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A fast screening method for the detection of the abuse of hemoglobin-based oxygen carriers (HBOCs) in doping control

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ABSTRACT

Artificial oxygen carriers (AOCs) can be abused by the athletes to improve their aerobic capacity. The AOCs produce a performance enhancing effect, especially in endurance sports. This article presents a method for the rapid screening of hemoglobin-based oxygen carriers (HBOCs) in blood samples. Common screening tests to reveal HBOC misuse by athletes are based on colorimetric detection since HBOC use causes discoloration of the plasma. In this communication we are presenting a different approach for HBOC detection using an hematological analyzer capable of measuring hemoglobin by two methods: a standard cyanmethemoglobin colorimetric method to calculate the amount of total hemoglobin (HGBtot) and a flow cytometric optical method to calculate the amount of hemoglobin within the red blood cells (HGBcell). Thanks to this dual contemporary hemoglobin measurement, the HGBdelta value (corresponding to free HGB) is automatically calculated by subtraction of HGBcell from HGBtot and can be used as a fast screening index of HBOC abuse. We tested the effectiveness of this approach using 68 normal blood samples with different basal HGB values fortified with three different HBOCs at varying concentrations. We evaluated the performance of the method by calculating the correlation between HGBcell and HGBtot values in normal samples. Finally we used a simple statistical approach to calculate a reliable HGBdelta cut-off value (0.35 g/dL) as a limit of decision to discriminate between a clear negative sample and a suspect sample to submit to a confirmation analysis.

1. Introduction

A recent development in doping is the use of artificial oxygen carriers (AOCs) by athletes. AOCs are chemical substances, which simulate the action of human hemoglobin (HGB). They are administered to improve the ability of blood to deliver oxygen to muscles. AOCs include perfluorocarbons and hemoglobin-based oxygen carriers (HBOCs). Recently HBOCs (and other oxygen delivery enhancers such as perfluorocarbon emulsions) have been included on the World Anti-Doping Agency (WADA) list of prohibited substances in sports. Common screening tests to reveal HBOC misuse by athletes are based on colorimetric detection since HBOC use causes discoloration of the plasma [1–3]. In this communication, a different approach for HBOC detection using an ADVIA 120 hematological analyzer (Bayer Diagnostics, Tarrytown, NY, USA) is presented. ADVIA 120 evaluates hemoglobin by two methods: a standard cyanmethemoglobin colorimetric method to calculate the amount of total hemoglobin (HGBtot) and a flow cytometric optical method to calculate the amount of hemoglobin within the red blood cells (HGBcell) [2]. Red blood cells become spherical in an isotonical environment and their volume does not change. This is particularly important since this spherical shape of the RBCs eliminates most of the common problems of the optical measurements including the effects of the orientation of the cells once they pass into the capillary and are hit by the laser. The light scattering produced by the cells is collected and measured over two angles: a 2–3° angle (forward scatter) and a 5–15° angle (side scatter). The refractive index of the cells gives informations about cell volume (V) and HGB concentration (CH). The individual cell HGB content is calculated as VxCH and the CH mean value (CHCM) is calculated as the sum of cell by cell measurements divided by #RBC/μl. In summary, the CHCM is calculated using the formula:

$$HGB\,cell = \frac{RBC \times MCV \times CHCM}{100}$$

Thanks to this dual hemoglobin measurement (colorimetric totalHGB and optical cellHGB), the HGBdelta value (corresponding to free HGB), is calculated by subtraction of HGBcell from HGBtot and can be used as fast screening index of HBOC abuse. The effectiveness of this approach has been tested using normal blood samples with different basal HGB values fortified with three different HBOCs at different concentrations. Initially, ADVIA 120

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Table 1
Summary of data obtained on whole blood samples spiked with different amounts of HBOCs.

Polyheme	Sample 1 (basal HGB 12.80 g/dL)			Sample 2 (basal HGB 14.30 g/dL)			Sample 3 (basal HGB 17.00 g/dL)		
(g/dL)	HGBtot	HGBcell	deltaHGB	HGBtot	HGBcell	deltaHGB	HGBtot	HGBcell	deltaHGB
0	12.80 (0.10)	12.80 (0.15)	0.00 (0.06)	14.30(0.06)	14.30 (0.10)	0.00 (0.06)	17.00(0.06)	17.00 (0.06)	0.00 (0.06)
0.5	12.80 (0.10)	12.30 (0.12)	0.50 (0.06)	14.20(0.06)	13.60 (0.10)	0.60 (0.12)	16.70(0.12)	16.20 (0.16)	0.50 (0.17)
1	12.50 (0.00)	11.50 (0.17)	1.00(0.17)	14.00(0.06)	13.00 (0.12)	1.00 (0.15)	16.30(0.12)	15.30 (0.06)	1.00 (0.06)
2.5	12.20 (0.12)	9.70 (0.06)	2.50(0.15)	13.40(0.06)	11.00 (0.06)	2.40 (0.06)	15.20(0.06)	12.70 (0.12)	2.50 (0.06)
5	11.50 (0.12)	6.50 (0.15)	5.00(0.21)	12.20(0.12)	7.40 (0.15)	4.80 (0.06)	13.40(0.17)	8.50 (0.06)	4.90 (0.06)
10	10.00 (0.10)	0.00 (0.00)	10.00(0.10)	9.90 (0.06)	0.00 (0.00)	9.90 (0.06)	10.00(0.10)	0.00 (0.00)	10.00 (0.10)
Hemopure	Sample 1 (basal HGB 13.00 g/dL)			Sample 2 (basal HGB 14.60 g/dL)			Sample 3 (basal HGB 17.70 g/dL)		
(g/dL)	HGBtot	HGBcell	deltaHGB	HGBtot	HGBcell	deltaHGB	HGBtot	HGBcell	deltaHGB
0	13.00 (0.06)	13.00 (0.06)	0.00 (0.06)	14.60(0.05)	14.60 (0.05)	0.00 (0.06)	17.70(0.05)	17.70 (0.10)	0.00 (0.15)
0.5	13.00 (0.06)	12.50 (0.10)	0.50(0.10)	14.50(0.00)	14.00 (0.05)	0.50 (0.11)	17.40(0.11)	16.90 (0.11)	0.50 (0.00)
1	12.90 (0.06)	11.90 (0.17)	1.00(0.20)	14.30(0.06)	13.30 (0.06)	1.00 (0.00)	17.30(0.11)	16.30 (0.11)	1.00 (0.10)
2.5	12.90 (0.17)	10.20 (0.15)	2.70(0.10)	14.30(0.10)	11.50 (0.23)	2.80 (0.15)	16.70(0.11)	14.00 (0.06)	2.70 (0.11)
5	12.90 (0.00)	7.40 (0.06)	5.50 (0.06)	13.90(0.00)	8.30 (0.06)	5.60 (0.00)	15.70(0.00)	10.00 (0.10)	5.70 (0.10)
10	10.00 (0.11)	0.00 (0.00)	10.00(0.11)	10.00(0.06)	0.00 (0.00)	10.00 (0.06)	10.00(0.05)	0.00 (0.00)	10.00 (0.05)
Oxyglobine	Sample 1 (basal HGB 13.00 g/dL)			Sample 2 (basal HGB 14.30 g/dL)			Sample 3 (basal HGB 17.60 g/dL)		
(g/dL)	HGBtot	HGBcell	deltaHGB	HGBtot	HGBcell	deltaHGB	HGBtot	HGBcell	deltaHGB
0	13.00 (0.00)	13.00 (0.08)	0.00(0.10)	14.30(0.06)	14.30 (0.06)	0.00 (0.06)	17.60(0.10)	17.60 (0.17)	0.00 (0.06)
0.5	13.00 (0.00)	12.40 (0.17)	0.60(0.17)	14.20(0.06)	13.70 (0.06)	0.50 (0.00)	17.30(0.10)	16.80 (0.10)	0.50 (0.20)
1	12.90 (0.12)	11.80 (0.15)	1.10(0.00)	14.10(0.06)	13.10 (0.06)	1.00 (0.06)	17.20(0.06)	16.10 (0.00)	1.10 (0.00)
2.5	13.00 (0.00)	10.40 (0.10)	2.60 (0.06)	14.00(0.06)	11.50 (0.10)	2.50 (0.06)	16.30(0.12)	13.70 (0.06)	2.60 (0.10)
5	13.00 (0.00)	7.50 (0.05)	5.50 (0.06)	13.90(0.06)	8.90 (0.06)	5.00 (0.10)	15.50(0.10)	10.10 (0.12)	5.40 (0.06)
10	10.00 (0.00)	0.00 (0.00)	10.00(0.00)	9.90 (0.06)	0.00 (0.00)	9.90 (0.06)	10.0(0.06)	0.00 (0.00)	10.0 (0.06)

performance was evaluated by calculating the correlation between HGBcell and HGBtot values in normal samples. Then, a simple statistical approach was used to calculate a reliable HGBdelta cut-off (or limit of decision) value to discriminate between a negative sample and a suspect sample to submit to a confirmation analysis, aimed to identify, usually by gel electrophoresis and LC/MS–MS, the identity of the non-endogenous hemoglobin(s) present in the sample.

2. Methods

Whole blood samples coming from 68 healthy athletes were analyzed to assess the correlation between HGBtot and HGBcell measurements. Samples were first analyzed with colorimetric method to be sure they were negative for HBOCs and then analyzed using ADVIA 120. Results are shown in Fig. 1.

To fortify whole blood sample with different amounts of HBOCs, (to the final concentrations indicated in Table 1), the following HBOCs were added to three Fresh K3-EDTA-anticoagulated blood samples: Oxyglobin and Hemopure from Biopure Corp. (Cambridge, MA, USA), and Polyheme kindly provided by Northfield Laboratories Inc. (Evanston, IL, USA). Varying amounts of HBOC solutions were mixed with whole blood to assess the linearity of this method using a wide range of HBOC concentrations shown in Table 1. Three hematologic parameters (HGBtot, HGBcell and Delta HGB) were determined in triplicate using the ADVIA 120. Additional parameters including mean corpuscular volume (MCV), number of eryhtrocytes (#RBC), hematocrit (HCT), mean cellular hemoglobin (MCH) and mean cellular hemoglobin concentration (MCHC) (data not shown) were also determined. For each parameter, the mean value and standard deviation were calculated. Statistical analysis of data was performed using Microsoft Excel

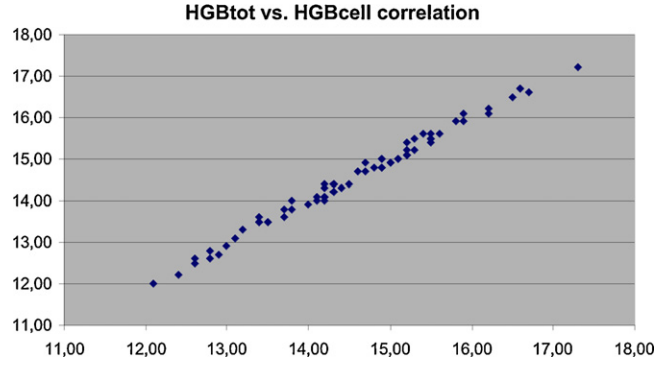

Fig. 1. Correlation between HGBtot and HGBcell in 68 healthy athletes' whole blood samples.

3. Results and discussion

Correlation between HGBcell and HGBtot values determined by ADVIA 120 was calculated. Fig. 1 shows the comparison of HGB values obtained with an optical method (HGBcell) and standard colorimetric method (HGBtot) on whole blood samples from 68 healthy athletes. The parameters are strongly correlated with $R^2 = 0.9905$. These results show flow cytometry as a pursuable method for HGB measurement.

Table 1 shows the mean values and standard deviations (in parenthesis) obtained for HGBtot, HGBcell and the Delta HGB. ADVIA 120 is able to determine the exogenous contribution of an HBOC in a sample. Each HBOC used in this study was detected. Delta HGB values are accurate and precise in the range of linearity we assessed. These results are important considering the different origins of the HBOCs used. Both Hemopure and Oxyglobin are made from bovine hemoglobin (with Hemopure used on humans and Oxyglobin intended for veterinary use). Polyheme however is produced from human haemoglobin; a solution of human haemoglobin is extracted from human red blood cells and modified using polymerization processes. It is interesting to note the reproducibility of the results using these different HBOCs because of their different matrices.

The method is fast and precise. The screening of a sample can be achieved in less than one minute (with a maximum performance of

Table 2
HGBtot and HGBcell values (in g/dL) of 68 whole blood samples negative for HBOCs. We calculated mean and standard deviation multiplied by three. The threshold we propose (0.35 g/dL) is the value of the mean added with the 3ds value.

	HGBtot	HGBcell	Delta
Mean	14.57	14.56	0.01
ds	1.15	1.18	0.11
Mean + 3ds			0.35

120 samples per minute). The analysis performed is a true hematological analysis. It is not only a visual, colorimetric determination but it also allows for the determination of more parameters. Apart from being a screen for HBOC abuse, the analysis also allows for the monitoring of the whole hematological status of an athlete. Additionally, this analysis can be performed without using open tubes of blood. There is no need to separate the serum from the sample therefore there is no direct contact with blood.

The data show a strong correlation between the colorimetric and optical methods using the ADVIA 120 for HGB determination. Therefore, with correct calibration, a normal blood sample has a delta value equal approximately zero, with a small and negligible contribution of free natural HGB. In a screening procedure for HBOC it is possible to establish a threshold value to discriminate between a negative and a suspect or a positive sample.

We measured the mean and standard deviation of 68 whole blood samples negative for HBOCs. Then we applied a 3ds rounded up criterion to the mean of the negative samples to obtain a threshold value to use as Limit of Decision. As shown in Table 2, a sample with a delta value below the cut-off 0.35 g/dL can be considered negative and a sample with a delta value higher or equal to 0.35 g/dL is considered suspicious and requires confirmation analysis. The addition of 3ds to the mean allows for a realistic and solid threshold value to reduce the number of false–positives and allows for confirmation analysis of a suspect sample (carried out by gel electrophoresis and LC/MS–MS) with reliable confidence.

4. Conclusions

We believe that the proposed approach can be followed for a fully automated, rapid, quantitative and reliable screening method for HBOCs in blood. The method can be used when other hematological parameters (including those that may be necessary for the longitudinal follow up of an athlete) have to be measured. The possibility of screening huge numbers of samples rapidly makes the method particularly useful for doping control analysis in major International sporting events, when the number of samples received by the laboratory increases dramatically and the time constraint becomes critical.

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