in bovine hemoglobin than those found in human hemoglobin. The method can confirm the presence of bovine sequences in samples from subjects who have been given Hemopure with a detection level below 2 g/L. The second LC–MS method is more general in its application in that it is based on the detection of changes in peptide sequences that result from the chemical reactions used in the preparation of all HBOCs. This method, using the ratio of the concentrations of modified and unmodified peptide sequences, can not only detect peptides that are of bovine origin but also can demonstrate that chemical modifications of the bovine hemoglobin have occurred. The method should also be able to detect and identify chemically modified human hemoglobins, but so far we have been unable to obtain samples of Polyheme or Hemolink to confirm this. Work is proceeding on development of the method using MS–MS with the aim of improving its selectivity and sensitivity. We are also hopeful of being able to directly detect the peptides that have been chemically modified. This will provide the means to determine which reactions have occurred and, thus, enable the identification of the particular HBOC present, be it bovine or human in origin.

Acknowledgments

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