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Detection of Hemoglobin-Based Oxygen Carriers in Human Serum for Doping Analysis: Confirmation by Size-Exclusion HPLC

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Background: Hemoglobin-based oxygen carriers (HBOCs) are being developed as potential substitutes for the oxygen-carrying functions of erythrocytes, but athletes may obtain and experiment with HBOCs as an illicit means of enhancing oxygen transport. An electrophoretic technique has been developed to screen for the presence of HBOCs in blood samples (Lasne et al. *Clin Chem* 2004;50:410–5). Interest has focused on complementary methods that can provide legally defensible scientific evidence for the presence of HBOCs in blood samples collected for doping control.

Methods: The aim of this research was to develop a size-exclusion SEC-HPLC technique to identify in plasma or serum samples the presence of HBOCs that are currently under development. This method was also used to detect a polymerized bovine hemoglobin (Hemopure[®]) after infusion in 12 healthy males.

Results: The chromatograms of all HBOCs tested were clearly separated from the 54-min peak associated with human hemoglobin dimers. It was possible to differentiate between the different HBOC products based solely on their chromatographic profiles, provided they were at high concentrations. Differences were discernible not only based on the presence (or absence) of peaks, but also the separation between respective peaks. The profiles for serum samples collected from the men immediately after infusion of Hemopure showed a distinctive

profile. The shape of the chromatographic profile remained consistent for at least 48 h.

Conclusions: Under the analytical conditions reported here, SEC-HPLC was able to separate native hemoglobin from the modified hemoglobin molecules present in each of the HBOC products studied. In tandem with electrophoretic screening, SEC-HPLC provides evidence of the presence of HBOCs and can therefore be regarded as a method that satisfies the criteria for use in an antidoping control setting.

Hemoglobin-based oxygen carriers (HBOCs)⁴ are being developed as substitutes to replace the oxygen-carrying functions of erythrocytes and thereby lessen the demand on donor blood supplies during surgery and trauma situations (1). Hemoglobin is the protein in erythrocytes responsible for the transport of oxygen from the lungs to other tissues in the body; it exists as a tetramer containing two α - and two β -subunits. If removed from the erythrocyte, hemoglobin dissociates into small α - β dimers, causing renal toxicity (2). Therefore, chemical modification of extracellular hemoglobin is essential, both to prolong its vascular half-life and to modify its oxygen affinity to allow efficient delivery of oxygen.

First-generation HBOCs were developed principally to serve as oxygen carriers and as a substitute for erythrocytes in certain clinical conditions, such as perioperative use (3). However, depending on the nature of the modification used to stabilize the protein, next-generation HBOCs could be useful in other clinical settings, such as enhancement of radiation therapy and nitric oxide scav-

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⁴ Nonstandard abbreviations: HBOC, hemoglobin-based oxygen carrier; SEC, size-exclusion chromatography; LOD, limit(s) of detection; mAU, milli-absorbance units; and AUC, area(s) under the curve(s).

enging (4). As academic and industrial research hastens the development and imminent commercial release of variants on the HBOC theme [Hemopure[®] has received approval for routine human clinical use in South Africa (5)], the likelihood increases that athletes could obtain access to and experiment with HBOCs as an illicit means of enhancing oxygen transport and thereby endurance performance.

Several HBOCs are currently under development (3, 6). Three principle approaches have been used to stabilize and modify the hemoglobin molecule: polymerization, binding of polymers to the surface of the hemoglobin, or cross-linking the α - and β -dimers. Glutaraldehyde has been used to polymerize bovine hemoglobin (Oxyglobin[®] and Hemopure; Biopure Corporation) as well as pyridoxylated human hemoglobin (PolyHeme[®]; Northfield Laboratories), although a different cross-linking agent, O-*raffinose*, has also been used to polymerize human hemoglobin (Hemolink[™]; Hemosol Ltd). Human hemoglobin has also been conjugated using polyoxyethylene (PHP[®]; Apex Bioscience) or maleimide-polyethylene glycol (Hemospan[®]; Sangart Inc.). Clinical trials have been halted on a diaspirin cross-linked tetrameric hemoglobin (DCLHemoglobin[®]; Baxter Healthcare), but a tetrameric human hemoglobin comprising two stable α - β -subunits derived from genetically engineered *Escherichia coli* is currently under development.

A commonality of all HBOCs is their resistance to dissociation when dissolved in a medium such as concentrated magnesium chloride solution. This is in contrast to native hemoglobin, which dissociates into two dimers under these nonphysiologic conditions. The resulting difference in molecular size should allow the two classes of hemoglobin to be separated by size-exclusion HPLC (SEC-HPLC).

The aim of this research was therefore to develop a SEC-HPLC technique able to identify the presence of HBOCs in plasma or serum samples. This method is intended to complement the electrophoretic technique for detection of HBOCs (7); these two methods together would provide legally defensible scientific evidence for the presence of HBOCs in blood samples collected for doping control.

Materials and Methods

ANALYTICAL SAMPLES

After establishment of appropriate Material Transfer and Confidentiality Agreements, HBOC products were provided by the respective pharmaceutical companies developing each product: Oxyglobin and Hemopure (glutaraldehyde-polymerized bovine hemoglobin; Biopure Corporation), PolyHeme (glutaraldehyde-cross-linked pyridoxylated human hemoglobin; Northfield Laboratories), PHP (pyridoxylated human hemoglobin-polyoxyethylene conjugate; Apex Bioscience Inc.), Hemospan (maleimide-polyethylene glycol-conjugated human hemoglobin; Sangart Inc.), and HemAssist[®] (diaspirin-cross-

linked human hemoglobin; Baxter Healthcare Co). We were unable to obtain the product Hemolink from its manufacturer, Hemosol Inc. All products were stored at 4 °C and diluted with human serum before analysis. Human serum was provided by the French Pyrenees-Mediterranean Establishment of Blood (Montpellier).

PRINCIPLE OF DETECTION METHODOLOGY

All of the HBOC compounds, as a result of the presence of the heme moiety, show the same absorbance spectrum as hemoglobin with a peak between 300 and 450 nm and maximum absorbance at 415 nm. They are separated from endogenous hemoglobin by SEC and are detected at 415 nm by a diode array detector. This enables the ultraviolet spectrum of the compound to be obtained. This method was applied to serum samples to which each of the HBOC products had been added, as well as to serum collected from study participants infused with 30 or 45 g of Hemopure. Although this methodology is equally applicable to the evaluation of plasma, serum was used in this study because this medium gave a notable extension of column life.

CHEMICALS AND REAGENTS

The mobile phase was prepared from bis-Tris {bis[2-Hydroxyethyl]imino-tris[hydroxymethyl]methane}, magnesium chloride hexahydrate, EDTA, and aqueous 5 mol/L HCl solution (all from Sigma Aldrich). Ultrapure water (Simplicity[®] System; Millipore) was used. Polyclonal rabbit anti-human haptoglobin (Dakocytomation S.A.) was used to remove haptoglobin from serum samples before analysis.

EQUIPMENT AND CHROMATOGRAPHIC CONDITIONS

The analysis was conducted with an Agilent HPLC system composed of a HP 1100 series injector, series 1050 degasser, series 1050 pump, and series 1100 diode array detector. The chromatographic separation was performed on a Superdex 200HR 10/30 column (Amersham Pharmacia Biotech; bed dimensions, 10 × 300–310 mm; bed volume, 24 mL; nominal bead size, 13 μ m) at 25 °C. The mobile phase consisted of 0.75 mol/L MgCl₂, 50 mmol/L bis-Tris buffer, and 0.1 mmol/L EDTA adjusted to pH 6.5 with 5 mol/L HCl solution. This mixture was filtered through a 0.45 μ m membrane (Interchim). A 0.5 μ m stainless steel frit (Upchurch Scientific Inc.) was placed between the injector and the column. Data acquisition and treatment were performed with a HP ChemStation 6.03.

Serum hemoglobin was also measured with a HemoCue[®] HEMOGLOBIN photometer (HemoCue AB) (8).

IMMUNOPRECIPITATION OF HAPTOGLOBIN

Free hemoglobin is rapidly bound by haptoglobin and subsequently removed from circulation. Preliminary research revealed that haptoglobin-hemoglobin complexes

were of comparable molecular size to the largest polymers found in some HBOC products and typically eluted between 26 and 36 min. Haptoglobin, as well as the hemoglobin-haptoglobin complex, can be successfully removed by treatment of the serum sample with an anti-haptoglobin antibody.

The reference interval for haptoglobin in serum is 0.5–2.2 g/L. The concentration increases in inflammation, collagen and vascular disorders, and infections as well as during treatment with androgens; however, concentrations >5 g/L are rare. Various permutations using different volumes of serum and antibody and various incubation conditions led us to propose the following simple protocol for the treatment of serum: 30 μ L of serum and 30 μ L of a 4 \times concentrated solution of anti-human haptoglobin (polyclonal rabbit anti-human haptoglobin antibodies from Dako; antibody titer, 5.1 g/L) are incubated at ambient temperature for 60 min, followed by centrifugation for 10 min at 16 000g. A 20- μ L volume of the supernatant was then injected into the HPLC system.

SPECIFICITY

The specificity of the method was verified against endogenous compounds present in serum. Different serum pools and serum samples from a different group of 12 healthy individuals were tested for the absence of interfering compounds.

LIMITS OF DETECTION

In most situations limits of detection (LOD) are derived by defining an acceptable single peak height in relation to the background noise (e.g., 3:1 ratio). However because of the broad, multiplex spectrum characteristic of HBOC products (associated with the different molecular size species typically evident in HBOC products), the absence of a single definable peak prevents the specification of a LOD derived from a simple signal-to-noise ratio (with the exception of HemAssist, which has only a single peak). An alternative approach to establish the LOD is to evaluate the ultraviolet-visible spectrum for evidence of absorbance by the heme moiety, which is present in all HBOCs, as well as hemoglobin and myoglobin. Because neither hemoglobin nor myoglobin elutes between 30 and 35 min, the absorbance in the typical characteristic ultraviolet-visible spectrum during this interval can be attributable only to nonendogenous heme moieties (i.e., HBOCs) and can therefore be used to define the LOD. A spectrum characteristic of heme with a maximum intensity of 3 milliabsorbance units (mAU) was considered as a LOD.

PARTICIPANTS

Twelve males [mean (SD) age, 24.4 (2.1) years; height, 177.8 (2.1) cm; weight, 69.2 (2.8) kg] were enrolled in and completed the study. All participants were healthy and engaged in aerobic exercise for at least 20 min three times per week. This study was conducted according to the

Declaration of Helsinki as amended in the 41st World Medical Assembly (Hong Kong 1989) and was reviewed and approved by the Regional Ethics Committee. Participants were included in the study after giving informed consent.

ADMINISTRATION TRIAL OF HEMOPURE

Serum samples were obtained from healthy volunteers who were infused with either 30 or 45 g of Hemopure (250 or 375 mL of a sterile solution of polymerized bovine hemoglobin in a solution similar to Ringer's lactate). The nominal hemoglobin concentration in Hemopure was 130 (10) g/L, and the percentage of methemoglobin was <15%. Hemopure was infused at a rate of 0.33 or 0.5 g/min over a 90-min period (for 30- and 45-g doses, respectively). Blood samples were collected into one 5-mL serum BD VacutainerTM Z Tube (BD Vacutainer Systems) from an antecubital vein before infusion, immediately at the end of the 90-min infusion procedure, and on days 1, 2, 3, 5, and 8 postinfusion. Samples were also collected on day 4 from six volunteers. Serum samples were frozen and kept at -20°C until they were analyzed, except for four samples, which were analyzed immediately before freezing.

CALIBRATION CURVE

Calibrators in serum were prepared at Hemopure concentrations of 0.406, 1.625, 3.250, 6.500, 13.000, and 26.000 g/L. Each concentration was injected twice. Peaks were integrated between 26 and 46 min, and a regression equation was derived for the relationship between the area under curve and the concentration of Hemopure in the serum sample.

PRECISION AND ACCURACY

Three concentrations of Hemopure (quality-control samples) in serum were prepared [high (10.00 g/L), medium (2.50 g/L), and low (0.81 g/L)] and stored at 5°C . Between-day reproducibility was determined by analyzing the quality-control samples against the calibration curve. The recovery was calculated as (mean measured concentration/theoretical concentration) multiplied by 100. Precision was determined by the CV.

STABILITY OF SAMPLES DURING STORAGE

The manufacturer's recommendations specify that Hemopure should be stored at room temperature ($2\text{--}30^{\circ}\text{C}$). To evaluate the stability of serum samples under conditions that might be encountered during antidoping evaluation, three samples with Hemopure added and three prepared serum samples (supernatant after addition of anti-haptoglobin antibody) were reanalyzed after 10 and 26 h of storage at ambient temperature. Using identical preparation procedures, we also reanalyzed four serum samples after 10 days of storage at -80°C .

STATISTICAL ANALYSIS

Calibration curves were obtained from unweighted least-squares linear regression analysis of the data. The resulting slopes and intercepts were used to obtain concentration values for the quality-control samples and the unknown samples. We evaluated the quality of the fit by comparing back-calculated concentrations with the nominal ones. The linearity of the method was confirmed by classic statistical evaluation, i.e., comparison of the intercept with 0 and the correlation coefficients with 1. In addition, the gaussian distribution of the residuals (difference between nominal and back-calculated concentrations) was verified.

Results

SPECIFICITY

The chromatogram of a pool of nonhemolyzed human serum revealed four peaks, including three of very low intensity (<5 mAU) at 27, 38, and 42 min, and a narrow peak of considerable intensity (50 mAU) at 47 min. However, the spectra of these different peaks, with a strong absorbance band around 260 nm and a weak one between 400 and 500 nm, were totally different from the spectra of the HBOCs. Serum samples from our 12 volunteers were treated with the same approach, and the chromatograms derived were quite similar. The peak at 47 min, although always present, was found to be quite variable among our volunteers, although it did not exceed 25 mAU in any sample. The height of these peaks decreased by one-half after treatment with an equal volume of antibody solution.

As illustrated in Fig. 1, when exposed to the dissociative medium the hemoglobin molecule is cleaved into two dimers, which demonstrate a peak at 54 min. The treatment of the hemolyzed serum sample with the anti-haptoglobin antibody solution makes it possible to remove the hemoglobin-haptoglobin complex, whereas the unbound hemoglobin dimers are still apparent at 54 min.

With one volume of the (4×) concentrated solution of the anti-haptoglobin antibody, we were able to remove all of the haptoglobin in an equal volume of serum with a haptoglobin concentration of 5.90 g/L.

CHROMATOGRAM OF HBOC PRODUCTS ADDED TO SERUM

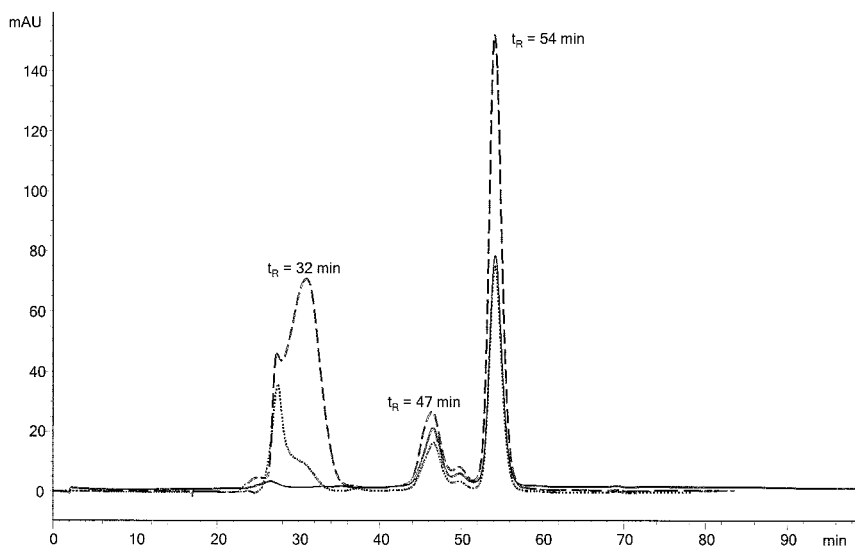
The chromatograms of all HBOCs tested were clearly separated from the 54-min peak associated with human hemoglobin dimers. The profiles from Oxyglobin, Hemopure, and HemAssist, which are either available commercially or have ceased development, are included in Fig. 2 (chromatograms of the remaining HBOCs were not published because of the confidential nature of ongoing research and development of these products). With the exception of HemAssist, which showed a major peak at 50 min that was preceded by a low-intensity peak at 44 min and a smaller peak at 40 min, each of the remaining HBOC products was found to have a broad but heterogeneous profile evident between 26 and 52 min. The narrow peak at 54 min corresponds with tetrameric hemoglobin (two dimers), which represents only a small portion of the molecular profile of most HBOC products (i.e., unmodified hemoglobin species). It was possible to differentiate between the different HBOC products based solely on their chromatographic profiles, provided they were at high concentrations (corresponding roughly with the concentration expected in serum 2 days postinfusion of 30–45 g of HBOC). Differences were discernible not only in the presence (or absence) of peaks, but also the separation between respective peaks.

LOD

The LOD for the different HBOC products were 0.2 g/L (Oxyglobin and Hemopure), 0.3 g/L (HemAssist and PolyHeme), 0.5 g/L (PHP), and 0.8 g/L (Hemospan).

Fig. 1. Chromatogram of a hemolyzed serum sample before (*dashed line*) and after (*dotted line*) treatment with an equal volume of anti-haptoglobin antibody and an equal volume of a 4× concentrated anti-haptoglobin antibody solution (*solid line*).

The peak at 54 min corresponds to human hemoglobin dimers; the broad peak at ~30 min corresponds to hemoglobin-haptoglobin complexes. t_R , retention time.



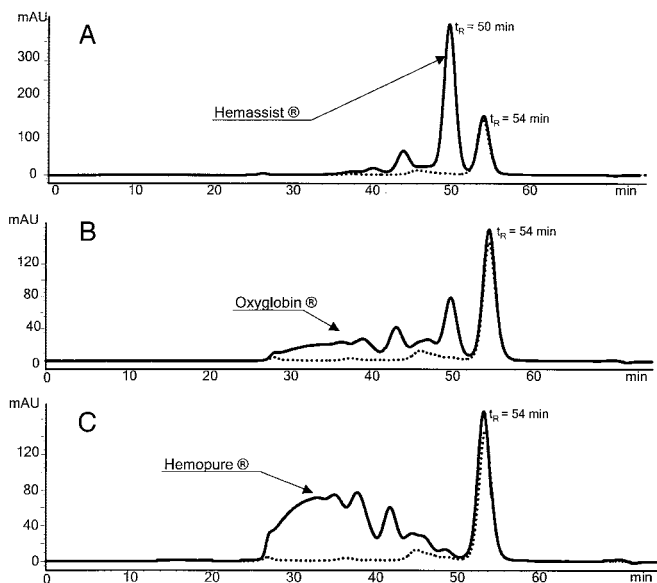


Fig. 2. Chromatograms of samples containing hemolyzed serum to which HemAssist (A), Oxyglobin (B), or Hemopure (C) was added (solid line) compared with the chromatogram of a sample containing only hemolyzed serum (dotted line).

t_R , retention time.

HEMOPURE CALIBRATION CURVES

For calibration curves prepared on different days ($n = 9$), linear relationships were obtained between the area under curve and the concentration of Hemopure in the serum sample ($y = 8328x - 378$; $r = 0.999$). The correlation coefficients (r) for calibration curves were ≥ 0.996 . Table 1 reports concentrations calculated from the corresponding calibration lines. The linearity of this method was statistically confirmed. For each calibration curve, linear regression of the calculated concentrations vs the nominal ones provided a unit slope and an intercept not statistically different from 0 (Student t -test). The distribution of the residuals (difference between nominal and calculated concentrations) showed random variations, the numbers of positive and negative values being approximately equal. Moreover, they were gaussian distributed and centered on 0.

Table 1. Interday precision and accuracy from calibration curves prepared on different days ($n = 9$).^a

Theoretical concentration, g/L	Calculated concentration, g/L	CV, %	Recovery, %
0.406	0.460	6.90	113.2
1.625	1.619	3.09	99.6
3.250	3.170	2.83	97.4
5.000	5.040	5.20	100.7
6.500	6.510	2.14	100.2
13.000	13.000	0.36	100.0
26.000	25.880	1.53	99.5

^a Linear relationships were obtained between the AUC and the concentration of Hemopure in the serum sample ($y = 8328x - 378$ g/L; $r = 0.999$).

PRECISION AND ACCURACY

For the calibrators, the precision around the mean value ranged from 0.36% to 6.90% (Table 1). For the QC samples, the results for the precision and accuracy (interday) of the method are given in Table 2. For each point on the calibration curves, the concentrations were recalculated from the equation of the linear regression curves (experimental concentrations), and the CV were computed.

STABILITY OF SAMPLES DURING STORAGE

The shapes and the areas under the curve (AUC) of the Hemopure peaks were unchanged after storage for 10 and 26 h at ambient temperature. After 10 days of freezing, the AUC was lower by 1–6%.

CHROMATOGRAM OF HEMOPURE IN BIOLOGICAL SAMPLES

The profiles from serum samples collected from volunteers immediately after infusion of either 30 or 45 g of Hemopure showed a distinctive profile. As demonstrated in Fig. 3, the shape of the broad chromatographic profile remained consistent for at least the first 2 days. As demonstrated by the peak at 54 min in Fig. 3B (corresponding to hemoglobin dimers), some serum samples were hemolyzed.

KINETICS OF HEMOPURE CLEARANCE IN HEALTHY INDIVIDUALS

For all 12 volunteers infused with (30 or 45 g) Hemopure, peaks associated with polymerized hemoglobin were clearly distinguishable in the chromatogram during the first 3 days postinfusion. The ultraviolet–visible absorbance spectra for 11 of the 12 volunteers contained a nonendogenously derived heme moiety in the 30–35 min elution zone on day 5 postinfusion, but the maximum intensity exceeded the 3 mAU threshold in only 2 of the volunteers (AUC was not measured where the signal was < 3 mAU; see Table 3).

The results of the elimination kinetics of Hemopure are given in Table 3 for an infusion of either 30 or 45 g. The mean (SD) elimination half-life was 20.1 (3.3) h (range, 15.4–23.7 h) for the six individuals who received 30-g infusions of Hemopure and 20.4 (2.7) h (range, 17.8–24.9 h) for the six individuals who received 45-g infusions. The two means were not significantly different and are comparable to the value reported by Hughes et al. (9).

Table 2. Assessment of the interday precision of the method ($n = 9$).

Theoretical concentration, g/L	Calculated concentration, g/L	CV, %	Recovery, %	Relative error, %
0.813	0.829	9.00	102.0	1.93
2.500	2.290	4.29	91.8	-8.22
10.000	9.690	7.47	96.9	-3.08

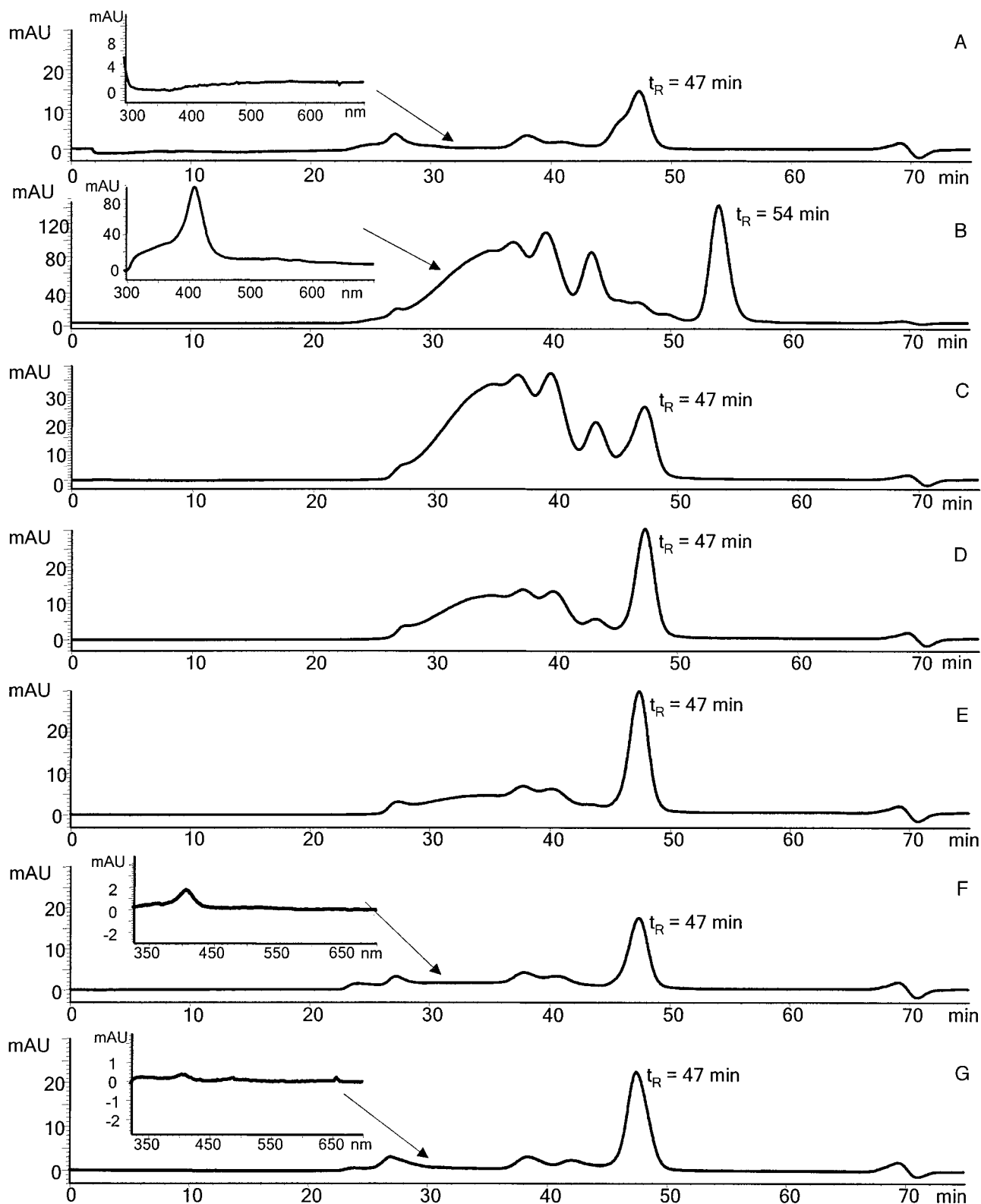


Fig. 3. Chromatograms of blood samples collected from a healthy volunteer before (A) and 1.5 h (B), 24 h (C), 48 h (D), 96 h (E), 120 h (F), and 192 h (G) after infusion of 30 g of Hemopure.

Insets depicts the ultraviolet-visible spectra showing evidence of absorbance by the heme moiety present in all HBOCs, as well as hemoglobin and myoglobin. Because neither hemoglobin nor myoglobin are eluted between 30 and 35 min, absorbance in the typical characteristic ultraviolet-visible spectrum during this interval is attributable only to nonendogenous heme moieties (i.e., HBOCs) and can therefore be used to define the LOD. t_R , retention time.

Table 3. Serum concentrations of Hemopure at various times after infusion of 30 g (volunteers 1–6) or 45 g (volunteers 7–12) of Hemopure in 12 healthy volunteers.

Time, h	Hemopure concentration (g/L) in samples from volunteer						Mean (SD) Hemopure concentration, g/L
	1	2	3	4	5	6	
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (0.00)
1.5	10.70	8.10	9.80	6.00	10.10	10.20	9.10 (1.80)
24	3.00	3.30	3.70	2.60	4.30	4.70	3.60 (0.79)
48	1.00	1.30	1.20	1.40	2.20	1.80	1.50 (0.42)
72	0.44	0.68	0.62	0.73	0.90	0.82	0.70 (0.16)
96		0.47			0.57		0.52 (0.07)
120	0.00	0.00	0.00	0.43	0.00	0.00	0.07 (0.18)
192	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (0.00)
	7	8	9	10	11	12	
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (0.00)
1.5	11.50	13.60	14.90	12.90	12.70	13.20	13.10 (1.12)
24	4.70	6.50	6.40	6.20	6.10	6.00	6.00 (0.68)
48	2.00	2.70	2.40	1.80	2.60	2.60	2.30 (0.38)
72	0.73	1.40	1.20	1.00	1.10	1.40	1.10 (0.27)
96		0.88			0.47	0.71	0.69 (0.21)
120	0.00	0.50	0.00	0.00	0.00	0.00	0.08 (0.20)
192	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (0.00)

COMPARISON BETWEEN HPLC AND HEMOCUE MEASUREMENTS

The HemoCue has been used previously to measure some HBOC products in plasma and serum (8). Comparisons of the values derived by the two methods are shown Table 4. The results are similar provided the concentration of Hemopure is higher than the concentration of free human hemoglobin in plasma. The values given by the HemoCue

were higher when the serum sample was hemolyzed (e.g., 13.8 vs 10.7 g/L for the sample in Fig. 2B).

Discussion

The present SEC-HPLC method was successful in detecting all of the HBOC products that had been, or were currently, under development and separating them from native hemoglobin through a single analytical procedure.

There were two main reasons to develop an analytical method for general use: (a) to simplify the implementation of this methodology in antidoping laboratories; and (b) to ensure that we did not inadvertently encourage the misuse of a product that remained undetectable through the implementation of an approach that was too selective. In this respect, the HPLC-mass spectrometric method reported recently for the identification of Hemopure in human plasma was based on the monitoring of slight differences between species-related peptides produced by tryptic digestion of bovine and human hemoglobin (10). This target method is of limited value given the heterogeneous nature of the various HBOCs. Similarly, the mass spectral analysis of peptide digests from Hemopure, which focused on its bovine origin and its glutaraldehyde cross-linking (11,12), is of limited scope. SEC-HPLC methods have been used under various analytical conditions to differentiate Hemopure and native hemoglobin (11–13). However, their relevance for general use is questionable because the analyses are performed in the presence of haptoglobin (11,12), which does not guarantee specificity, and they use a special SEC column that does not separate the different subunits of Hemopure (13).

Table 4. Comparison of plasma hemoglobin concentrations measured by the HemoCue photometer and by the described HPLC methodology.^a

Hemopure infusion	Time, h	Hemoglobin (HemoCue), g/L	Hemopure (HPLC), g/L
30 g	0	0.5	0.0
	1.5	10.6	9.8
	24	4.4	3.7
	48	1.7	1.2
	72	1.1	0.6
	96	0.4	0.0
	120	0.4	0.0
	144	0.3	0.0
45 g	0	0.4	0.0
	1.5	12.9	12.6
	24	6.7	6.1
	48	2.8	2.6
	72	1.5	1.1
	96	0.9	0.5
	120	0.6	0.0
	144	0.4	0.0

^a Data are from two different volunteers who received either 30 or 45 g of Hemopure.

The success of the present SEC-HPLC method relies on three points: (a) use of a dissociative medium was necessary to cleave human hemoglobin into dimers and therefore distinguish native hemoglobin and all HBOC products; (b) hemoglobin-haptoglobin complexes were precipitated out and therefore did not confound the detection of HBOCs (if there is concern about the complete precipitation of haptoglobin, it is possible to increase the quantity of anti-haptoglobin antibody by use of a more concentrated antibody solution); and (c) the method was sufficiently sensitive to detect Hemopure for several days postinfusion (which coincides with the period during which athletes would seek to obtain a meaningful performance advantage from the surreptitious use of HBOCs). Although there is a dearth of published data to confirm the performance benefits associated with HBOC infusion, a logical hypothesis is that the performance advantage will diminish in parallel with the clearance of HBOCs from the circulation. On the basis of the clearance rates we observed in our physically active volunteers, it seems that to obtain the maximum performance advantage, HBOCs would need to be infused either the night before or on the day of competition. The biological significance of the diminished Hemopure concentrations evident 2 or 3 days after infusion has yet to be elucidated, but they are unlikely to offer a substantial oxygen-carrying advantage.

The chromatograms of each HBOC obtained with the present SEC-HPLC method were sufficiently distinctive to enable identification of the different products based solely on their chromatograms, especially at high concentrations (unpublished results). However, this SEC-HPLC method should be regarded as an analytical tool to detect various HBOC products and separate them from native hemoglobin rather than as a method for HBOC characterization. In this respect, the current list of prohibited substances from the International Olympic Committee provides a "blanket ban" on the use of "modified hemoglobin products" (14) and thus does not require the identification of the precise product used.

As a consequence of this International Olympic Committee recommendation, additional layers of scientific certainty might be required to produce evidence that can withstand legal challenge. One possibility is the simultaneous analysis of hemoglobin derived from the red blood cells of the athlete being investigated. Provided that whole blood, or at least the red cell fraction of blood samples, is sent together with the serum sample to the antidoping laboratory, the athlete's red cells can be lysed and the free hemoglobin reanalyzed together with the corresponding serum sample. Separate chromatographic peaks for the endogenous hemoglobin and the heme moiety, which elutes between 30 and 35 min, would be unequivocal evidence of the presence of a nonendogenous modified hemoglobin product and therefore irrefutable evidence of a doping offense. This solution is very attractive but might face serious difficulties in practice. A

second way might be to consolidate the HBOC results obtained from the SEC-HPLC method applied to plasma or serum alone, using an equivalent method based on a different analytical principle. A sodium dodecyl sulfate-based electrophoretic method capable of resolving native hemoglobin and HBOC products in plasma or serum, which could be used in conjunction with SEC-HPLC, is currently under investigation.

This study represents a watershed in antidoping deterrents in that it required multiparty industry support of a single research initiative, necessitating the altruistic contribution of sensitive intellectual property from each industry competitor to just one research entity. Although this has enabled antidoping authorities to anticipate future doping trends and provided an opportunity to proactively implement a test for HBOCs, one of the inevitable limitations of this strategy has been the justifiable restrictions on the release of proprietary information into the public forum. Perhaps most notably this includes the inability to disclose in the current report the chromatographic profiles of those HBOCs still under development.

In conclusion, the SEC-HPLC method developed in the present work is suitable for general use and confirmation purposes in doping analysis. Its use in tandem with the electrophoretic method recently developed to screen for HBOC products in serum or plasma (7) offers a robust deterrent to the misuse of these substances by athletes.

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