PROJECT REVIEW

"Detection of the Abuse of Haemoglobin based Blood Substitutes in Sport"

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Blood substitutes based on stabilised or polymerised haemoglobins are already approved for use in animals eg Oxyglobin is approved for use in dogs in the USA. Due to increasing demand for blood during surgical procedures and the perceived risk from blood borne diseases there is demand for such products to be made available for human use. At least two companies Biopure and Hemosol have products in stage three clinical trials. The haemoglobin based blood substitutes have several advantages over whole blood which makes their use by athletes highly probable. At present athletes who seek to increase their performance in endurance sports can do so by blood transfusions or by the injection of recombinant EPO. Both procedures increase the red blood cell concentration in the blood but with blood transfusions there is always the risk of mismatching unless homologous blood is used. Also blood has a relatively short storage life and must be kept refrigerated. With EPO there are no particular health risks but the rate of increase of red blood cells is quite slow requiring a doping regime over several weeks prior to an event. Also since the Sydney 2000 Olympic Games there is a risk of detection by the combined blood and urine test (Parisotto et al, 2001 and Lasne and de Ceaurriz, 2000). The haemoglobin based blood substitutes at present have none of the disadvantages of either blood or EPO. They have shelf lives of up to two years at room temperature, their effect on increasing the oxygen carrying capacity is almost immediate, and there is no risk of mismatching. Thus unless detection methods are developed and put in place soon then it is likely that haemoglobin based blood substitutes will be keenly sought after by athletes who are willing to go beyond ethical means to improve performance (Birkeland and Hemmersbach, 1999).

Three sources of haemoglobin have been considered for use in HBOCs namely human haemoglobin, bovine haemoglobin, and recombinant haemoglobin (Chapman, 1998). Each has advantages and disadvantages but one problem that must be overcome with any type is the kidney toxicity problem caused by the rapid dissociation of cell-free haemoglobin into its dimer and monomer sub-units (Bunn, 1995). To overcome this the haemoglobin molecule must be stabilised and four approaches have been investigated (Chang, 1999). The four types of stabilised haemoglobin are intramolecular cross-linked haemoglobin, polymerised haemoglobin, conjugated haemoglobin, and recombinant haemoglobin. Problems with vasoconstriction leading to increased blood pressure (Alayash, 1999) have limited the development of the Intramolecular cross-linked haemoglobins, and products based on polymerised human or bovine haemoglobin are the furthest advanced in clinical trials.

Detection of the abuse of haemoglobin-based blood substitutes in sport.

Results and conclusions

Blood substitutes are oxygen-carrying therapeutics developed for use in operations and emergencies in place of donated blood. Increased oxygen-carrying capacity through the use of blood substitutes can help elite athletes to improve their performance and lengthen endurance capacity. Only one product, Hemopure™ (Biopure Corporation), a glutaraldehyde-polymerised bovine haemoglobin solution with a high molecular weight range (130-500 kDa), has limited approval for human use but there are others in phase III clinical trials. As blood substitutes become more readily available, it is essential that a detection method, for their abuse in sport, is available.

The aim of this study was to investigate methods that could be used as screening procedures for polymerised haemoglobin in plasma and to identify tests that can unequivocally confirm their presence. Direct visual screening of plasma discolouration was the most appropriate screening method with detection limits lower than 1% haemoglobin-based oxygen carrier (HBOC) in plasma. Three methods have been shown to confirm the presence of exogenous haemoglobin in plasma samples: size-exclusion chromatography with photodiode array detection (SEC-PDA), native-polyacrylamide gel electrophoresis (native-PAGE) with luminol exposure and enzymatic digestion with detection by mass spectrometry.

SEC separates the plasma proteins according to size with the largest molecules eluting first. Size exclusion separation coupled with the spectral analysis of the HBOCs compared to human and bovine haemoglobin standards uniquely identified the polymerised haemoglobin molecules by their retention time and spectral match to haemoglobin standards. The SEC-PDA method was able to determine the presence of HBOCs at a 1% spiking level in plasma. Native-PAGE separates the plasma proteins by both molecular weight and conformation. Haemoglobin and modified haemoglobin were identified on gels by their production of chemiluminesence after exposure to luminol and their migration point relative to each other. Hemopure™ and Oxyglobin™ were clearly detectable down to a 1% level in plasma samples. The LC/MS and LC/MS/MS analysis of tryptic digests of Hemopure™ and Oxyglobin™ identifies peaks which are from polymerised bovine haemoglobin and not from human haemoglobin origin.

The methods have been validated by demonstrating their ability to detect and confirm the presence of Hemopure™ in incurred plasma samples. Furthermore, the methods which have been developed are not specific to Hemopure™ and should be capable of detecting and confirming the presence of any HBOC which is chemically cross-linked and has an average molecular weight significantly higher than human haemoglobin. All HBOCs which are in advanced stages of development meet these criteria.

Publications and poster presentations

1) C. Alma et al., The Detection of Haemoglobin Based Oxigen Carriers. In: Schänzer W, Geyer H, Gotzmann A, Mareck U, editors. Recent Advances in Doping Analysis (10) Köln: Sport und Buch Strauss, 2002:169-177.

2) C. Goebel et al., Methodologies for detection of haemoglobin based oxygen carriers, J. Chromatogr. Sci., *in press*.