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Detection of Hemopure® and Hemoglobin in Human Plasma by HPLC/UV and Different Types of Columns – an Approach to Screen for this Substance in Doping Analysis

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Abstract

A first approach is presented to detect the misuse of Hemopure®, a hemoglobin based oxygen carrier (HBOC) in athletes' blood samples. After visual screening of plasma samples, which show broad red coloration at concentrations expected after administration of a recommended single dose, a second step is a screening for cell free human hemoglobin (hHb) with the ADVIA 120 hematology system, followed by a high performance liquid chromatographic (HPLC) separation in order to distinguish between hHb and Hemopure®. The type of HPLC column, size-exclusion- or ion-exchange-chromatography as well as the HPLC conditions have been optimised.

Introduction

Endogenous hemoglobin circulates within red blood cells as a tetramer. When free hemoglobin is transfused, the tetramers rapidly break down into dimers and monomers. These small molecules then freely diffuse into the renal tubules and the subendothelium. In order to decrease the toxicity of hemoglobin solutions, manufacturers have had to develop methods to stabilize the hemoglobin tetrameric structure and increase its size. Several procedures have been established, and one is used for Hemopure®, a product of the Biopure Corporation (Cambridge, Massachusetts, USA). Hemopure® is a new generation of blood substitutes which belongs to the group of hemoglobin based oxygen carriers. It consists of glutaraldehyde-polymerized bovine hemoglobin formulated in modified Ringer's lactate (Figure 1) [1].

Hemopure® is indicated for the treatment of adult surgical patients who are acutely anaemic (i.e. blood loss in result of an accident). These non-cellular hemoglobin molecules circulate in plasma, are much smaller and have lower viscosity and more readily release oxygen to tissues than red blood cells. They provide oxygen to areas of the body that red blood cells cannot

reach. The product is currently being evaluated for human use in a multinational Phase III clinical trial. It is recommended to administer Hemopure® by *i.v.* infusion at a flow rate of 1g/minute. One infusion bag contains 30 g crosslinked bovine hemoglobin in 250 ml Ringer's lactate. The plasma elimination half-life is approximately 24 hours. [1]

Hemopure® and all other HBOCs might be misused in endurance sports to improve the maximal oxygen capacity which is one of the limiting factor of endurance performance. There is one study which describes a greater oxygen uptake and lower lactate levels in submaximal exercise when Hemopure® is compared to an autologous transfusion [2].

Since 2003, HBOCs are banned in sport, as they belong to the list of prohibited methods (enhancement of oxygen transfer; IIAb) in the antidoping regulations of the International Olympic Committee [3].

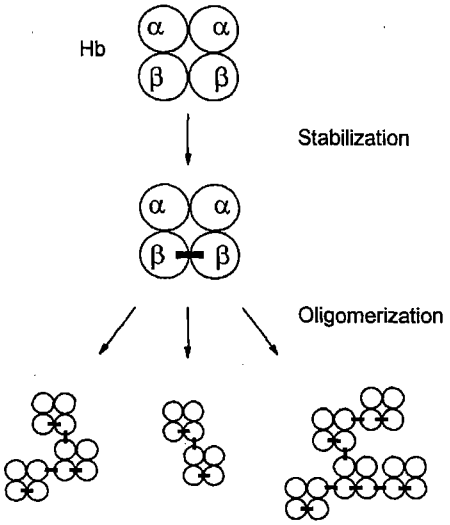

Figure 1: Stabilization and oligomerization of Hemoglobin (Hb). Stabilization involves intramolecular cross-linking of the β globin chains. (from [4])

Experimental

Chemicals and supplies:

In order to test the separation capacity of the size exclusion columns, a gel filtration standard from BIO RAD (München, D) containing Thyroglobulin, bovine 670 kDa; Gamma globulin, bovine 670 kDa; Ovalbumin, chicken 44 kDa; Myoglobin, horse 17 kDa and Vitamine B12, 1.35 kDa, was analysed with each batch of samples. Human hemoglobin (hHb) was obtained

from SIGMA (Deisenhofen, D). Formic acid, magnesium chloride, Bis-Tris (all from SIGMA, Deisenhofen, D), sodium chloride, ethylenediaminetetraacetic acid (EDTA; MERCK, Darmstadt, D) and methanol (J.T.BAKER, Deventer, NL) were used for the preparation of the different mobile phases. All chemicals were of p.a. quality. Purified water was obtained from a Milli-Q reagent-grade water system (MILLIPORE, Eschborn, D). Hemopure® was a kind gift of the BIOPURE Corporation (Cambridge, Massachusetts, USA). Human blood samples were taken by vene punction of healthy male volunteers.

HPLC Columns

The following HPLC columns were tested:

Size exclusion column

- VA 300/7.7 Nucleogel GFC 1000-8, (MACHEREY&NAGEL, Düren, D)
  for molecules with a molecular weight ranging from 20 kDa to 2.000 kDa
- TSKgel G 3000 SW, 7,5 mm ID x 300 mm (TOSOH BIOSEP, Stuttgart, D)
  for globular proteins with a molecular weight ranging from 10 kDa – 500 kDa

Ion exchange column

- EC 250/4 Nucleosil 4000-7, 4mm ID x 25cm (MACHEREY&NAGEL, Düren, D)

Sample preparation and HPLC parameters:

The lyophilised BIO RAD gel filtration standard was prepared following the manufacturer's instructions. To prepare a Hemopure® standard at a concentration of 1 mg/ml, 20 µl of the original Hemopure® solution (0.13 g/ml) were added to 2.58 ml H_20 dest. A hemoglobin standard was prepared by solving 1,3 g hemoglobin in 10 ml H_2O dest. and diluted in same way.

For the preparation of human plasma standards, blood was centrifuged 20 minutes at 2000 g at 4° Celsius and spiked with Hemopure® at concentrations of 12, 21 and 31 mg/ml. All standards and plasma samples were filtered through a 0,45µm filter unit (Spartan 13/0,45 RC, SCHLEICHER+SCHUELL, Einbeck, D) before injection into the HPLC system.

The analyses were performed on a liquid chromatograph HP1090, Series II, with diode array detector (DAD) and automatic injection system (HEWLETT PACKARD, Böblingen, D). The LC and DAD conditions were as follows: flow rate 1 ml/min, injection volume 10 µl, mobile

phase (1) 0,75 M MgCl2, 0,05 M Bis-Tris, 0,1 mM EDTA or (2) 0,1 M formic acid, 0,05 M NaCl, 5% methanol, isocratic. The mobile phase was degassed and filtered through a nylon filter membrane, pore size 0.45 µm, under vacuum prior use. The analysis was monitored at two different wavelengths: 280 nm for the gel filtration standard and 420 nm for hHb and Hemopure®. Cell-free Hb was estimated with the ADVIA 120 hematology system (BAYER, Leverkusen, D).

Results

Table 1 lists the results, which were obtained with the hematology system ADVIA 120. Plasma samples, which were spiked with Hemopure® at 3 different concentrations (12, 21 and 31 mg/ml) showed a cell-free hHb value comparable to the added amount. In all 3 plasma samples the red colour was obvious.

Table 1: Comparison of cell-free Hb, estimated by ADVIA 120 hematology system, and visual colour test of plasma samples spiked with different amounts of Hemopure®

Hemopure® added to plasma [mg/ml]	Estimated cell-free Hb [mg/ml]	Visual plasma colour test
0	0	-
12	11	+
21	21	+
31	31	+

The HPLC-UV chromatograms in Figure 2 shows the BIO RAD gel filtration standard containing Thyreoglobulin 670 kDa, Gamma globulin 158 kDa, Ovalbumin 44 kDa, each at a concentration of 10 mg/ml, Myoglobulin 17 kDa, at a concentration of 5 mg/ml and Vitamine B12 1,35 kDa at a concentration of 1 mg/ml. This gel filtration standard was analysed under the same conditions as the hHb and Hemopure® standard solutions (Figure 3) as well as the blank plasma sample (Figure 4) on the size exclusion column from Tosoh Biosep, 10 kDa – 500 kDa. Figure 5 compares the HPLC-UV chromatograms of the blank human plasma, the hHb standard solution and the Hemopure® standard solution. All 3 compounds were clearly separated under the described analytical conditions. The size exclusion column from Macherey&Nagel shows only one sharp peak for Hemopure® and its subunits (Figure 6), which is also separated from hHb.

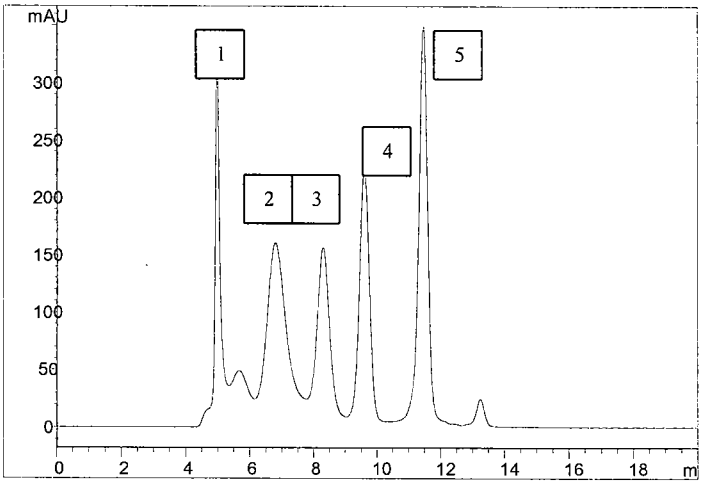

Figure 2: HPLC-UV chromatogram of the BIO RAD gel filtration standard: [1] Thyreoglobulin 670 kDa, [2] Gamma globulin 158 kDa, [3] Ovalbumin 44 kDa, each at a concentration of 10 mg/ml, [4] Myoglobulin 17 kDa, at a concentration of 5 mg/ml and [5] Vitamine B12 1,35 kDa at a concentration of 1 mg/ml. Column: Tosoh Biosep (size exclusion, for details see text), injectionvol.: 20 µl, solvent: 0.1M NaH_2PO_4, 0.1M Na_2SO_4, 0.05% NaN_3, pH: 6.8, isocratic at a flow rate of 1 ml/min, DAD: 280 nm

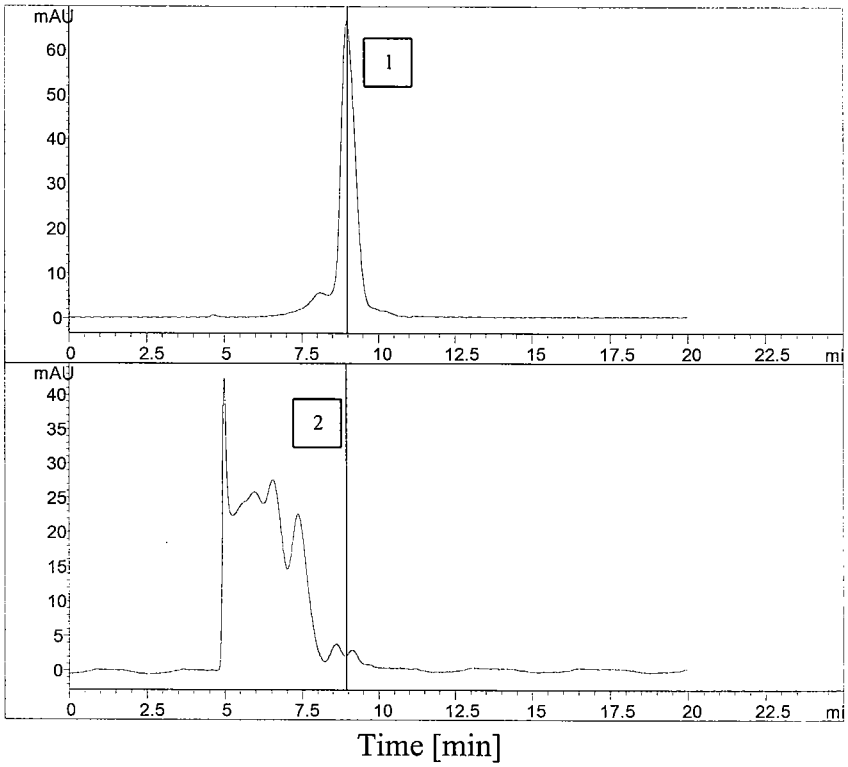

Figure 3: HPLC-UV chromatogram of [1] hHb standard solution (1 mg/ml) and [2] Hemopure® standard solution (1 mg/ml). Column: Tosoh Biosep (size exclusion, for details see text), injectionvol.: 20 µl, solvent: 0.1M NaH_2PO_4, 0.1M Na_2SO_4, 0.05% NaN_3, pH: 6.8, isocratic at a flow rate of 1 ml/min, DAD: 420 nm.

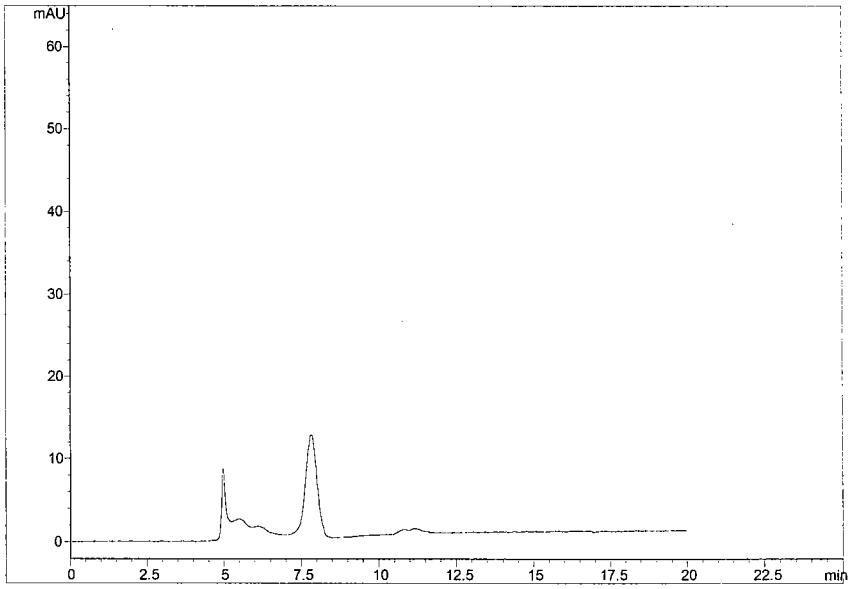

Figure 4: HPLC-UV chromatogram of human plasma. Column: Tosoh Biosep (size exclusion, for details see text), injectionvol.: 20 µl, solvent: 0.1M NaH_2PO_4, 0.1M Na_2SO_4, 0.05% NaN_3, pH: 6.8, isocratic at a flow rate of 1 ml/min, DAD: 420 nm

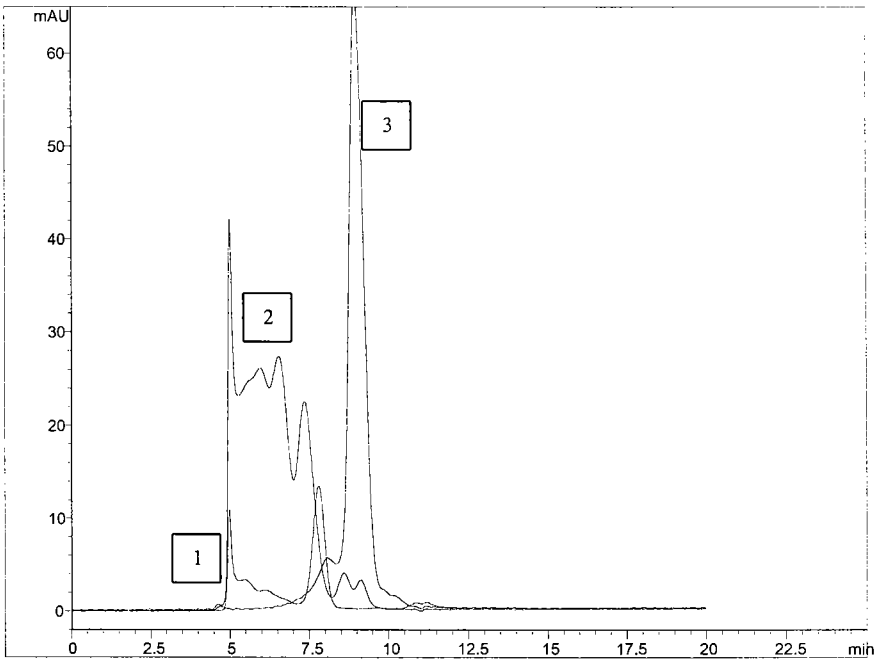

Figure 5: Comparison of 3 HPLC-UV chromatograms [1] human plasma, [2] Hemopure® standard solution (1 mg/ml) and [3] hHb standard solution (1 mg/ml). Column: Tosoh Biosep (size exclusion, for details see text), injectionvol.: 20 µl, solvent: 0.1M NaH_2PO_4, 0.1M Na_2SO_4, 0.05% NaN_3, pH: 6.8, isocratic at a flow rate of 1 ml/min, DAD: 420 nm

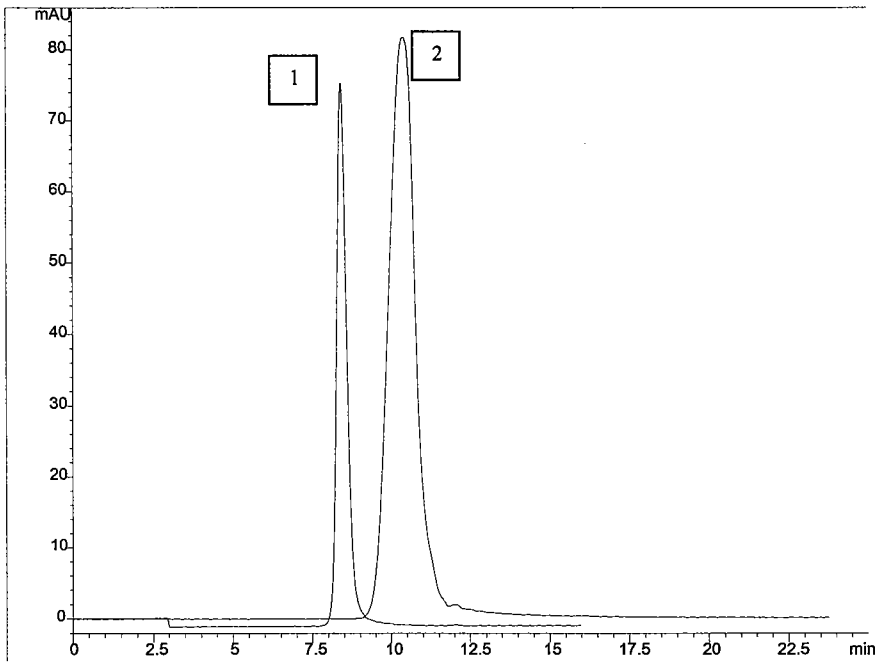

Figure 6: Comparison of 2 HPLC-UV chromatograms [1] Hemopure® standard solution (1 mg/ml) and [2] hHb standard solution (1 mg/ml). Column: Macherey&Nagel (size exclusion, for details see text), injectionvol.: 20 µl, solvent: 0.1M NaH_2PO_4, 0.1M Na_2SO_4, 0.05% NaN_3, pH: 6.8, isocratic at a flow rate of 1 ml/min, DAD: 420 nm

Discussion

The approach to detect doping abuse with Hemopure® is divided into three parts: the first check is an optical proof of the plasma sample and judging its possible red coloration. Hemopure® solutions will make the plasma specimens appear hemolyzed. This red coloration was observed in concentrations as low as 0.2 mg/ml (not shown). This concentration is below the concentrations, which are expected shortly after an *i.v.* infusion of one bag of Hemopure® (250 ml/30 g HBOC) [5].

- 5000 ml blood in human body
- 30 g crosslinked bovine hemoglobin in 5250 ml = 5.7 mg/ml

In a second step, the attained plasma of the blood sample is tested by the ADVIA 120 hematology system to estimate the cell free hemoglobin concentration. The results indicate that it is possible to distinguish clearly between cell free hemoglobin, in our case Hemopure®, and the cell hemoglobin. The third step, HPLC size-exclusion-chromatography

and UV detection, is necessary to separate hHb and Hemopure®. The size exclusion column TSKgel G 3000 SW, 7,5 mm ID x 300 mm from TOSOH BIOSEP showed the best results in separating both compounds [6]. Different mobile phases have been tested without any significant difference in separation of the compounds. The mobile phases with the lowest ionic concentration have been chosen in order to minimize interference in any following confirmatory step. The BIO RAD gel filtration standard is a helpful tool to appraise the separation capacity of the size exclusion columns if it is analysed daily prior to each batch of samples. An interesting approach was the Macherey&Nagel size exclusion column which did not separate the different subunits of Hemopure®, but resulted in one sharp peak which could be easily fractionated for further follow-up steps. There was also a clear separation between hHb and Hemopure®. The results obtained with a HPLC column of the ion exchange type, were not satisfying, and no further investigations have been performed.

Analytical methods to confirm the presence of the fractionated Hemopure® after HPLC separation, e.g. LC-MS-MS analysis after a tryptic digestion have to be developed in a future project. Further investigation will be necessary.

Acknowledgement

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