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Doping Control Analysis of Bovine Hemoglobin-Based Oxygen Therapeutics in Human Plasma by LC-ESI-MS/MS

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Introduction

The quest for artificial oxygen carriers as a substitute for stored blood is being actively pursued because of shortages of blood especially in the case of rare blood types[1]. In addition, the need to test blood samples before use for possible disease vectors and to check the suitability in terms of type and rhesus factor has been a time-consuming task. Thus, the development of blood substitutes with more convenient storage characteristics, compatibility and availability, has been performed for years, and besides oxygen therapeutics based on synthetic perfluorocarbons (PFCs), hemoglobin (Hb) derivatives have been investigated intensively[2, 3]. Several preparations have been investigated using either bovine or human hemoglobin crosslinked by different molecules such as *O*-raffinose (e.g. Hemolink™), bis(3,5-dibromosalicyl) fumarate (e.g. HemAssist™) or glutaraldehyde (e.g. PolyHeme™, Hemopure®), and some have been approved for animal or human application[3]. Hemopure®, in particular, which is based on bovine hemoglobin[4] that is intra- and intermoleculary crosslinked by glutaraldehyde units at the amino acid residue Lys 99 as demonstrated in an earlier study[6], is approved for administration to humans in South Africa. An initial application volume of 250-500 mL (1-2 bags) containing 30-60g of the crosslinked bovine hemoglobin is recommended by the manufacturer[4].

Besides the indicated medical purpose of oxygen therapeutics, abuse of those compounds is possible, especially in sports with enormous endurance performance in order to improve oxygen transport during competition. Owing to this fact, artificial oxygen carriers have been added to the list of prohibited substances of the International Olympic Committee (IOC) in

January 2000[5]. In the present study, we demonstrate a method to identify bovine hemoglobin in human plasma samples[7], the presence of which can result from administration of remedies such as Hemopure®, which is prohibited according to the anti-doping code of the IOC. The assay is based on the differences in amino acid sequences of bovine and human hemoglobin that allow a distinction after enzymatic degradation of proteins and subsequent liquid chromatography-tandem mass spectrometry.

Experimental

Materials and chemicals. Filter units with a molecular weight cut-off (MWCO) of 100 kDa (Centricon) or 10 kDa (Ultrafree®-MC) were purchased from Millipore (Bedford, MA). Modified trypsin (sequence grade) was obtained from Promega (Madison, WI). Ammonium bicarbonate, carbonic anhydrase (bovine erythrocytes), human and bovine hemoglobin were from Sigma (Deisendorf, Germany). Trifluoroacetic acid and glacial acetic acid were bought from Merck (Darmstadt, Germany). All solutions and buffers were prepared using deionized water (MilliQ grade). Hemopure® was a generous gift from Biopure® Corporation (Cambridge, MA).

Plasma samples. Plasma specimens pooled from eight different healthy volunteers were obtained from the Institute of Cardiology and Sports Medicine of the German Sport University, Cologne, Germany. Furthermore, 68 blood samples of high performance athletes were kindly provided by the Fédération Internationale de Ski (FIS) and the World Anti-Doping Agency (WADA). Blood specimens (delivered at 4°C in 3mL Vacutainer™ tubes) were centrifuged for 10 min at 1150 *g* upon arrival, the plasma was subsequently transferred into 2 mL glass vials and sealed properly. All plasma samples were stored at 4°C until preparation and analysis.

Liquid chromatography-mass spectrometry. Analyses were performed on an Agilent 1100 Series liquid chromatograph (Waldbronn, Germany) interfaced to an Applied Biosystems API 2000 triple quadrupole mass spectrometer (Darmstadt, Germany). The LC was equipped with a Zorbax 300SB C_{18} column (i.d. = 2.1 mm; length = 50 mm; particle size = 3.5 µm). The eluents used were A: 0.2% acetic acid containing 0.02% TFA, and B: 80% acetonitrile/20% 0.2% acetic acid containing 0.02% TFA. The flow was 300µL/min, and a

gradient was utilized from 5% B to 100% B in 9 minutes. The column was finally reequilibrated for 5 min. Positive ionization was accomplished by electrospray at 5500V, declustering potential was set to 30 V, and collision energies were optimized individually for each ion transition. Nitrogen (obtained from a Whatman K75-72 nitrogen generator) was used as collision gas at a collision cell pressure of 2.5e-5 torr.

Sample preparation. Aliquots of 50µL of plasma were transferred to 1.5mL Eppendorf tubes and diluted with 450µL of deionized water. The solutions were applied to Centricon 100kDa filter units and centrifuged for 40 min at 2000 *g*. A volume of 250µL of deionized water was added to the retentate and the samples were centrifuged again at 2000 *g*. After 30 min, a volume of 20µL of the retentates was transferred to a fresh Eppendorf tube, 100 µg of carbonic anhydrase (dissolved in 10 µL of 50mM ammonium bicarbonate), 1µg of trypsin (dissolved in 50µL of 50mM ammonium bicarbonate) and 30µL of acetonitrile were added, and the samples were incubated in an aluminium heating block at 37°C overnight. A volume of 5µL of glacial acetic acid was added, the mixtures were vortexed shortly and applied to Ultrafree®-MC 10 kDa filter units and centrifuged at 4650 *g* for 10 min. The filtrates were diluted by an equal amount of deionized water, transferred to HPLC vials, and 5µL were injected into the LC-ESI-MS/MS system.

Fortified plasma samples and specificity. In order to analyze 'positive' plasma samples, specimens were fortified with 50-400µg of Hemopure® before sample preparation. In addition, plasma aliquots of 50µL were spiked either with 200µg of human hemoglobin, 100µg of human Hb and 200µg of Hemopure®, or 200µg of human Hb and 50µg of Hemopure® demonstrating the specificity of the method.

Results and discussion

Trypsin digestion of human hemoglobin, bovine hemoglobin, carbonic anhydrase and hemopure provide mixtures of peptides, which can be separated and analyzed by means of LC-ESI-MS/MS. The amino acid sequence homology of human and bovine hemoglobin is 85%, and thus a differentiation between both oxygen carriers can be accomplished by identification of peptides with, concerning hemoglobins, unique molecular weights and amino acid compositions.

a)

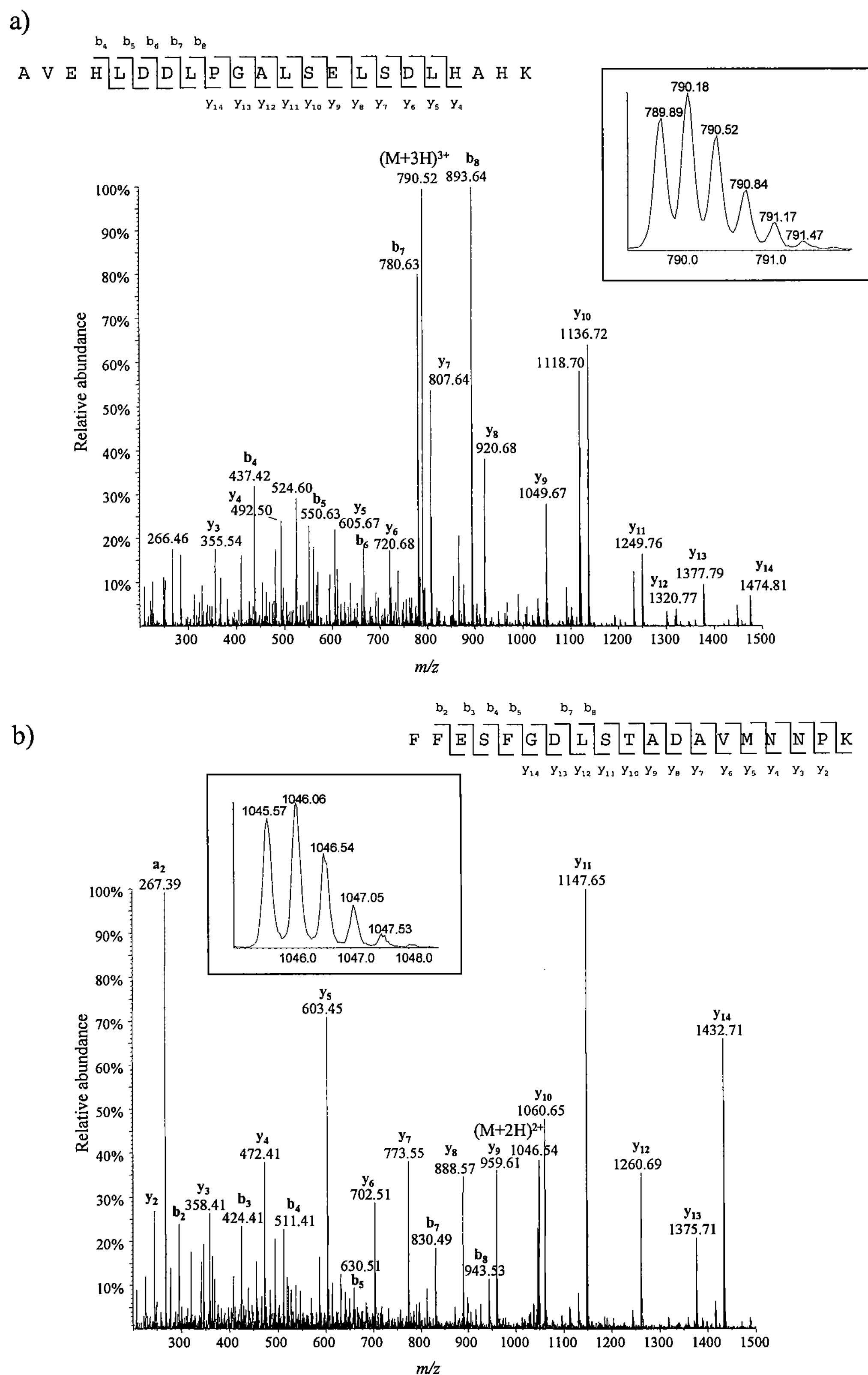

Figure 1: a) ESI-Product ion spectrum of *m/z* 790 of the triply charged peptide $A_{69\text{-}90}$ (2367.2 Da), and b) ESI-Product ion spectrum of *m/z* 1046 of the doubly charged peptide $B_{40\text{-}58}$ (2089.9 Da) of bovine hemoglobin.

Table 1: Predicted peptide masses of alpha- and beta-chains of human and bovine hemoglobin after tryptic digestion. Only peptides with masses higher than 500 Da or m/z greater than 300 without missed cleavage are listed.

	mass (Da)	peptides after ESI (*m/z*)		position	amino acid sequence	bovine Hb only
		$(M+2H)^{2+}$	$(M+3H)^{3+}$			
alpha-chains						
	2996,49	1499,24	999,83	62-90	VADALTNAVAHVDDMPNALS ALSDLHAHK	
	2969,61	1485,80	990,87	100-127	LLSHSLLVTLASHLPSDFTP AVHASLDK	*
	2967,61	1484,81	990,20	100-127	LLSHCLLVTLAAHLPAEFTP AVHASLDK	
	2367,19	1184,60	790,06	69-90	AVEHLDDLPGALSELSDLHA HK	*
	1833,89	917,95	612,30	41-56	TYFPHFDLSHGSAQVK	
	1529,73	765,87	510,91	17-31	VGAHAGEYGAEALER	
	1279,73	640,86	427,58	128-139	FLANVSTVLTSK	
	1252,71	627,36	418,57	128-139	FLASVSTVLTSK	
	1071,55	536,78	358,18	32-40	MFLSFPTTK	
	818,44	410,22		93-99	VDPVNFK	
	729,41	365,71		1-7	VLSPADK	
	703,40	352,70		1-7	VLSAADK	*
	532,29			12-16	AAWGK	
	526,31			57-61	GHGKK	
beta-chains						
	2089,95	1045,98	697,65	40-58	FFESFGDLSTADAVMNNPK	*
	2058,95	1030,47	687,32	41-59	FFESFGDLSTPDAVMGNPK	
	1719,97	860,99	574,32	105-120	LLGNVLVCVLAHHFGK	
	1669,89	835,95	557,63	67-82	VLGAFSDGLAHLDNLK	
	1422,73	712,36	475,24	120-131	EFTPVLQADFQK	*
	1421,67	711,84	474,89	83-95	GTFATLSELHCDK	
	1391,66	696,83	464,89	82-94	GTFAALSELHCDK	*
	1378,70	690,35	460,57	121-132	EFTPPVQAAYQK	
	1314,66	658,33	439,22	18-30	VNVDEVGGEALGR	
	1274,73	638,36	425,91	31-40	LLVVYPWTQR	
	1265,83	633,92	422,94	104-115	LLGNVLVVVLAR	*
	1177,68	589,84	393,56	132-143	VVAGVANALAHR	*
	1149,67	575,84	384,22	133-144	VVAGVANALAHK	
	1126,56	564,28	376,52	96-104	LHVDPENFR	
	1101,55	551,78	368,18	19-29	VDEVGGEALGR	*
	1098,56	550,28	367,19	95-103	LHVDPENFK	*
	1097,53	549,76	366,84	66-75	VLDSFSNGMK	*
	952,51	477,25	318,50	1-8	VHLTPEEK	
	950,51	476,26	317,84	8-16	AAVTAFWGK	*
	932,52	467,26	311,84	9-17	SAVTALWGK	
	821,41	411,71		1-7	MLTAEEK	*
	740,39	371,20		76-81	HLDDLK	

In Table 1, peptides predicted for tryptic digestions of human and bovine Hb are listed, and those naturally not occurring in human blood are labelled by an asterisk. Peptides specifically originating from bovine hemoglobin as well as those commonly generated by bovine and human Hb were selected as target compounds and detected by LC-ESI-MS/MS. Bovine Hb specific peptides can originate from both subunits, alpha and beta-chain, such as the fragments A_{69-90} (2367.2 Da) or B_{40-58} (2089.9 Da) that were detected by ESI-MS as the triply charged (*m/z* 790) or doubly charged (*m/z* 1046) molecules, respectively, the product ion spectra of which are shown in Figure 1.

The combination of liquid chromatogtaphy, ESI-MS/MS of unique peptides of bovine and human Hb, and those commonly generated by both proteins enables the identification of Hb originating from either human or bovine species. The efficiency of digestion is determined by analysis of the carbonic anhydrase peptide comprising the amino acid residues 36-56 with a molecular mass of 2198.2 Da. Its doubly charged molecule is observed in ESI mass spectra at *m/z* 1100.1 proving enzyme activity and hydrolysis of proteins during sample preparation. In Figure 2, a typical test result of a 50 µL plasma sample fortified with ISTD and 200 µg of Hemopure® is shown, containing the doubly charged molecules at *m/z* 1046 and its product ions after collisionally activated dissociation (CAD), the unique alpha-chain peptide at *m/z* 790, common Hb peptides at *m/z* 638, 766 and 918, and the ISTD at *m/z* 1100. Furthermore, the peptide specifically indicating human Hb at *m/z* 1030 and one of its fragment ions after CAD at *m/z* 547 are monitored, giving evidence for the presence or absence of human Hb in investigated plasma samples. Samples fortified with human Hb at concentrations of 2-4 mg/mL yielded signals at characteristic peptide ion traces but were lacking peaks at *m/z* 1046 and 790 at appropriate retention times. An example of a test result for a plasma specimen fortified with 4mg/mL of human Hb is shown in Figure 3.

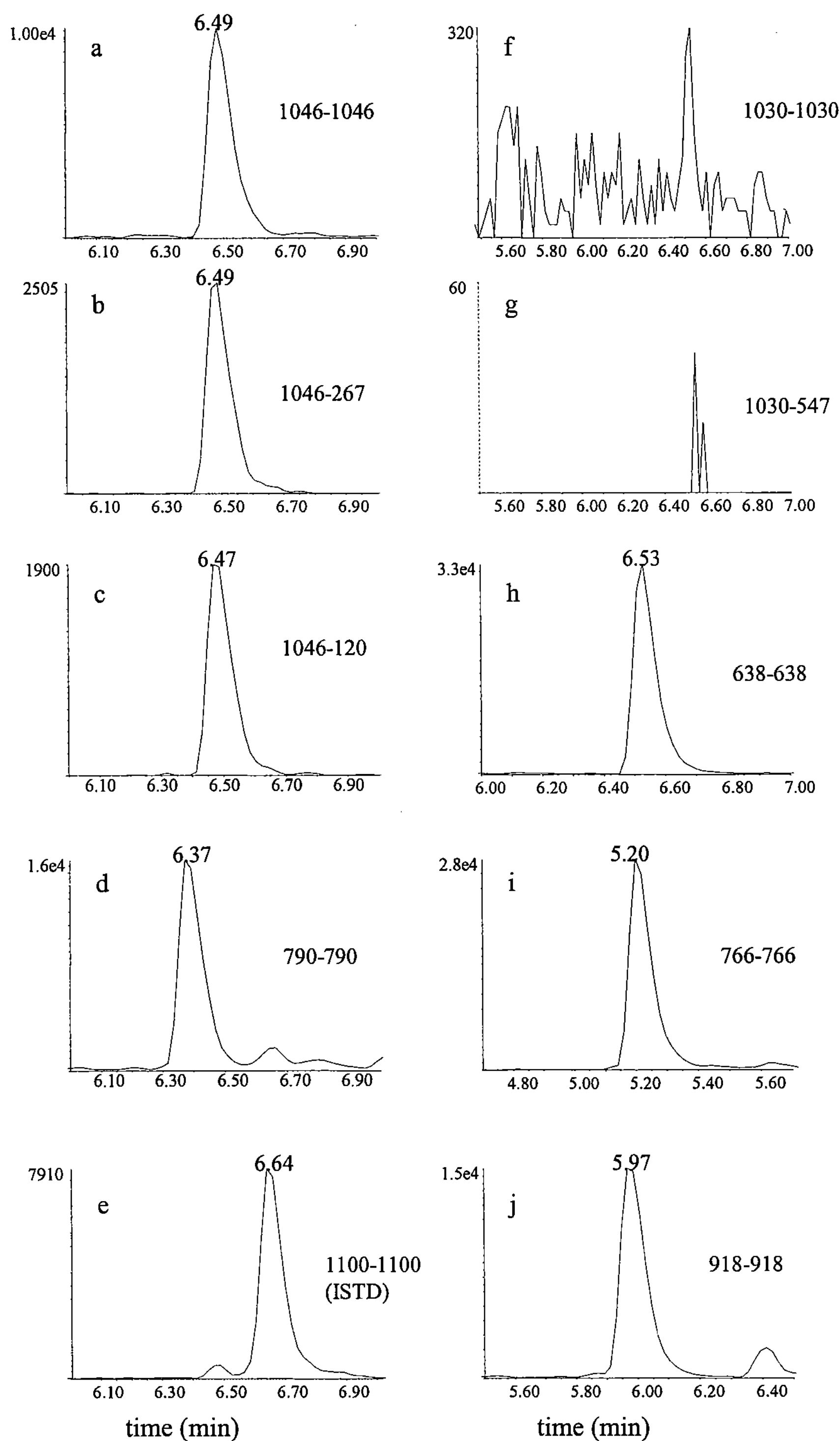

Figure 2: Test result of a plasma sample fortified with Hemopure® to 4 mg/mL. The peptides at *m/z* 1046 (a), its product ions at *m/z* 267 (b) and 120 (c), and 790 (d) are bovine hemoglobin related. Peptides monitored at *m/z* 638 (h), 766 (i) and 918 (j) are commonly generated by human and bovine Hb, while *m/z* 1030 (f) and its product ion at 547 (g) are specifically originating from human Hb

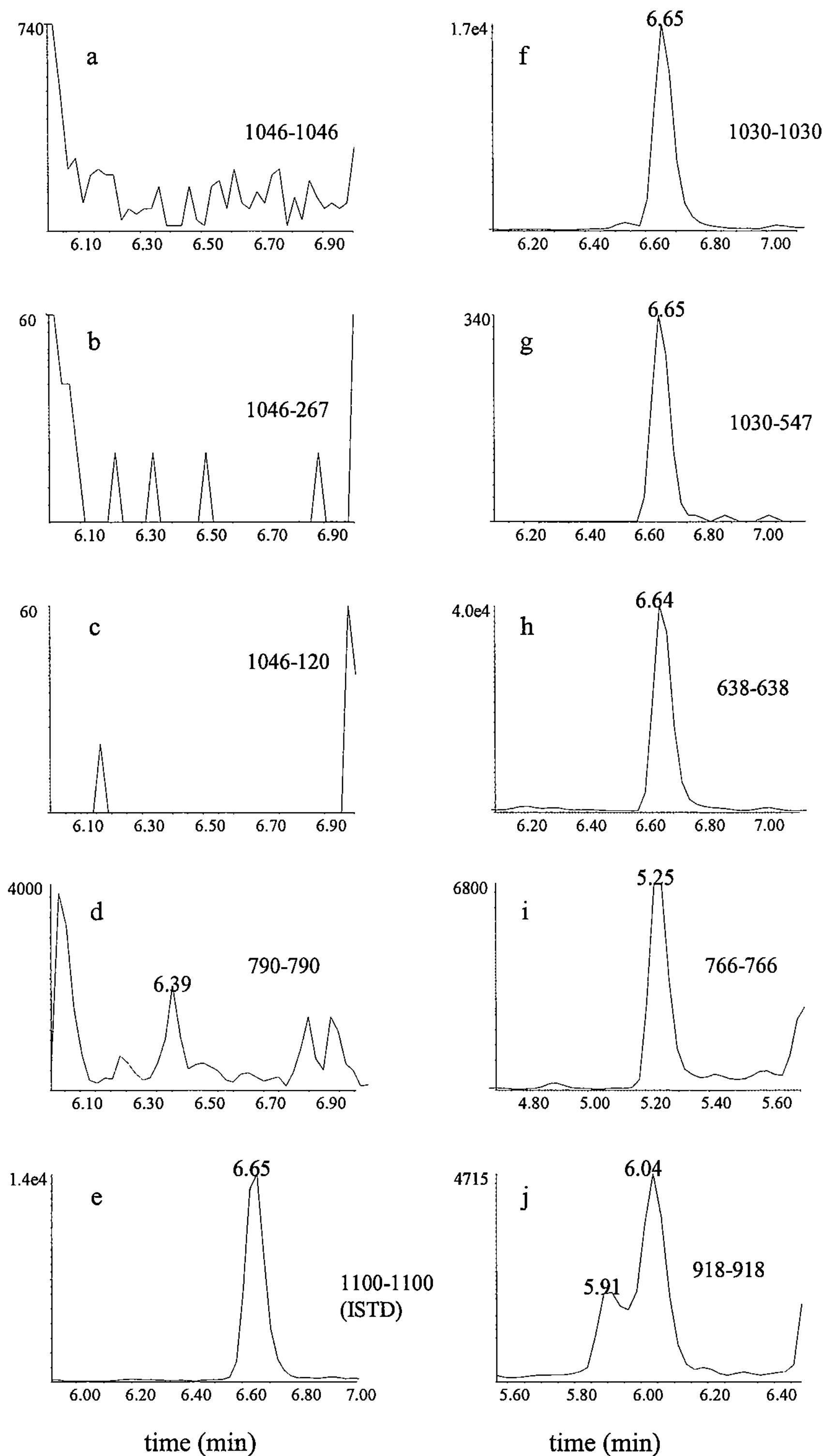

Figure 3: Test result of a blank plasma sample fortified with human hemoglobin to 4mg/mL. No signals for bovine Hb are observed at expected retention times (a-d), but peaks indicating the presence of human hemoglobin at *m/z* 1030 (f), its product ion *m/z* 547 (g) and common peptides of human and bovine Hb (h-j).

Specificity and detection limits. Plasma samples fortified with Hemopure® and human Hb at the ratios 1:4 and 2:1 demonstrated the possibility to unambiguously identify the bovine hemoglobin-based oxygen therapeutic in the presence of high amounts of human Hb. Peptides characteristic for bovine Hb were identified by tandem mass spectrometry at Hemopure® concentrations of 1mg/mL. The estimated detection limit of 0.25 mg/mL can be improved by injection of greater sample volumes into the LC-ESI-MS/MS system, tryptic digestion of the entire sample washed initially on a 100kDa filter unit, and concentration of the final filtrate. Given the recommended administration of at least 250 mL of Hemopure® containing 30g of crosslinked bovine hemoglobin, and an estimated blood volume of 6 L, blood concentrations of Hemopure® of approximately 5 mg/mL are expected, hence plasma concentrations of 10 mg/mL are likely. With pharmacokinetic studies after administration of 45g of Hemopure®, the half-life of this remedy was determined as 20h[4]. Owing to the presumably high concentrations of oxygen therapeutics such as Hemopure®, and its limited half-life after intravenous application, detection limits of 1 mg/mL are sufficient for doping control analysis, as a putative misuse is expected only at the day of competition. Thus, no further investigations were performed in order to identify peptides of bovine hemoglobin at lower concentrations.

Analysis of blood samples of high performance athletes. In order to estimate influences of high physical stress and common nutrition of high performance athletes on blood analysis for bovine hemoglobin, 68 blood samples of endurance sport athletes were analyzed as described above. No positive test result was obtained, and specificity was maintained also with samples containing high amounts of human hemoglobin (about 20 mg/mL) owing to severe hemolysis of the blood specimens.

Conclusion

The determination of bovine hemoglobin-based oxygen therapeutics in human plasma for doping controls is performed with aliquots of 50 µL. Employing common filtration and enzymatic degradation techniques, an identification of tryptic peptides generated from bovine hemoglobin is accomplished by liquid chromatography interfaced by ESI to tandem mass spectrometry. In the present study, a triple quadrupole mass spectrometer was used, which

provides sufficient sensitivity for the detection of multiply charged molecules and enables MS/MS experiments facilitating the identification of bovine hemoglobin-based peptides.

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