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Detection of extracellular hemoglobin from *Arenicola marina* in doping control serum samples by means of liquid chromatography and high-resolution tandem mass spectrometry

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Abstract

The manipulation of blood and blood components in sports is prohibited at all times, and besides blood transfusions, also hemoglobin-based oxygen carriers (HBOCs) can be employed to artificially improve the oxygen transport capacity of the blood. But while most drug candidates based on stabilized hemoglobin (Hb) were found to be characterized by serious side effects, the natural giant extracellular Hb from the marine invertebrate *Arenicola marina* (lugworm) could be another candidate for transfusion medicine and cheating athletes, as it was found to be well tolerated in preclinical animal studies. Within this research project, lugworm Hb was implemented into the existing doping control detection method for bovine HBOCs based on ultrafiltration, tryptic digestion, and liquid chromatography coupled with high-resolution tandem mass spectrometry (LC-HRMS/MS). For the mass spectrometric identification of lugworm Hb, two precursor–product ion pairs for a total of four tryptic peptides originating from subunits hbA2 (T_6), hbB1 (T_3 and T_6), and the linker chain (T_{16}) were employed. The modified approach was comprehensively characterized and found to allow for the specific and sensitive detection of lugworm Hb down to concentrations of 10 µg/mL from 50 µL of serum/plasma. Therefore, it can serve as confirmation procedure for lugworm Hb following visual or electrophoretic screening. Moreover, a proof-of-concept rat administration study was conducted, and the observed detection windows of at least 4 (dose: 200 mg/kg) and 8 h (dose: 600 mg/kg) suggest that the approach can be readily employed to efficiently test in-competition doping control samples for the presence of the drug candidate.

KEYWORDS
blood substitute, doping, hemoglobin, hemoglobin-based oxygen carrier (HBOC), LC-HRMS/MS, lugworm, ultrafiltration

1 | INTRODUCTION

Especially in endurance disciplines, athletic performance significantly depends on the oxygen delivery capacity of the blood.[1] Consequently, blood manipulation represents an important strategy for performance enhancement potentially misused by cheating athletes. Besides blood transfusions and erythropoiesis-stimulating agents (ESAs), also hemoglobin-based oxygen carriers (HBOCs) are potential doping agents, whose misuse in sports is prohibited both in and out of competition.[2]

As cell-free hemoglobin (Hb) is not only very ineffective in reversible oxygen binding and release but also characterized by a high (nephro-)toxicity—mainly due to its rapid degradation in vivo, HBOCs developed as blood substitutes are based on human, bovine, or recombinant Hb stabilized by polymerization, intra-molecular cross-linking, conjugation, or encapsulation in liposomes.[3] Unfortunately, the in vivo use of most products was found to be still associated with serious side effects such as vasoconstriction and hypertension due to NO scavenging, which led to an early termination of clinical development.[3–5]

A promising alternative both for transfusion medicine and cheating athletes is the natural giant extracellular Hb from the marine invertebrate *Arenicola marina* (lugworm). This large protein complex comprises a total of 156 globin and 42 linker chains, resulting in a molecular mass of 3682 kDa, and can carry up to 156 oxygen molecules.[5–7] Animal studies demonstrated that it has no vasoactivity, is generally well tolerated, and has a high oxygenation potency, especially in poorly vascularized tissue.[8,9] Additionally, it has potential anti-oxidant, anti-inflammatory, and anti-bacterial effects.[5] The company Hemarina (Morlaix, France) has developed different preparations with lugworm Hb as active ingredient M101[10,11]: HEMO2life® is an additive to hypothermic graft preservation solutions, which has recently obtained the CE marking allowing its marketing as medical device for ex vivo usage in Europe. By contrast, HEMOXYCarrier® is a therapeutic oxygen carrier still in preclinical development.

Even though no adverse analytical findings (AAFs) with HBOCs have been reported so far, several athletes have confirmed to have misused such compounds for performance enhancement in the past.[12] Compared with blood transfusions and ESAs, the use of HBOCs as doping agents has several advantages: There is no need for blood group matching, the risk for pathogen transmission is significantly reduced, logistic requirements are simple, and effects occur immediately following infusion.[13] Therefore, it is of utmost importance to implement novel HBOC drug candidates into existing doping control methods. The applicability of a detection method based on native-gel agarose electrophoresis at basic pH (9.2), Western blotting, and heme-specific chemiluminescence detection to lugworm HB was already demonstrated by Marchand et al. in 2017 by analyzing plasma and serum samples collected from mice treated with single doses of 20 and 200 mg/kg.[14] Surprisingly, the target analyte was only found in the first samples collected 4 h following administration in the group injected with 200 mg/kg. This short detection window suggests a fast dissociation and elimination of lugworm Hb from the system. It was assumed that a mass spectrometric assay could potentially increase detection times, as it can also detect non-human peptides originating from degraded lugworm Hb. Therefore, the aim of this research project was to implement M101/lugworm Hb into the existing doping control detection method for bovine HBOCs based on ultrafiltration (cut-off: 100 kDa), tryptic digestion, and liquid chromatography coupled with high-resolution tandem mass spectrometry (LC-HRMS/MS).[15] Additionally, a rat administration study was to be conducted to obtain authentic samples which can serve as proof of concept for the assay and additionally provide important information about the detection window of the drug.

2 | MATERIAL AND METHODS

2.1 | Reference material, chemicals, and consumables

For method development and validation, an aliquot of HEMO2life® containing M101/lugworm Hb at a concentration of 50 mg/mL was kindly provided by WADA (Montreal, Canada). For the animal administration studies, an additional vial with 1 g of M101/lugworm Hb was bought from Hemarina (Morlaix, France).

Amicon® Ultra 0.5 mL centrifugal filters (cut-offs: 100 and 10 kDa) were obtained from Merck Millipore (Darmstadt, Germany), and sequencing grade modified trypsin (V5111) was purchased from Promega (Walldorf, Germany). All chemicals/solvents were from Sigma-Aldrich (Taufkirchen, Germany) or Merck Millipore and of analytical grade. Carbonic anhydrase employed as internal standard (ISTD) was also bought from Sigma-Aldrich.

2.2 | Human samples

Human serum obtained from male AB plasma was purchased from Sigma-Aldrich (Munich, Germany) and used for method development and characterization. For the determination of certain validation parameters such as specificity and robustness, additional serum and plasma samples were collected from 10 healthy volunteers (four male and six female). Written informed consent was provided by all participants, and the study approved by the local ethics committee of the German Sport University Cologne (DSHS No. 139/2021).

2.3 | Sample preparation

For sample extraction, a previously published protocol[15] was slightly modified as follows: 50 µL of serum/plasma were mixed with 450 µL of 50 mM NH_4HCO_3 and subjected to ultrafiltration using Amicon® Ultra 0.5 mL centrifugal filters with a cut-off of 100 kDa (10 min at 14,000 × *g*). After washing the retentate with 250 µL of 50 mM NH_4HCO_3 (10 min, 14,000 × *g*), 20 µL were transferred to a fresh tube, mixed with 100 µg of the internal standard (10 µL of a solution with 10 mg/mL in 50 mM NH_4HCO_3), 1 µg of trypsin (50 µL of a solution containing 20 ppm in 50 mM NH_4HCO_3), 10 µL of 50 mM NH_4HCO_3, and 10 µL of acetonitrile, and incubated overnight at 37°C. Proteolysis was stopped by adding 5 µL of glacial acetic acid,

and to separate tryptic peptides from undigested proteins, ultrafiltration was performed again with Amicon® Ultra 0.5 mL centrifugal filters with a cut-off of 10 kDa (20 min, 14,000 × *g*). Finally, filtrates were transferred to high-performance liquid chromatography (HPLC) vials and subjected to LC-HRMS/MS analysis.

2.4 | LC-HRMS/MS

LC-HRMS/MS analysis was conducted on an Orbitrap Exploris™ 480 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to a Vanquish™ UHPLC (Thermo Fisher Scientific, Bremen, Germany). The liquid chromatography (LC) system was equipped with an Accucore™ Phenyl-Hexyl trapping column (3 × 10 mm, 2.6 µm; Thermo Fisher Scientific, Bremen, Germany) and Poroshell EC-C18 column analytical column (3 × 50 mm, 2.7 µm; Agilent Technologies, Santa Clara, CA, USA) and operated with a column temperature of 30°C and an autosampler temperature of 10°C. Injection volumes were 2–10 µL, and 0.1% formic acid with 1% dimethyl sulfoxide (DMSO) in water (A) and 0.1% formic acid with 1% DMSO in ACN (B) were used as LC solvents. The following LC gradient with a flow rate of 400 µL/min and a total run time of 15 min was employed: First, trapping was performed for 2 min with 5% B. Then, the amount of solvent B was increased to 40% within 6 min and 80% within another 4 min. Finally, the system was re-equilibrated for 5 min with 5% of B.

The mass spectrometer was operated in positive mode with an ionization voltage of 4 kV, and the temperature of the ion transfer tube was set to 320°C. Data were acquired by full MS (*m/z* 400–1700, resolution of 30,000 full width at half maximum [FWHM] at *m/z* 200) and targeted MS^2 experiments (isolation window of *m/z* = 2 at a resolution of 15,000 FWHM at *m/z* 200, *m/z* 100–1800). For MS^2 experiments, the normalized collision energy (NCE) was set to 30%, and nitrogen obtained from a N_2-generator (CMC, Eschborn, Germany) was used as collision gas. Moreover, an inclusion list with the accurate mass-to-charge ratios for the most abundant charge states of the target peptides was employed. According to the manufacturer's recommendations, the system was calibrated with a mixture of caffeine, the tetrapeptide MRFA (methionine-arginine-phenylalanine-alanine), and Ultramark 1621 (Thermo Fisher Scientific). For the evaluation of the acquired MS data, Thermo Xcalibur Software (Version 4.0.27.10, 2015) was employed. As shown in Table 1, two precursor–product ion transitions were evaluated for each target peptide.

2.5 | Method development: Mass spectrometric detection of lugworm Hb

According to the literature,[6] lugworm Hb is composed of eight different globin subunits (A1, A2, B1, B2, B3, C, D1, and D2) and two non-globin linker chains (L1 and L2). Unfortunately, not all of the corresponding amino acid sequences are listed in online protein databases. To allow for a mass spectrometry-based identification of lugworm Hb, the available amino acid sequences of lugworm Hb subunits A2, B1, B2, and L2 were obtained from UniProtKB database[16] and subjected to in silico tryptic digestion using GPMAW software (version 8.00sr1, Lighthouse Data, Odense, Denmark). The resulting diagnostic peptides were then blasted against different protein databases to ensure specificity for lugworm Hb and verified by in-solution tryptic digestion and LC-HRMS/MS analysis of M101 reference material. Eventually, peptides T_6 from hbA2 (accession #: Q53I65), T_3 and T_6 from hbB1 (accession #: Q2PAD4), and T_{16} from the linker chain L2 (accession #: Q4A1S6) were selected for an unambiguous mass spectrometric detection of M101/lugworm Hb (Table 1).

2.6 | Method validation

The method was comprehensively characterized according to current WADA guidelines,[17] and the following parameters were determined:

- Specificity:

To demonstrate the specificity of the method, a total of 10 blank serum samples were fortified with the ISTD only and analyzed as described above.

- Linearity:

The assay's linearity was assessed by analyzing 13 serum samples including one blank and 12 specimens fortified with 5, 10, 15, 20, 50, 100, 150, 200, 500, 1000, 1500, and 2000 µg/mL of lugworm Hb. For the construction of a calibration curve, the absolute peak areas were used, and linearity was determined by regression analysis.

- Limit of detection (LOD):

Six serum specimens were fortified with 2, 5, 10, and 15 µg/mL and prepared as described above. To estimate the method's LOD, a detection rate of >95% was applied.

- Reliability:

The reliability was demonstrated by analyzing 10 different serum samples fortified with 100 µg/mL of lugworm Hb. As acceptance criterion, a detection rate of 100% for all precursor–product ion transitions was applied.

- Robustness 1—Biological matrix:

To investigate the robustness of the approach, 10 different plasma samples (instead of serum) were fortified with 100 µg/mL of lugworm Hb and analyzed as described in Sections 2.3 and 2.4. Again, a detection rate of 100% was employed as acceptance criterion.

- Robustness 2—Complex samples:

In a second robustness experiment, the detectability of lugworm Hb in plasma collected from hemolyzed blood (*n* = 2) and serum

simultaneously fortified with 100 µg/mL of lugworm Hb and 2000 µg/mL of the bovine HBOC Hemopure ($n = 2$) was studied.

- Robustness 3—Sample preparation:

The robustness of the sample preparation protocol was investigated by preparing 10 serum samples fortified with lugworm Hb at a concentration of 100 µg/mL without the final ultrafiltration step (cut-off: 10 kDa). As in the other experiments, a detection rate of 100% for all precursor–product ion transitions had to be fulfilled.

- Carryover:

Sample carryover was determined by injecting a blank serum extract immediately after the extract of a sample fortified with 2000 µg/mL of lugworm Hb.

- Stability 1—Serum samples:

A serum sample was fortified with lugworm Hb at a concentration of 100 µg/mL, and aliquots (in duplicate) were stored for 1, 2, and 7 days at RT as well as 1, 2, 7, 14, and 28 days at 4°C. At the respective time points, samples were frozen at −80°C until the experiment was completed and finally extracted and analyzed as described above.

- Stability 2—Sample extracts:

A total of five linearity sample extracts containing between 10 and 1500 µg/mL of lugworm Hb were stored for 5 days in the autosampler of the liquid chromatography–mass spectrometry (LC–MS) system at a temperature of 10°C. Then, they were re-analyzed as described in Section 2.4.

2.7 | Animal study

The rat administration study was conducted in cooperation with Pig For Life (PFL) and CER Groupe in Marche-en-Famenne, Belgium, as follows: HEMO2life® single doses of 200 and 600 mg/kg were administered to three male Wistar rats (age and weight at the beginning of the study: 6–7 weeks, 272–283 g) after gaseous anesthesia (isoflurane box) via a catheter placed in the femoral vein (VAB system). Intravenous injection was performed over several minutes with a dilution of HEMO2life® containing 40 mg/mL of the active ingredient M101. Blood samples with a volume of 250 µL were collected both before and 2, 4, 8, 24, 48, and 72 h following administration, centrifuged at 3000 × g for 15 min, and the resulting serum aliquots were stored frozen until analysis in the Cologne Anti-Doping Laboratory. Between both studies, a wash out period of 10 days was implemented. Clinical evaluation of the animals was performed at arrival, before injection, before blood sampling, and once a week during the wash out period. All rats were daily checked for mortality, morbidity, and evident signs of toxicity, general appearance, viability, mobility, and stools. The animal study was compliant with the respective Belgian regulations[18] and approved by the local ethics committee.

3 | RESULTS AND DISCUSSION

3.1 | Mass spectrometric detection of lugworm Hb

Employing in silico tryptic digestion of the lugworm Hb amino acid sequences available in online protein databases and in-solution tryptic digestion of M101 reference material, peptides T_6 from hbA2 (accession #: Q53I65), T_3 and T_6 from hbB1 (accession #: Q2PAD4), and T_{16} from the linker chain L2 (accession #: Q4A1S6) were selected for an unambiguous mass spectrometric detection of the target protein. The product ion mass spectra of the chosen diagnostic peptides are shown in Figures S1–S4. For each peptide, two precursor–product ion pairs were employed for identification purposes (Table 1). For peptide T_6 from hbB1, the conducted protein blast yielded a 100% sequence homology to the ATP-dependent RNA helicase DbpA from the bacterium *Steroidobacter soli*, which is currently listed in the nr database with non-redundant protein sequences. Consequently, this peptide is not entirely specific for lugworm Hb but can support its detection/identification in combination with the other diagnostic peptides.

3.2 | Method validation

To demonstrate the applicability of the approach to sports drug testing, it was comprehensively characterized according to current WADA guidelines.[17] Validation results are summarized in Table 2. Overall, the method was found to allow for a both specific and sensitive detection of lugworm Hb down to concentrations of 10 µg/mL from 50 µL of serum. In Figure 1, exemplary extracted product ion chromatograms of a blank and a serum sample fortified with 100 µg/mL of lugworm Hb

TABLE 1 Details on the diagnostic peptides for lugworm Hb detection.

Peptide	Amino acid sequence	Amino acid positions in the mature protein	Monoisotopic mass (Da)	Precursor ion (*m/z*)	Charge state	Product Ion 1		Product Ion 2	
						ID	*m/z*	ID	*m/z*
hbA2: T_6	DEAGHVLWK	25–33	1053.52	527.77	2	b_5	510.19	b_6	609.26
hbB1: T_3	AQWNSLWNTPDSSTSK	14–29	1820.83	911.42	2	y_7	721.34	y_9	936.43
hbB1: T_6	FFEVDPESK	40–48	1096.51	549.26	2	y_4	460.24	a_2	267.15
Linker: T_{16}	SVDTVPFFTTHPK	150–162	1474.75	738.38	2	y_8	974.51	b_4	403.18

TABLE 2 Results of method validation.

Validation parameter	*n*	Amount (µg)	Concentration (s) (µg/mL)	Target peptide: hbA2: T_6	hbB1: T_3	hbB1: T_6	Linker: T_{16}
Specificity	10	-	-	0/10	0/10	0/10	0/10
Linearity	13	0–100	0–2000	0–100 µg/mL (R^2 > 0.98)	0–200 µg/mL (R^2 > 0.98)	0–200 µg/mL (R^2 > 0.99)	0–20 µg/mL (R^2 > 0.99)
LOD	6	0.1–0.75	2–15	10 µg/mL (6/6)	10 µg/mL (6/6)	10 µg/mL (6/6)	10 µg/mL (6/6)
Reliability	10	5	100	10/10	10/10	10/10	10/10
Robustness (Plasma)	10	5	100	10/10	10/10	10/10	10/10
Robustness (Ultrafiltration)	10	5	100	10/10	10/10	10/10	10/10
Robustness (Hemolysis)	2	5	100	2/2	2/2	2/2	2/2
Robustness (Hemopure)	2	5	100	2/2	2/2	2/2	2/2
Carryover	-	100	2000	<1%	<1%	<1%	<1%
Sample stability at RT	2	5	100	>7 days	>7 days	>7 days	>7 days
Sample stability at 4°C	2	5	100	>28 days	>28 days	>28 days	>28 days
Extract stability at 10°C	5	0.5–75	10–1500	>5 days	>5 days	>5 days	>5 days

are displayed. In the blank specimen, no interfering signals were present at the retention times of the different target peptides. The linearity significantly differed between the diagnostic peptides—while the signals measured for peptides T_3 and T_6 from subunit hbB1 were linear up to a concentration of 200 µg/mL as reflected by correlation coefficients (r^2) higher than 0.98, those for peptides T_6 from subunit hbA2 and T_{16} from the linker were only linear between 5 and 100 µg/mL (r^2 > 0.98) and 5 and 20 µg/mL (r^2 < 0.99), respectively. Moreover, the intensity of the ISTD signal decreased with increasing analyte concentration. Both can probably be attributed to saturation of proteolytic cleavage: According to the literature,[6] the copy numbers of hbA2, hbB1, and the linker L2 in the lugworm Hb protein complex are highly different: It contains 72 copies of hbA2 (total molecular mass: ~1150 kDa), seven copies of linker L2 (total molecular mass: ~187 kDa), and four copies of hbB1 (total molecular mass: ~64 kDa). Therefore, saturation of proteolysis is potentially more rapidly achieved for the linker and hbA2. To estimate the LOD, a detection rate of at least 95% was applied to six serum samples fortified with 2, 5, 10, and 25 µg/mL, and the detection limit was set to 10 µg/mL for all four diagnostic peptides. In animal studies, lugworm Hb was administered at doses between 200 and 600 mg/kg,[8,9] which would be equivalent to an absolute therapeutic amount of 14–42 g in a 70 kg adult. The resulting maximal plasma levels (assuming a total plasma volume of 3 L) would therefore be in the range of 4.6–14 g/L. Consequently, an LOD of 10 µg/mL (10 mg/L) should allow for sufficiently long detection windows, especially as a mass spectrometric bottom-up approach does not differentiate between the intact molecule and its dissociated subunits. The reliability was demonstrated by achieving a 100% detection rate for all precursor—product ion pairs in 10 different serum samples containing 100 µg/mL of lugworm Hb. To assess the robustness of the method, plasma instead of serum was used as biological matrix, and also, the detectability of the target peptides from hemolyzed plasma and serum simultaneously fortified with lugworm Hb and Hemopure, a bovine HBOC, was investigated. The diagnostic peptides of lugworm Hb were unambiguously identified in all samples, and also, omission of the final ultrafiltration step did not affect their detection. Sample carryover was below 1%, and the sample extracts were found to be stable in the LC autosampler (10°C) for at least 5 days. Moreover, the stability of lugworm Hb in unextracted serum samples was investigated, and sample analysis yielded comparable mean absolute peak areas over the investigated periods of 7 days at RT and 28 days at 4°C (Figure S5). This would be fully compatible with current protocols for the collection, transportation, and storage of doping control blood samples[19] requiring transportation in refrigerated condition within a certain period of time under continuous monitoring by a data logger.

In 2006, Rousselot et al. showed that the slightly alkaline pH and low salt concentration of human/murine plasma in combination with a physiological temperature of 37°C (compared with ~15°C in *A. marina*) lead to a rapid dissociation of the lugworm Hb megacomplex into dodecamers with a molecular mass of approximately 205 kDa.[7] However, the resulting fragments were found to remain functional and freely circulating without any side effects for several hours.[7,9] Consequently, it can be expected that these dodecamers instead of intact lugworm Hb are present in doping control serum samples. However, this is not an analytical issue as the described MS assay is not specific for the intact protein complex but proteolytic peptides derived from its subunits.

3.3 | Animal study

The applicability of the approach to authentic post-administration samples was demonstrated by analyzing serum specimens collected within two HEMO2life® rat studies (see Section 2.7). During the study period, no anomalies regarding rat vitality were observed and the serum samples collected 2 and 4 h following administration were

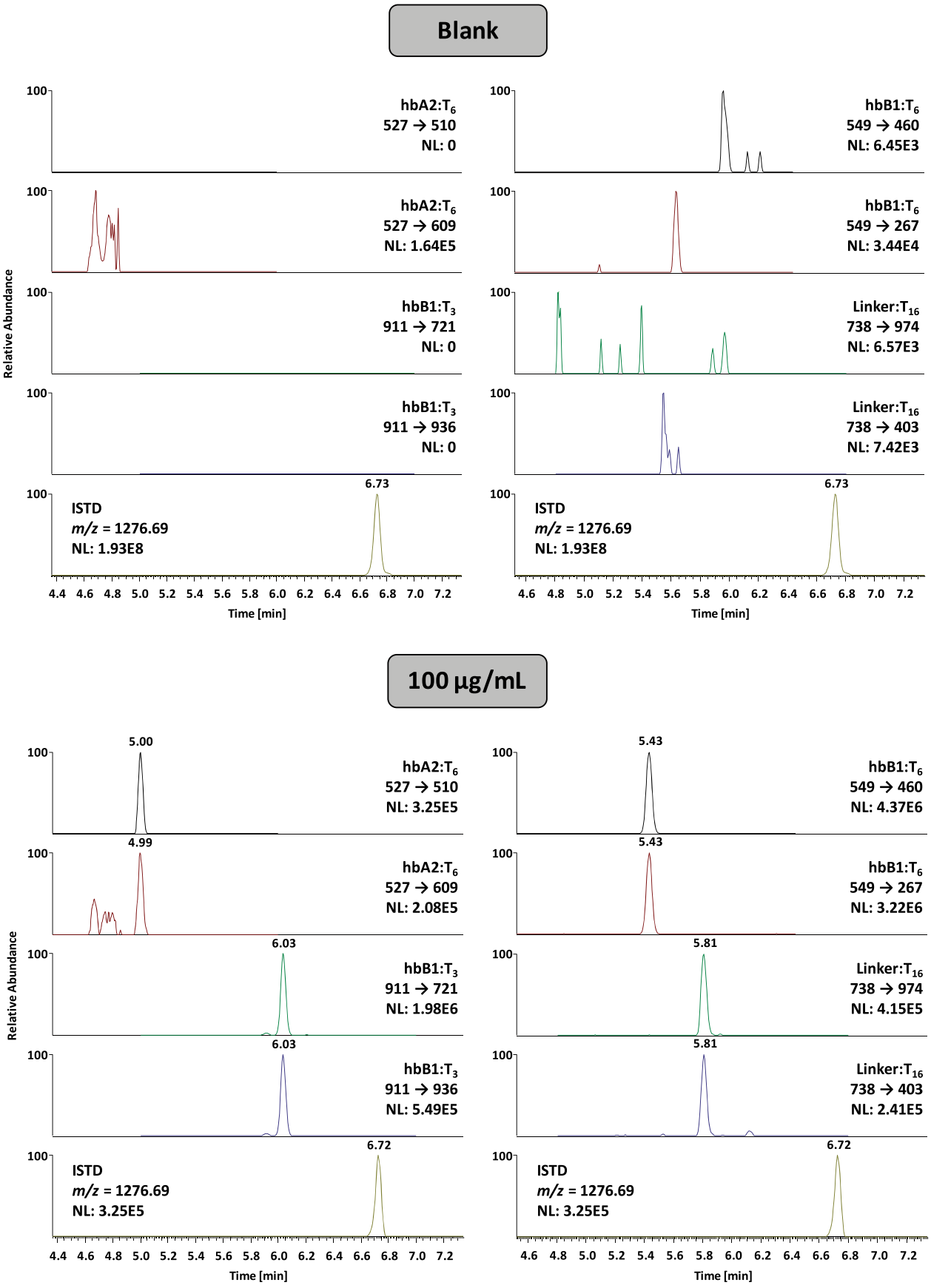

FIGURE 1 Exemplary extracted product ion chromatograms of a blank and a serum sample fortified with 100 µg/mL of lugworm Hb. Samples were analyzed on an Orbitrap Exploris™ 480 mass spectrometer coupled to a Vanquish™ UHPLC.

characterized by a significant red coloring, especially after injecting the high drug dose (Figure S6). Therefore, also a visual screening as suggested by Goebel et al.[20] would be applicable to identify samples for a subsequent mass spectrometric confirmation.

After injecting lugworm Hb at a dosage of 200 mg/kg, all diagnostic peptides were unambiguously detected for 4–8 h (Figure 2). The absolute signal intensities were found to significantly decrease from 2 to 4 h, indicating a rapid degradation and/or elimination of the protein complex. In Figure 3, the extracted product ion chromatograms of serum samples collected before and 8 h following injection of 200 mg/kg are exemplarily shown. No interfering signals were observed in the blank specimens, and all diagnostic peptides were unambiguously detected in the post-administration sample. At the higher dose of 600 mg/kg, the maximum detection time was found to be at least 8 h, but all serum specimens collected 24 h and one sample collected 48 h following injection were still found to contain traces (≥2) of some diagnostic peptides (Figure 2). Interestingly, it varied from sample to sample which precursor–product ion transitions could be detected for the longest period. As to be expected, the absolute signal intensities at 600 mg/kg were significantly higher than at 200 mg/kg, and a rapid degradation of lugworm Hb was observed again. Exemplary product ion chromatograms of a serum sample collected 24 h following injection of 600 mg/kg still containing traces of the oxygen carrier are displayed in Figure S7.

Overall, these results are in accordance with an earlier study describing a rather short plasma detection window of only 4 h in mice injected with 200 mg/kg of lugworm Hb.[17] Nevertheless, detection times were found to correlate well with serum coloring and the presumed active window of the drug. Consequently, the presented doping control assay should be well suited to test in-competition samples for the presence of this HBOC. As mentioned above, blood substitutes show their therapeutic effect immediately following administration,[13] so it can be assumed that cheating athletes would misuse such compounds shortly prior to a competition. Therefore, a detection window of 4–8 h should be sufficient to uncover doping with lugworm Hb. However, no data on the administration of M101 to humans have been published so far, so the results of this study still need to be confirmed in human subjects.

As shown by Zal et al.,[6] Lugworm Hb has a characteristic hexagonal bilayer structure where 12 subunits are arranged in two six-membered rings. Each of these subunits represents a dodecamer composed of 12 globin chains with a total molecular mass of approximately 205 kDa. Another dodecamer can be found in the center of the molecule between both hexagons. Molecule assembly is mediated by 42 linker chains, resulting in a protein mega complex with a total mass of 3682 kDa. Both the intact protein complex and the large dodecameric subunits should be covered by the presented sample extraction procedure, however, potential degradation products with a molecular mass below 100 kDa are discarded with the flow through of the first ultrafiltration step. Due to the observed detection windows of only 4–8 h, which are indicative of a rapid in vivo dissociation/degradation of the molecule, also the flow throughs from one animal obtained after the first ultrafiltration step (cut-off: 100 kDa) were concentrated in Amicon® Ultra 0.5 mL centrifugal filters with a cut-off of 10 kDa and subjected to tryptic digestion and LC-HRMS/MS analysis as described above. In the serum specimens collected after injecting 200 mg/kg of lugworm Hb, only the diagnostic peptides derived from subunits hbA2 and hbB1 could be detected for 2–4 h. After a dose of 600 mg/kg, all diagnostic peptides were present for 4–8 h. These findings indicate that no degradation products with a molecular mass between 10 and 100 kDa can be employed to prolong detection times in serum. Additionally, they emphasize the need to further investigate the in vivo metabolism and elimination of this drug and also test urine as biological matrix for the detection of lugworm Hb. Le Gall et al. studied the in vivo biodistribution of the oxygen carrier in mice treated with 200 mg/kg of fluorescence-labeled M101.[9]

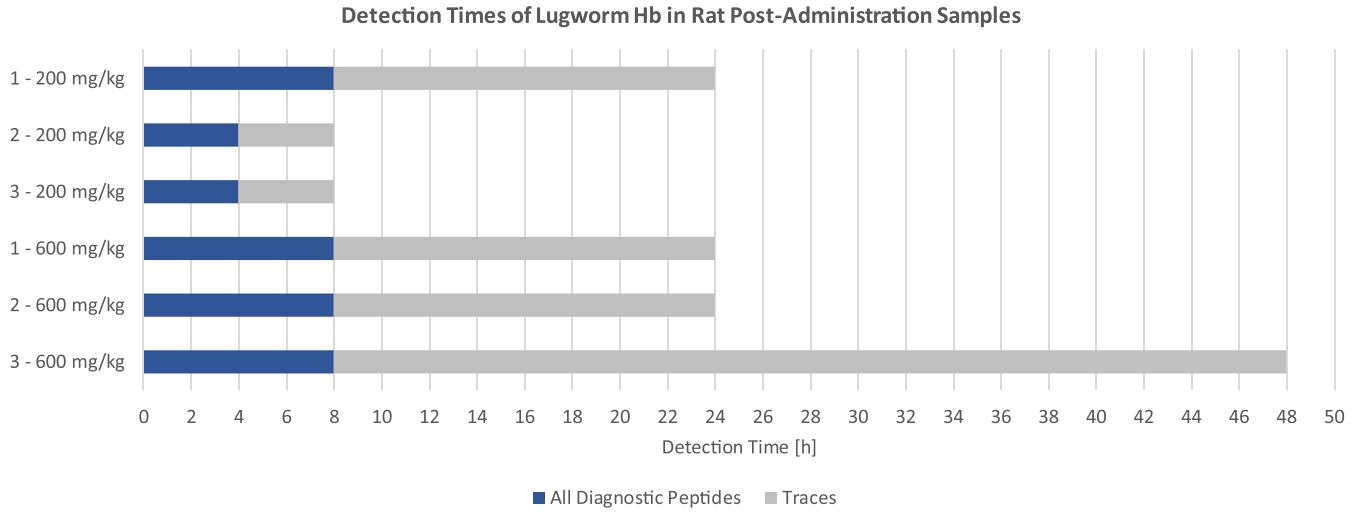

FIGURE 2 Detection times of lugworm Hb in post-administration serum samples collected from rats treated with 200 and 600 mg/kg of HEMO2life®. Blue bars are indicative of a successful detection of two precursor–product ion transitions for each of the four diagnostic peptides, grey bars for the detection of trace amounts as reflected by less than 4 × 2 precursor–product ion transitions.

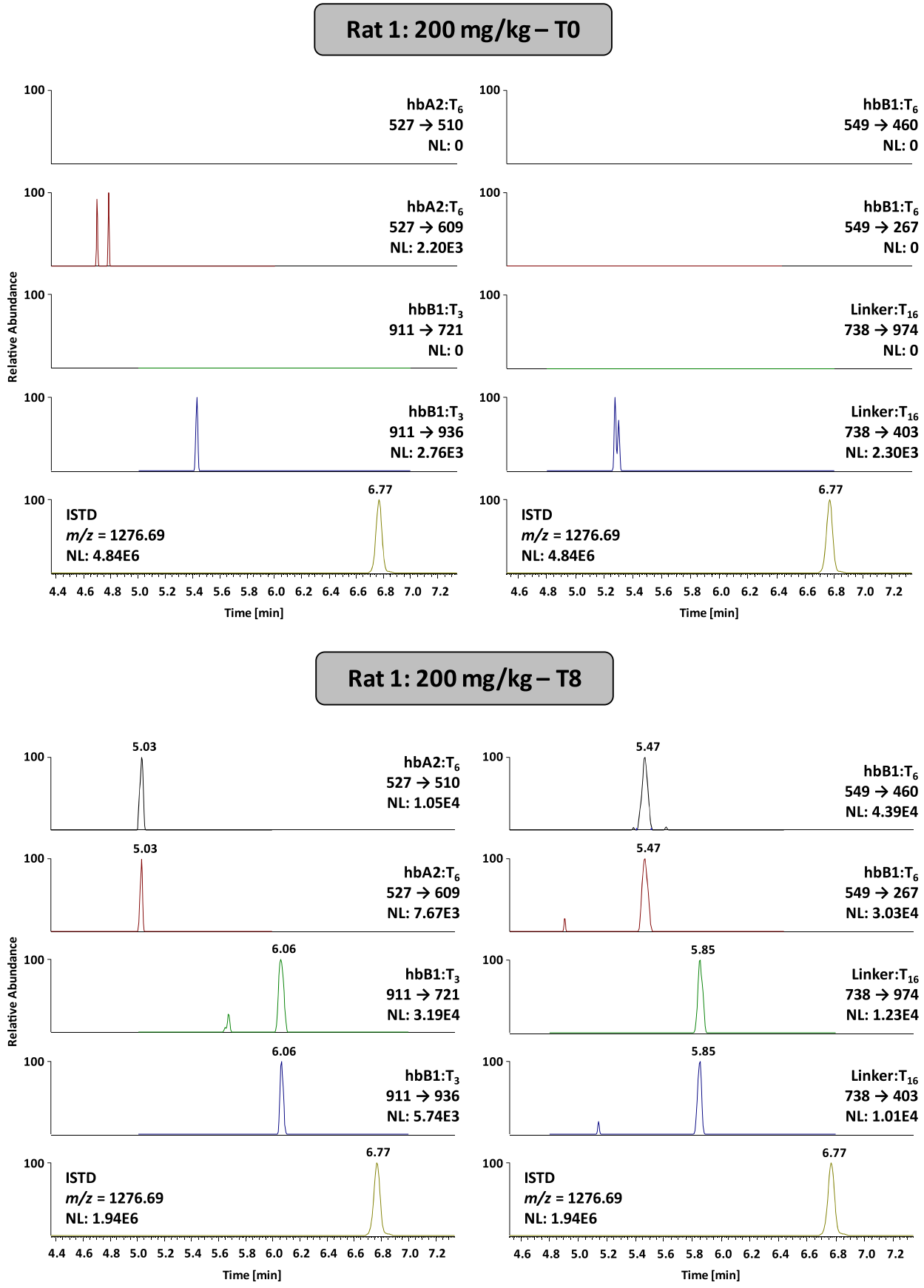

FIGURE 3 Extracted product ion chromatograms of rat serum samples collected before and 8 h following administration of lugworm Hb at a dosage of 200 mg/kg. Samples were analyzed on an Orbitrap Exploris™ 480 mass spectrometer coupled to a Vanquish™ UHPLC.

While the corresponding fluorescence signal could be detected in plasma for up to 19 h post-injection, urine specimens were fluorescent for up to 4 days, probably due to the presence of degradation products filtered through the renal glomerulus.

4 | CONCLUSIONS

Due to its promising therapeutic properties, lugworm Hb represents an emerging doping agent which can potentially be misused in sports to improve the oxygen delivery capacity of the blood. Even though clinical approval for in vivo use as oxygen carrier is still missing, a graft preservative for transplant procedures containing lugworm Hb as active ingredient M101 has recently obtained the CE marking allowing its marketing as medical device for ex vivo usage in Europe, which makes the drug readily available for cheating athletes. Therefore, it was of utmost importance to implement lugworm Hb into existing doping control detection methods for HBOCs, which was successfully accomplished within this study. The presented assay employing ultrafiltration, tryptic digestion, and LC-HRMS/MS was found to be highly specific and sensitive, and the analysis of post-administration serum specimens collected from rats could demonstrate its applicability to authentic samples. Moreover, the in vivo data collected within this study are of great value for sports drug testing, as—due to the lack of clinical approval—the access to human post-administration samples is currently very difficult.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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