

Blood doping with hemoglobin-based oxygen carriers (HBOC): analysis by CE-UV/Vis and CE-ESI-TOF/MS

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Hemoglobin-based oxygen carriers (HBOC)



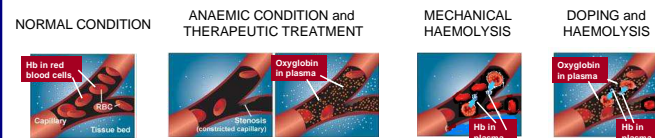
HBOC were initially developed as artificial blood replacement products to substitute the oxygen-carrying functions of erythrocytes. However, they are now misused in sport as performance enhancers. These products consist of **bovine or human hemoglobin (Hb)**, which is **polymerized**. Polymerizing of Hb molecules is necessary, otherwise Hb tetrameric structure dissociates into dimers, leading to nephrotoxicity.

	approved for veterinary use in US and Europe	} polymerized bovine hemoglobin
	phase III trials	
	phase III trials	} polymerized human hemoglobin
	phase III trials	

Analytical challenge

Oxyglobin as well as Hb can be found simultaneously in plasma since the former is a circulating polymer and the latter can be released from erythrocytes due to mechanical haemolysis (may occur from physical effort, such as running) and/or sampling conditions.

False positive results have to be eliminated.



Adapted from www.oxyglobin.com (accessed 10 August 2009)

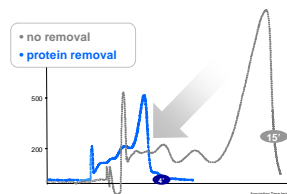
Sample preparation

Endogenous proteins, such as albumin and immunoglobulins, are present at high concentrations in plasma and could interfere with the separation and the detection.

Immunodepletion can remove interfering proteins with the following features:

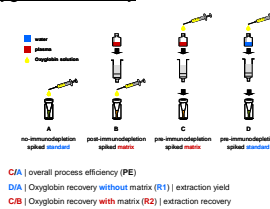
- specific interactions with **antibodies**
- the **20** most abundant proteins removed from plasma | haptoglobin (Hp) and Hp-Hb complexes totally removed
- spin column format | only **40 µL** of plasma
- **CE compatible** | direct injection, no desalting

1 Protein removal



50% protein removal
 no more CE adsorption and ESI suppression

2 Oxyglobin recovery

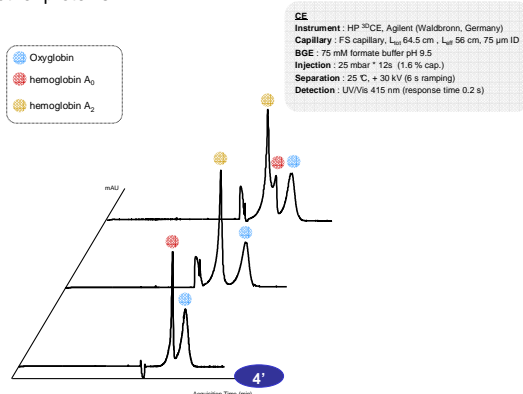


CA | overall process efficiency (PE)
 DA | Oxyglobin recovery without matrix (R1) | extraction yield
 CB | Oxyglobin recovery with matrix (R2) | extraction recovery

Process efficiency (PE) = 72%
 Extraction yield (R1) = 82%
 Extraction recovery (R2) = 75%

UV/Vis detection

Capillary electrophoresis (CE) appears to be an interesting technique for HBOC analysis since different methods have already been developed for the analysis of Hb variants. Sufficient electrophoretic selectivity between Oxyglobin and main Hb variants was reached with a BGE at pH 9.5. An interesting selectivity was obtained with the UV/Vis detection at **415 nm**. At this wavelength, hemoproteins such as Oxyglobin and Hb were easily observed due to their heme moiety in contrast to other proteins.

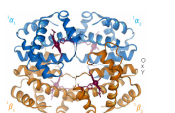


LOD (UV/Vis)
 0.20 g/dL in plasma | 13 µM
 1 pmol (16 ng) injected

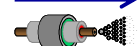
ESI-TOF/MS detection

Although Hb and Oxyglobin migrated under their intact form, they were detected according to their respective dissociated chains. Monomers were detected because **in-Taylor cone dissociation** of the tetramers occurred afforded by the sheath liquid.

CE migration
 tetramers of 4 globins

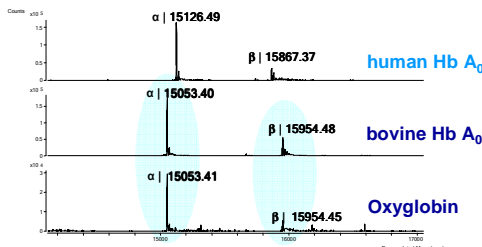


Alcoholic and acidic conditions



ESI-TOF/MS detection
 individual globins

Because human and bovine Hb possess different polypeptide sequences, discrimination between both species was possible. Oxyglobin being constituted of bovine Hb, it presented a different deconvoluted mass spectra than human Hb.



CE Instrument: HP 100CE, Agilent (Waldbronn, Germany)
 Capillary: FS capillary, L₀ 80 cm, 75 µm ID
 BGE: 75 mM formate buffer pH 9.5
 Injection: 50 mbar * 10s (1.5% cap.)
 Separation: 25 °C, + 30 kV (6 s ramping)
 ESI Sheath liquid: iPrOH:H₂O (50:50) + 3% formic acid, 3 µL·min⁻¹
 Nebulizing gas: 4 psi
 Drying gas: 4 L·min⁻¹, 150 °C
 Ionization voltage: + 4500 V
 MS Instrument: 6210 LC/MS TOF mass spectrometer, Agilent (Waldbronn, Germany)
 Detection: scan range 900-2500 m/z, scan rate 1 spectrum/s

LOD (TOF/MS)
 0.45 g/dL in plasma | 29 µM
 2 pmol (36 ng) injected

Human Hb presented α and β chains of 15126 and 15867 amu respectively, while bovine Hb and Oxyglobin presented α and β chains of 15053 and 15954 amu respectively. **Unambiguous identification of Oxyglobin was thus possible.**

Conclusions

Immunodepletion prior to the CE-UV/Vis-ESI-TOF/MS analysis of Oxyglobin in plasma was achieved, providing four complementary analytical steps.

The methodology could be implemented in doping control.

