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HBOC Detection – Progress since 2000

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Introduction

Since 2000 there have been some significant changes in the development of artificial oxygen carriers (AOCs). As of 2004 only two AOCs, Polyheme and Hemopure or HBOC-201, continue in phase III clinical trials in the USA. Hemopure has been approved for limited clinical use in South Africa. A phase III clinical trial of Hemolink was suspended due to "*an imbalance in the incidence of certain adverse events between the Hemolink and control groups*".

No perfluorocarbons, or liposome encapsulated or recombinant haemoglobin AOCs are currently undergoing human trials or have commercial sponsors in the USA. The effect of this is that there are really two AOCs that are likely to be used by athletes, namely Hemopure and Polyheme, with Hemopure being the more likely to be abused owing to its greater availability.

Since the 2000 Olympics we have received over 2000 blood samples from elite athletes as part of Australia's anti-doping program. The samples have been collected primarily out of competition to detect and deter doping with EPO. All these samples have also been screened for the presence of haemoglobin based oxygen carriers (HBOC) using a simple visual screen with confirmation methods available using size exclusion chromatography and LC-MS. Details of these methods have been recently published (Goebel et al 2005). A simpler procedure using gel electrophoresis has also been developed in this laboratory to enable us to readily distinguish extra-cellular haemoglobin from the use of an HBOC (Alma 2001). The publication of a paper describing the use of a four step process to detect HBOCs in serum using electrophoresis (Lasne et al 2004) led us to review our simple two step method to determine if there could be problems with the presence of haptoglobin which we had

overlooked. The procedure we use to distinguish higher molecular weight haemoglobins (64kDa to >500kDa) from natural extra-cellular haemoglobin (<64kDa) is based on native-polyacrylamide gel electrophoresis (PAGE) followed by selective detection of the bands on the gel.

Experimental

All reagents were of AR or HPLC grade. Water was from a Milli-Q water purification system. The sample buffer consisted of 1 mL of 0.5 M Tris-HCl, pH 6.8, 2 mL of glycerol, 1 mL of 0.1% bromophenol blue, and 4 mL of Milli-Q water. The running buffer consisted of Tris-Base (15 g) and glycine (72 g) made up to 500 mL with Milli-Q water. The gels were run using a Ready Gel Cell (Bio-Rad, Hercules CA, USA). The human haemoglobin and human haptoglobin were purchased from Sigma (Sydney, Australia). The Hemopure and plasma samples from subjects administered Hemopure were kindly supplied by the Biopure Corporation (Cambridge MA, USA).

The serum or plasma samples were diluted one to two with sample buffer and 10 uL of each was loaded into the individual wells on the 4-15% Tris-HCl Ready Gel (Bio-Rad, Hercules CA, USA). The gel was run at 200V with 70mA for 30 minutes. The gel was then placed in 0.5% potassium ferricyanide solution and shaken for 15 minutes. Two more 15 minute washes were performed in 3% sodium carbonate. The dish was then emptied and to it was added 40 mL of sodium carbonate solution followed by 2 mL of Pierce Supersignal West Pico Luminol solution (Pierce, Rockford IL, USA) and the gel shaken for 10 minutes before adding 2 mL of Supersignal West Pico Peroxide solution (Pierce, Rockford IL, USA). The chemiluminescent images were captured using a Fuji LAS-1000 Imaging System (Fuji, Tokyo, Japan).

Results and Discussion

The selective detection of the heme-containing proteins in the serum is shown in Fig 1 where results from the non-specific staining of a gel with Gelcode modified Coumassie stain (Pierce, Rockford IL, USA) are compared with staining with Luminol. It can be seen that the Hemopure and native haemoglobin are clearly resolved on the basis of their molecular weights using the Bio-Rad 4-15% Tris HCl Ready Gel when visualised with the non-specific stain. However, when plasma samples containing Hemopure are run the Hemopure and haemoglobin are not able to be seen amongst the mass of other proteins present in the plasma. When the selective staining is used only the heme-containing proteins are detected and a normal plasma sample can be clearly distinguished from one containing Hemopure either from spiking or from an administration study. A number of spiked and incurred plasma samples have been run with this method and no problems have been encountered with distinguishing these from samples which are heavily contaminated with extra-cellular haemoglobin from lysed red blood cells. In view of the recently published four step method which includes the removal of haptoglobin prior to gel electrophoresis we investigated the effect of added haptoglobin on our method. Haptoglobin is a naturally occurring protein in blood with a molecular weight of approximately 45,000, which forms a one to two complex with any free haemoglobin. The resulting complex is then removed by the liver. The haptoglobin-haemoglobin complex has a molecular weight in the range of those found in HBOCs such as Hemopure and hence might be expected to interfere with their detection. To investigate whether this might be occurring a series of standards and mixtures were run on our two step method. The results are shown in Figure 2 where it can be seen that the complexes formed with haptoglobin are outside the molecular weight band used for the detection of Hemopure. The absence of any detrimental effect of added haptoglobin was confirmed when spiked plasma samples were analysed (Figure 3). Use of the method for detecting Hemopure in the presence of heavily lysed blood is shown in Figure 4. It can be seen that the boxed region of the gel used for the identification of Hemopure is clear of any bands in either normal plasma or plasma containing large amounts of free haemoglobin from the lysis of red blood cells.

Conclusions

The simple two step gel electrophoresis method is capable of distinguishing Hemopure from free haemoglobin in those very few samples which have shown to be suspect from the simple visual screen. The method is simple to use and cheap to set up using commercially available gels which cost less than $20 each. The electrophoresis cell is less than $700 and can use virtually any power supply. Other than centrifugation no sample pretreatment is required. The presence of excess haptoglobin does not affect the efficacy of the method. It should be possible to digest the bands for LC/MS confirmation if required.

References

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Acknowledgements

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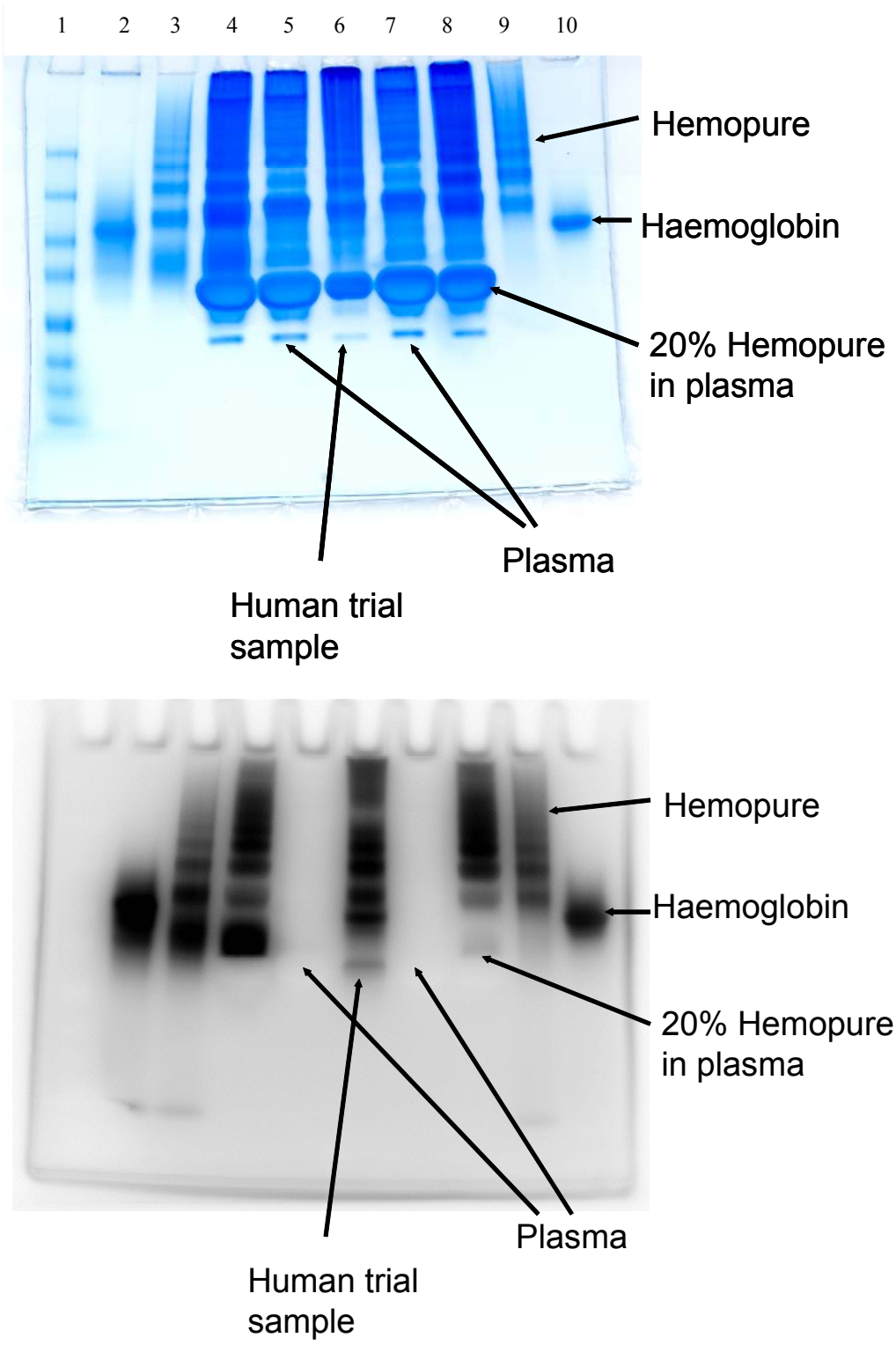

Figure 1. Images of gels stained with Gelcode non-selective stain (top image) and the same samples visualised using Luminol chemiluminescence (bottom image). Lane 1 (left to right) molecular weight marker, Lane 2 Haemoglobin, Lane 3 Oxyglobin, Lane 4 Oxyglobin in plasma, remaining lanes labelled on the diagram.

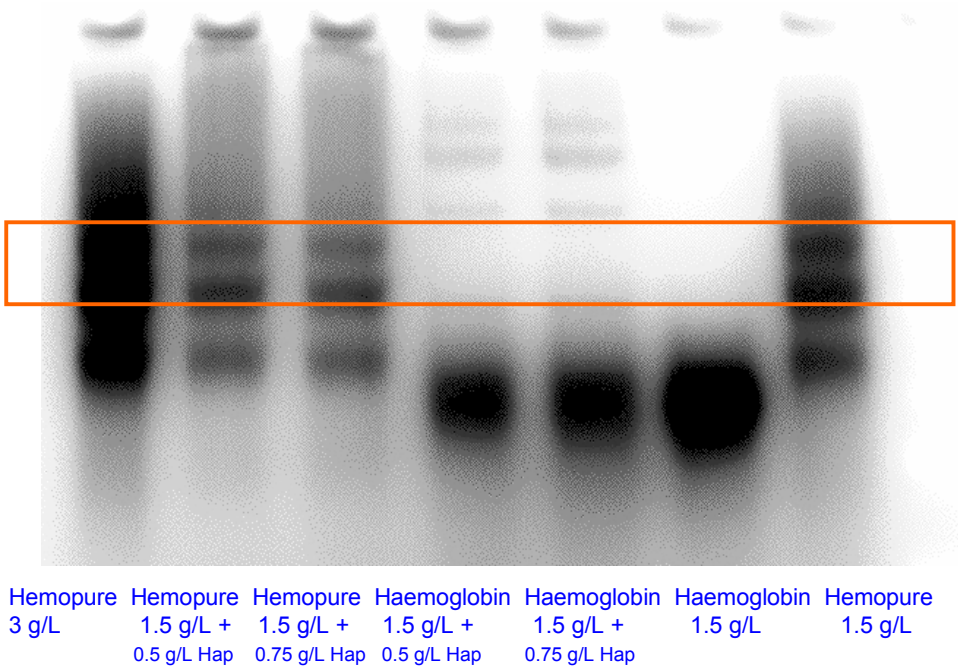

Figure 2. Chemiluminescent image of standards run on Ready gel showing that haptoglobin/haemoglobin complexes do not interfere with the primary region used for distinguishing extra-cellular haemoglobin from Hemopure (boxed region).

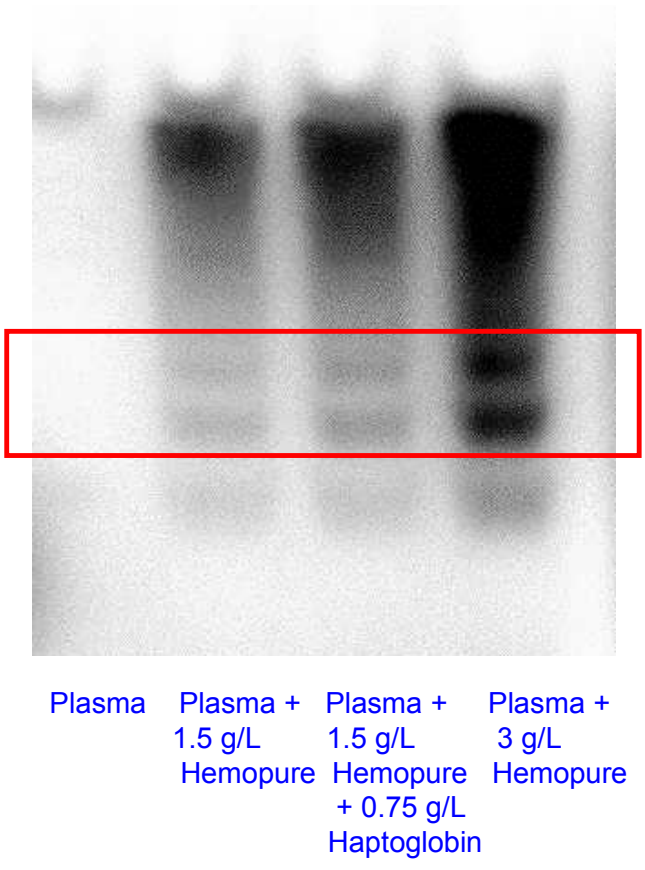

Figure 3. Chemiluminescent image of plasma samples spiked with Hemopure with and without added haptoglobin.

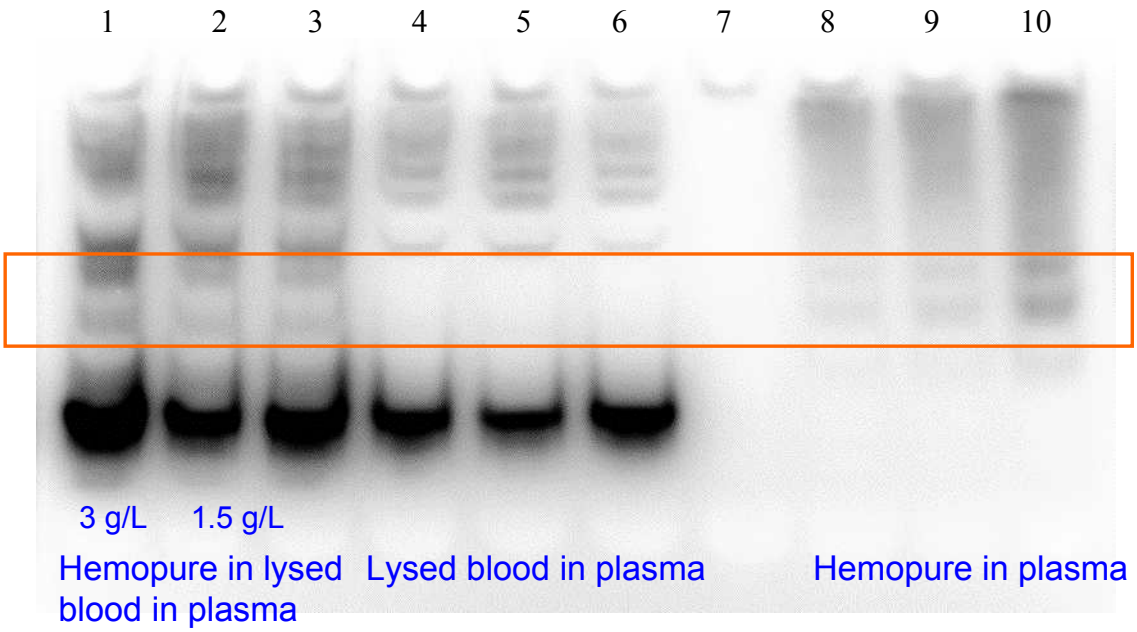

Figure 4. Chemiluminescent image of normal plasma sample (lane 7), plasma spiked with Hemopure at 1.5 and 3 g/L (lanes 8 to 10), plasma from heavily lysed blood (lanes 4 to 6), and plasma from heavily lysed blood spiked with Hemopure at 1.5 to 3 g/L (lanes 1 to 3).