F. Donati, M. Mazzarino, X. de la Torre, F. Botrè

A fast pre-confirmation method for the detection of HBOCs by capillary electrophoresis and UV detection

Laboratorio Antidoping, Federazione Medico-Sportiva Italiana, Rome, Italy

Introduction

HBOCs are banned by the World Anti-Doping Agency (WADA) as they are enhancers of the oxygen delivering to tissues [1]. Detection of HBOCs is made on serum or plasma and screening methods are routinely performed using colorimetric assays since their abuse causes miscoloration of serum or, as is the case in our laboratory, using automated haematological analyzers (in our case Advia 120, Siemens) that are capable to detect both free and cellular haemoglobin, providing a delta value used as index of misuse [2-3]. These methods are simple and fast but affected by red blood cell hemolysis as a result of mechanical stress and/or sampling conditions. In every case of strong hemolysis, the screening analysis leads to a "suspect" result and, in turn, to time-consuming confirmation procedures by gel-electrophoresis, SEC-LC and LC-MS/MS techniques [2,4-7]. To avoid the need to confirm a sample as a consequence of a strong hemolysis, in this study we propose the use of the capillary zone electrophoresis (CZE) technique with UV detection to discriminate between an hemolyzed sample and a HBOCs positive sample starting from the capillary electrophoresis procedures already developed [8-9]. This strategy could be applied to all samples giving a "suspect" result in the screening procedure with the aim to avoid to perform whole and futile confirmation analyses, eliminating in this way the additional workload for the antidoping laboratories.

Materials and Methods

Samples, Chemicals and Reagents

Hemopure® and Oxyglobin® were provided by Biopure Corporation (Cambridge, MA, USA). Polyheme® was provided by Northfields Laboratories (Evanston, Illinois, USA).Distilled water was from a MilliQ water purification system (Waters, Italy). Whole blood samples were obtained from routine samples analysed in our laboratory. These samples were used also to simulate the haemolysis, using a pH 9.0 taurine/arginine buffer. Stock solutions and plasma spiked samples with the various HBOCs, were prepared at different

concentration from 1 mg/mL to 10 mg/mL. The Ceofix HbA2 kit system, developed by Analis SA, Belgium, consists of two buffers: an initiator containing a polycation and a second buffer (accelerator) consisting of a polyanion and of the background electrolyte was purchase from Beckman Coulter (Torino, Italy).

Analytical procedure

All CE experiments were performed using a P/ACE system MDQ (Beckman Coulter, Torino Italy), equipped with a 25 μm (i.d.) 30 cm (effective length 24 cm) uncoated fused silica capillary (Beckman Coulter, Torino Italy) thermostated. Experiments were carried out in normal polarity, with the anode at the inlet and the cathode at the outlet. The capillary was first rinsed for 0.75 min under a pressure of 25 psi with the "initiator" solution (solution of a polycation in a malic acid buffer, pH 4.7). This process coats the capillary wall with the polycation because a large number of anchor points are formed between the negative charges of the silanol groups of the capillary glass and the positive charges of the polycation. This rinse was followed by a 0.75 min rinse with the "acelarator" solution (solution of a sulphated polyanion in arginine buffer, pH 8.9), which adds a second layer of coating because a large number of anchor points form between the polycation and the polyanion. This double layer of polymer provides a large number of negative sulphated charges facing the inside of the capillary, creating a zeta potential higher than the original charge from the silanol groups. The sample was injected for 10 s by pressure (0.5 psi). The buffer solution was then injected by pressure (10 s) to clean the outside of the capillary and to push the sample inside. Separation of HBOCs was performed for 8.5 min in the same buffer solution (accelerator). Different separation voltage (17, 20 and 25 KV) and initial ramping 0.1, 1 and 1.5 KV/s) were tested. After separation, the coating was removed by first rinsing the capillary with the "conditioner" solution (0.2 M NaOH) for 1.0 min under pressure (25 psi) and then rinsing with distilled water for 0.5 min under pressure (25 psi). Detection was made at 405 nm using an on-line UV diode-array detector. Electropherograms were analyzed with the Beckman P/ACE Station 32 Karat software, Ver. 5.0.

Before analysis blood samples are pre-treated adding anti human haptoglobin antibody as described in a previous publication [4].

Results and Discussion

The method developed here, enables rapid analysis of the three HBOCs tested, offering a pre-confirmation alternative to the time-consuming, complex and expensive confirmation

procedures currently used by the antidoping laboratories. Sufficient resolution between the endogenous haemoglobin and the HBOCs tested was confirmed, allowing the discrimination between a normal haemolysed samples and a sample in which HBOCs are present. The optimum separation voltage was 25 KV (normal polarity) with an initial ramping of 1 KV/s and a separation time of 8.5 min at a capillary temperature maintained at 25 °C. The reliability of the method, in terms of specificity and repeatability of migration time (CV% < 1) was confirmed (see Figure 2). No significant interference was found at the expected migration time of each analyte (see Figure 1). Carryover signal was not detected in blank plasma samples that were injected in sequence after the analysis of the fortified plasma samples at the concentration of three times the LOD. In addition, LODs (1 mg/mL for Hemopure®, 2.5 mg/mL for Oxyglobin® and Polyheme®) was generally comparable with the gel electrophoresis and SEC-LC reference confirmation methods presently followed by the accredited sports anti-doping laboratories.

Additional experiments are currently in progress to verify the suitability of the proposed method for the detection of other HBOCs and to optimized the Hemopure® and Oxyglobin® separation. Finally, we are confident that this approach could be positively applied to the screening and confirmation analysis of other important peptides and proteins in doping control by linking CE separation properties with the powerful MS detection system.

References

1. The World Anti-Doping Code. The 2010 Prohibited List International Standard. World Anti-Doping Agency, Montreal, Canada, 2010. http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2010_EN.pdf
2. Simitsek PD, Giannikopoulou P, Katsoulas H, Sianos E, Tsoupras G, Spyridaki M-H, Georgakopoulos C.(2007) Electrophoretic, size-exclusion high-performance liquid chromatography and liquid chromatography-electrospray ionization ion trap mass spectrometric detection of hemoglobin-based oxygen carriers. Analytica Chimica Acta 583, 223-230.
3. Donati F, Botrè F. (2010) A fast screening method for the detection of the abuse of hemoglobin-based oxygen carriers (HBOCs) in doping control. Talanta 81, 252-254.
4. Lasne F, Crepin N, Ashenden M, Audran M, de Ceaurriz, J (2004) Detection of hemoglobin-based oxygen carriers in human serum for doping analysis: screening by electrophoresis. Clinical Chemistry 50, 410–415.
5. Varlet-Marie E, Ashenden M, Lasne F, Sicart M-T, Marion B, De Ceaurriz, J, Audran M. (2004) Detection of hemoglobin-based oxygen carriers in human serum for doping analysis: confirmation by size-exclusion HPLC. Clinical Chemistry 50, 723–731.
6. Trout G J, Kazlauskas R. (2004) Sports drug testing--an analyst's perspective. Chemical. Society. Review. 2, 1-13.

7. Thevis M, Ogorzalek Loo R R, Loo J A, Schänzer W. (2003) Doping control analysis of bovine hemoglobin-based oxygen therapeutics in human plasma by LC-electrospray ionization-MS/M. Analitycal Chemistry 75, 3287–3293.
8. Doelman C J, Siebelder C W M, Nijhof W A, Weykamp C W, Janssens J, Penders T J.(1997) Capillary electrophoresis system for hemoglobin A1c determinations evaluated. Clinical Chemistry 43, 644–648.
9. Staub A, Rudaz S, Saugy M, Veuthey J L, Schappler J. (2010) Analysis of hemoglobin-based oxygen carriers by CE-UV/Vis and CE-ESI-TOF/MS. Electrophoresis 31, 1241-1247

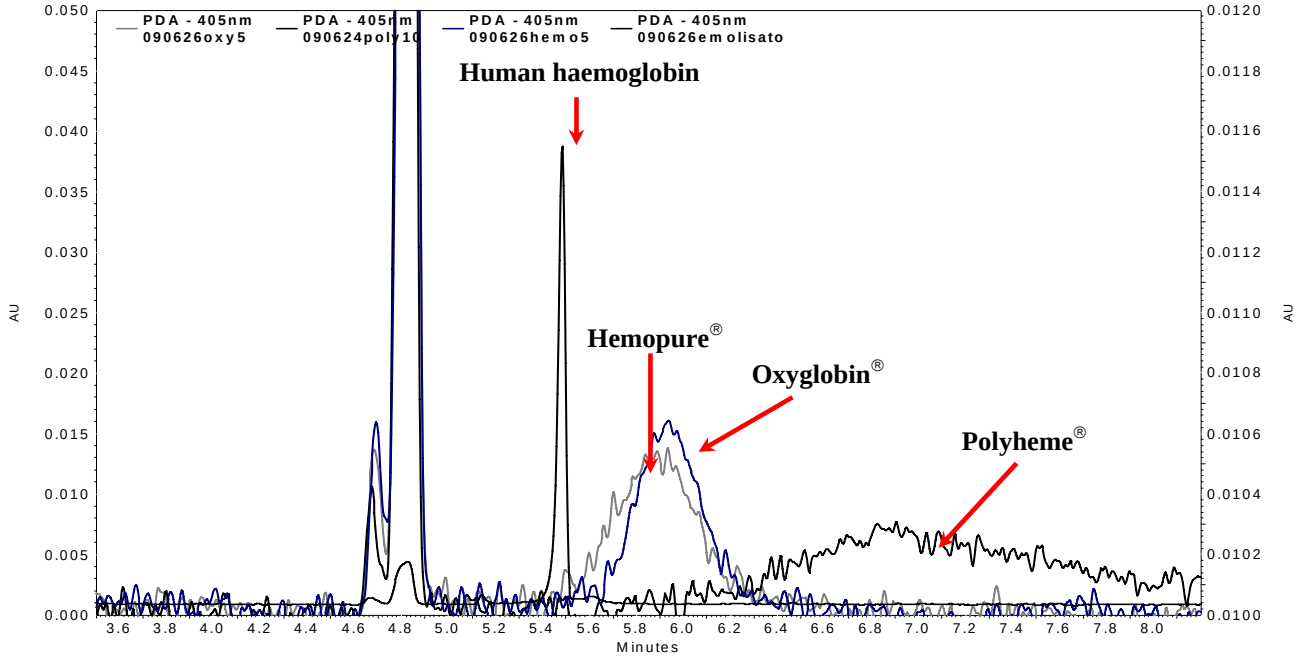

Figure 1

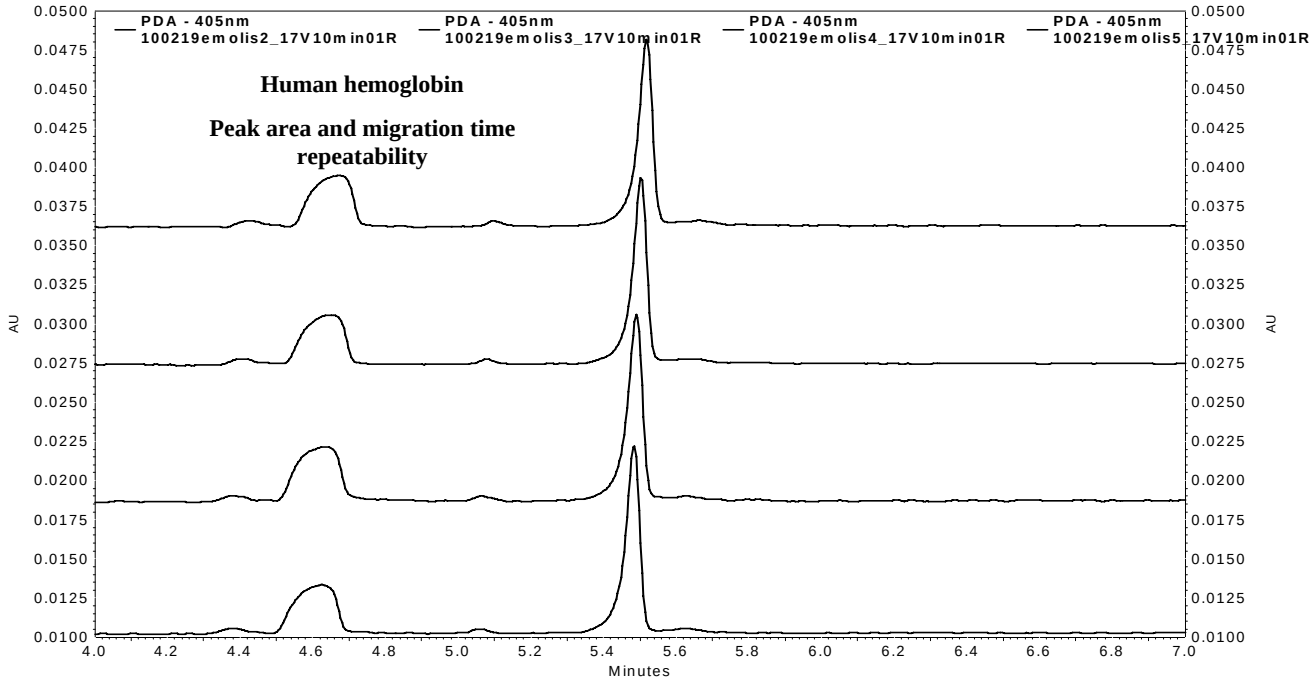

Figure 2