Identification of Recombinant Equine Growth Hormone in Horse Plasma by LC−MS/MS: A Confirmatory Analysis in Doping Control

ARTICLE *in* ANALYTICAL CHEMISTRY · OCTOBER 2008

Impact Factor: 5.64 · DOI: 10.1021/ac801234f · Source: PubMed

Anal. Chem. **2008,** *80,* 8340–8347

Identification of Recombinant Equine Growth Hormone in Horse Plasma by LC−MS/MS: A Confirmatory Analysis in Doping Control

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Equine growth hormone (eGH) has been available since 1998 as an approved drug (EquiGen-5, Bresagen) containing recombinant eGH (reGH). It is suspected of being illegally administered to racehorses in order to improve physical performance and to speed-up wound healing. Thus it may be considered a doping agent which would require a sensitive and reliable method of identification and confirmation in order to regulate its use in racehorses. reGH differs from the native eGH by an additional methionine at the N-terminal (met-eGH) and has never been unambiguously detected in any type of biological matrix at trace concentrations (1−10 μg/L). A plasma sample (4 mL) was treated with ammonium sulfate at the reGH isoelectric point and the pellet was purified by solid-phase extraction. Specific peptides were generated by trypsin digestion and analyzed by LC−MS/MS. The detection limit was 1 μg/L. The method was validated according to European Union regulation (DEC/2002/657/EC) and the Association of Official Racing Chemists (AORC) requirements. Furthermore, it was successfully applied to determining the plasma concentrations of reGH with time using linear ion trap mass analyzer. The presence of this prohibited hormone (reGH) was also successfully detected by triple quadrupole mass spectrometry up to 48 h postadministration of reGH to a horse. The present LC−MS/MS method is the first with adequate sensitivity and specificity for detection of reGH, rbGH, and endogenous eGH. Hence, an efficient analytical tool is proposed as a means to fulfilling the regulation of reGH abuse in the horse racing industry.

Growth hormone (GH) or somatotropin (ST) comprises 190 amino acids produced by the anterior pituitary gland.[1] Its has many biological functions such as growth, developmental, and reproductive physiology.[2,3] Recombinant equine growth hormone (reGH) was first synthesized as a research topic[4,5] before being marketed in 1998 as a licensed drug in Australia (EquiGen-5). This recombinant molecule differs from the native form by one additional amino acid, methionine, at the N-terminal (met-eGH). It was indicated for improvement of a positive nitrogen balance in horses older than 15 years.[6] However, reGH can be diverted from its intended clinical use to improving both athletic performance and wound healing.[7] The possibility of worldwide illicit use and abuse of reGH cannot be ruled out despite the ban enacted by racing rules (International Federation of Horseracing Authorities 2007, IFHA). The need to regulate the use of reGH in racehorses has led to the development of various analytical methods aimed at detecting its misuse. To date, no direct method with adequate sensitivity for the detection of reGH in plasma at the required level (1−10 μg/L)[2,8,9] has been reported. Consequently, various alternative methods for the screening of GH abuse have been developed, such as quantification of secondary markers of rGH administration: insulin-like growth factor-1 (IGF-1),[10−13] IGF binding protein 3 (IGFBP-3),[14] and more recently, the long-term detection of specific antibodies anti-reGH, produced as a consequence of repeated reGH administrations.[15]

On the basis of differences in amino acid sequence, the molecular weight discrimination by mass spectrometry between endogenous GH and recombinant forms is well documented in

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10.1021/ac801234f CCC: $40.75
Published on Web 09/25/2008

Table 1. LC Gradient for the Separation of reGH Tryptic Peptides

time (min)	flow rate (mL/min)	water, 0.2% FA	acetonitrile, 0.2% FA
0	0.300	70	30
5		50	50
12		40	60
17		0	100
22		0	100
27		70	30
35		70	30

several species such as bovine,[16−18] pig,[19] and equine.[20,21] Moreover, GH detection can be improved in terms of sensitivity and specificity by peptide mass mapping,[17,18,21−24] involving liquid chromatography−tandem mass spectrometry (LC−MS/MS) for the detection of the respective N-terminal peptides specific to growth hormones. Nonetheless, all data reported were obtained from reference drug standards in clean buffer matrix. Therefore, the main focus in this method development was rGH isolation from biological matrixes. So far, reported methods for GH purification were on tissue samples containing high concentrations of GH, e.g., in bovine,[25−27] pig,[28] or horse[29] pituitary gland. An attempt was made to extract and identify rGH from 10 mL of bovine plasma, spiked with 10 μg/L of recombinant porcine growth hormone (rpGH).[30] This method successfully used ultracentrifugation and immunoaffinity steps for analyte enrichment prior to LC−MS detection.

In this study, the analytical challenge was to develop a method capable of detecting reGH in equine plasma at low concentration (1 μg/L or 45 pmol/L) in a sample <5 mL. Plasma matrix contains abundant proteins, such as albumin or immunoglobulin which further complicate reGH extraction and purification. Nevertheless, plasma was still the preferred test medium to urine because the concentration of GH in the former is nearly 1000 times higher than that in the latter due to glomerular filtration.[31,32] All these critical points were considered in setting up a dedicated cleanup protocol leading to the successful confirmation of reGH in plasma by LC−MS^n. Even if a qualitative test would have been sufficient for the assessment of the abused hormone, this study was conducted with addition of an internal standard for quantification purposes and validated according to the European Union DEC/2002/657/EC[33] and Association of Official Racing Chemist (AORC) requirements.[34]

MATERIALS AND METHODS

Chemicals and Reagents. Recombinant equine growth hormone, reGH (EquiGen-5) was purchased from Bresagen Ltd. (Thebarton, Australia). Recombinant bovine growth hormone (rbGH) and equine growth hormone (eGH) were obtained from the Harbor-UCLA Medical Center, National Hormone and Pituitary Program (Torrance, CA). Synthetic peptides with amino acid sequences ^{1}MFPAMPLSSLFANAVLR^{17}, ^{1}FPAMPLSSLFANAV-LR^{16}, and ^{1}MFPAMSLSGLFANAV-LR^{17} corresponding, respectively, to reGH, eGH, and rbGH N-terminal tryptic peptides were purchased from Millegen (Labège, France). Trypsin (sequencing grade) was purchased from Promega (Charbonnières-les-Bains, France). HPLC-grade methanol and acetonitrile were purchased from Carlo Erba (Val de Reuil, France). Formic acid (FA), trifluoroacetic acid (TFA), ammonium bicarbonate, pepstatin A, and EDTA were obtained from Sigma-Aldrich (St. Louis, MO). Ammonium sulfate, sodium dihydrogenophosphate, and disodium hydrogenophosphate were purchased from VWR International (Leuven, Belgium). All buffers and solutions were exclusively prepared with high purity water with conductivity of >18 MΩ cm and filtered online through a 0.22 μm membrane (Millipore, Bedford, MA).

Standard solutions of GH and rGH were prepared in EPAB buffer (pH 7.9, 10 mM EDTA, 1 μM pepstatine A, 50 mM ammonium bicarbonate) at a concentration of 1 mg/mL and stored at 4 °C. Standard solutions of synthetic peptides were prepared in water/acetonitrile (70/30 v/v, 0.2% FA) at a concentration of 1 mg/mL and stored at 4 °C. Working standard solutions were prepared by consecutive 1/10 dilutions and used within 1 month with storage at 4 °C.

Drug Administration. Drug administration protocol was established in agreement with animal welfare rules and performed at the administration and sampling Center of Fédération Nationale des Courses Françaises (FNCF). A 17 year-old, 557 kg Thoroughbred stallion, identified as H563, was subcutaneously administered with reGH (25 μg/kg bw), according to the protocol supplied. Whole blood (50 mL) was collected from the jugular vein into lithium heparinate tubes (Greiner Bio-One SAS, Courtaboeuf, France) from which nearly 25 mL of plasma was obtained. Samples were collected at 35 and 15 min before drug administration and at 8 min, 25 min, 40 min, 60 min, 90 min, 120 min, 180 min, 4 h, 5 h 30 min, 6 h 30 min, 7 h 30 min, 8 h 30 min, 9 h 30 min, 11 h 30 min, 13 h 30 min, 15 h 30 min, 24 h, 26 h, 36 h, 48 h, Day$_{+4}$, Day$_{+5}$, and Day$_{+7}$ after drug administration. All plasma samples were stored at −80 °C until analysis. A total of 20 blank plasma samples were collected from horses at an experimental breeding

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Table 2. Parameters for the Detection of eGH, reGH, and rbGH N-Terminal Peptides in Product Ion Scans Mode Using Linear Ion Trap Mass Analyzer

N-terminal	$[M + 2H]^{2+}$ average m/z	collision energy (%)	Q_z	activation time (ms)	isolation windows (m/z)	scan windows (m/z)	fragment ions (m/z)
eGH	868.0	20	0.260	45	3	400−1500	447.0 1287.5 1288.5
reGH	933.5	20	0.260	45	3	500−1500	578.0 794.0 1287.5 1288.5
rbGH	913.0	18	0.260	45	3	600−1600	774.0 790.0 960.0 1047.5 1247.5

Table 3. Amino Acid Sequences of N-Terminal Peptides of eGH, reGH and rbGH, Based on Trypsin Cleavage, And Experimental Average Mass-to-Charge Ratio of the Doubly-Charged Peptide

growth hormones	N-terminal amino acid sequences	molecular mass (Da)	$[M + 2H]^{2+}$ average m/z
eGH	^{1}FPAM-PLSS-LFANAVLR^{16}	1733	868.0
reGH	^{1}M-FPAM-PLSS-LFANAVLR^{17}	1864	933.5
rbGH	^{1}M-FPAM-SLSG-LFANAVLR^{17}	1824	913.0

farm of Haras Nationaux (Chambéret, France) for method validation.

Analytical Procedure. *Ammonium Sulfate Precipitation.* To 4 mL of plasma, 18 mL of phosphate buffer (0.1 M, pH 6.9) were added and mixed by slowly inverting the tube. A saturated solution of ammonium sulfate (pH 6.9, 18 mL) was then added in a dropwise manner with gentle stirring until 45% saturation was achieved. Stirring continued for 90 min at room temperature after which the mixture was stored overnight at 4 °C to induce protein precipitation. Following centrifugation at room temperature (3000*g*, 20 min), the supernatant was discarded and the protein pellet was dissolved in 4 mL of phosphate buffer (0.1 M, pH 6.0).

Solid-Phase Extraction. C_4 SPE cartridges (500 mg, 6 mL, 300 Å), obtained from Interchim (Montluçon, France) were sequentially conditioned with 10 mL each of methanol, water, and phosphate buffer (0.1 M pH 6.0), using a VacElut vacuum manifold (Sigma-Aldrich, St. Louis, MO). Sample was then loaded onto the column and allowed to pass through the column bed, followed by washing with 5 mL of water containing 0.1% TFA and then with 5 mL of 70/30 water/acetonitrile containing 0.1% TFA. Analytes of interest were eluted with 7 mL of 20/80 water/acetonitrile containing 0.1% TFA. The eluate was dried to 1 mL under a gentle stream of nitrogen at 48 °C in a TurboVap LV Evaporator (Zymark, Hopkinton, MA).

Methanol Precipitation. Cold methanol (5 mL, −20 °C) was slowly added to the eluate and left at −20 °C for 90 min prior to centrifugation (4 °C, 3000*g*, 15 min), then 5 mL of the supernatant was discarded, and the remainder was dried as described above.

Trypsin Digestion. EPAB (120 μL, pH 7.9) and 20 μL of acetonitrile were added to the dry extract and gently mixed with repeated aspiration in a pipet tip. The mixture was carefully transferred into a glass HPLC insert. Trypsin (2 μg) in EPAB was added, and extract solution was digested at 37 °C for 17 h. Prior to LC−MS/MS analysis, the peptides obtained were dried under nitrogen at 48 °C and then dissolved in 40 μL of water/acetonitrile (70/30 v/v, 0.2% FA). Samples could be stored for 1 week at 4 °C before analysis without loss of sample integrity.

LC−MS/MS Measurements. LC−MS/MS analyses were carried out, either on a linear ion trap (LTQ XL) or on a triple quadrupole (TSQ Quantum Ultra), by ThermoFisher Scientific (San Jose, CA) with an electrospray ionization (ESI) source operated in the positive mode, interfaced with either a 1200 series pump (Agilent, Palo Alto, CA) or a Surveyor pump (ThermoFisher Scientific, San Jose, CA) equipped with a standard autosampler, a vacuum degasser, and a column oven. Separations were performed on a wide-pore UP5WC$_4$ column (300 Å, 2.0 mm i.d. × 250 mm, 5 μm particle size, Interchim, Montluçon, France) with guard column (2.0 mm i.d. × 10 mm). The LC flow was 300 μL/min. The mobile phase comprised water/acetonitrile + 0.2% formic acid. The LC gradient is shown in Table 1. The sample injected for analysis was 20 μL.

The linear ion trap and the triple quadrupole instruments were calibrated monthly according to the instrument manual. ESI source parameters were optimized with GH, rGH, and peptides standards (10 ng/mL) in methanol/water (50:50, v/v) containing 0.1% FA and directly introduced into the analyzer at 8 μL/min by syringe pump incorporated within the mass spectrometer instrument.

Parameters of the linear ion trap mass spectrometer were 270 °C and 4.9 kV for ion transfer capillary temperature and ESI needle voltage, respectively. Sheath gas and auxiliary gas were 45 and 10 arbitrary units, respectively. Activation Q_z of 0.26 with activation of 45 ms was for isolation of the three specific precursor ions $[M + 2H]^{2+}$. The collision-induced dissociation (CID) of $[M + 2H]^{2+}$ precursor ions was in the MS^2 mode, and the resulting fragment ions were monitored (Table 2).

Settings of the triple quadrupole mass spectrometer were 350 °C and 4.0 kV for ion transfer capillary temperature and ESI needle voltage, respectively. Sheath, auxiliary, and sweep gases were 95, 5, and 15 arbitrary units, respectively. Argon collision gas pressure was 1.0 mTorr. Collision energy of 28 V was used for fragmentation of the N-terminal reGH peptide precursor ion $[M + 2H]^{2+}$ (m/z 933.5). MS^2 was performed in selected reaction monitoring (SRM) mode, with two ion transitions: m/z 933.5 → m/z 1287.5 and m/z 933.5 → m/z 794.0. The Q1 and Q3 peak widths were 0.70 m/z. To improve detection selectivity, the Q3 scan width was

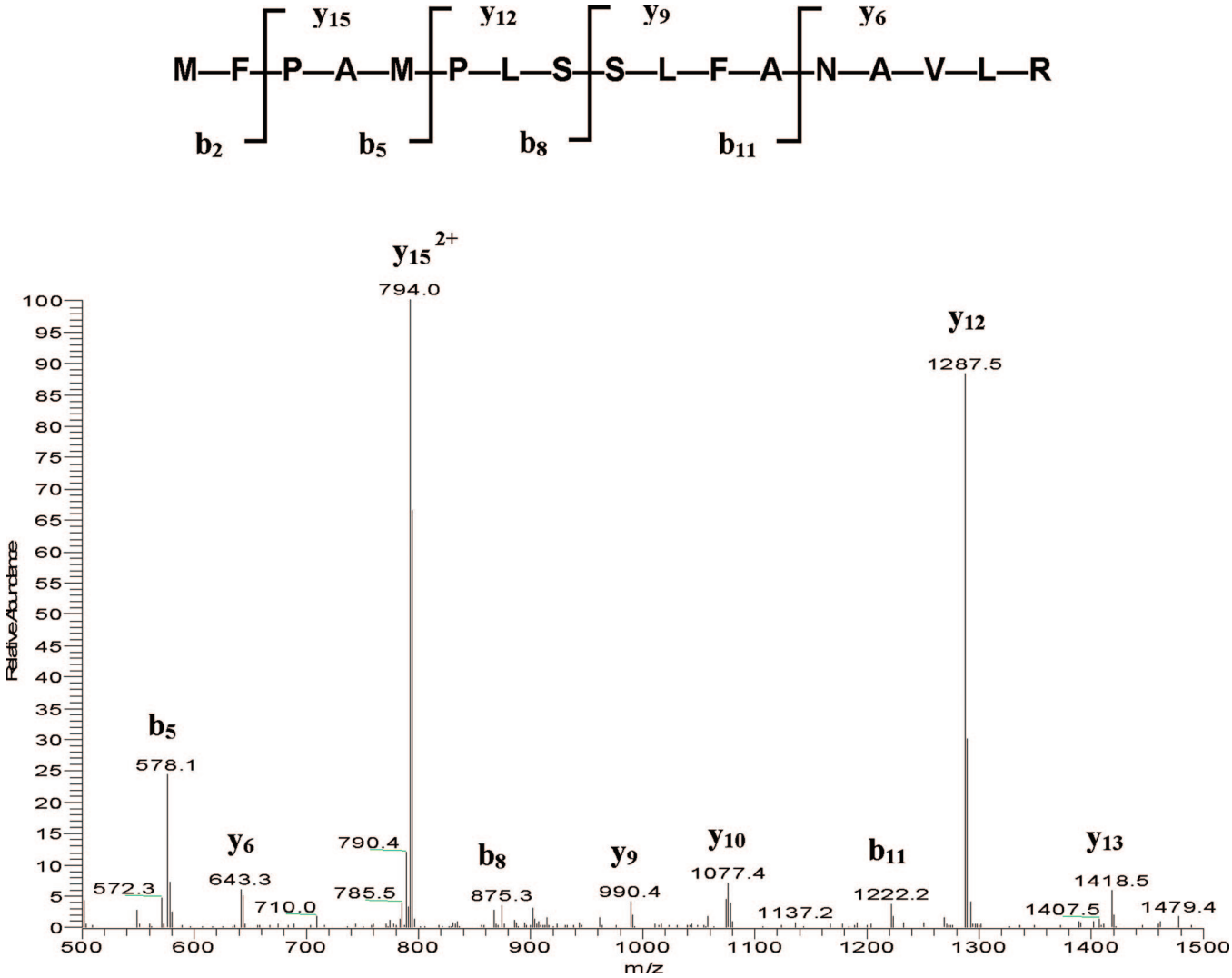

Figure 1. Product ion spectrum at low-energy CID (20%) of the characteristic doubly charged synthetic N-terminal peptide of reGH ($[M + 2H]^{2+}$, m/z 933.5) displayed the partial sequence information from b and y ion series in the linear ion trap between 500 < m/z < 1500. Identification of the three most abundant ions resulting from cleavage at the N-terminal side of prolines: m/z 794.0 (y_{15}^{2+}), m/z 1287.5 (y_{12}), and m/z 578.1 (b_5).

set at 0.01 m/z. All data were acquired and analyzed by Xcalibur software v. 2.0 (ThermoFisher Scientific).

RESULTS AND DISCUSSION

MS and MS/MS Parameters Optimization. reGH, eGH, and rbGH standards were characterized by linear ion trap mass analyzer. At least 10 multiply charged ions were observed and, after deconvolution,[35] 21 885 Da was calculated for reGH, 21 756 Da for eGH, and 21 874 Da for rbGH. The results were in agreement with data previously reported for equine GH[19,20] and bovine GH.[18,19]

For enhancement of GH detection, sensitivity, and specificity, we focused on specific peptides, particularly at the N-terminals, where slight differences in amino acid sequence occur. Each N-terminal synthetic peptide was directly introduced into the linear ion trap mass analyzer with resulting data (Table 3). Peptides were characterized by their doubly charged state $[M + 2H]^{2+}$, as is common with peptides resulting from trypsin digestion.[36] Upon storage at 4 °C in excess of 30 days, slight modification in amino acid composition in N-terminal peptides consisting mainly of deamidation of Asn (N) to Asp (D) was observed (roughly 10% of the total amount). The consequence of this chemical reaction was both an increase of 1 Da in all N-terminal peptide ions and a slight decrease of the retention time.

MS/MS experiments were performed on each synthetic peptide to optimize CID for production of abundant specific fragments. Figure 1 shows the product ion spectrum of the characteristic doubly charged synthetic N-terminal peptide of reGH ($[M + 2H]^{2+}$, m/z 933.5). Identification of the most abundant fragment ions: m/z 794.0, m/z 1287.5, and m/z 578.1 corresponding to y_{15}^{2+}, y_{12}, and b_5, respectively, was achieved. These fragments resulted from cleavage at the N-terminal of the two proline residues. The presence of proline induces a significant proline effect.[36] Indeed, proline is unusual in that it is the only naturally occurring amino acid in which the side chain participates

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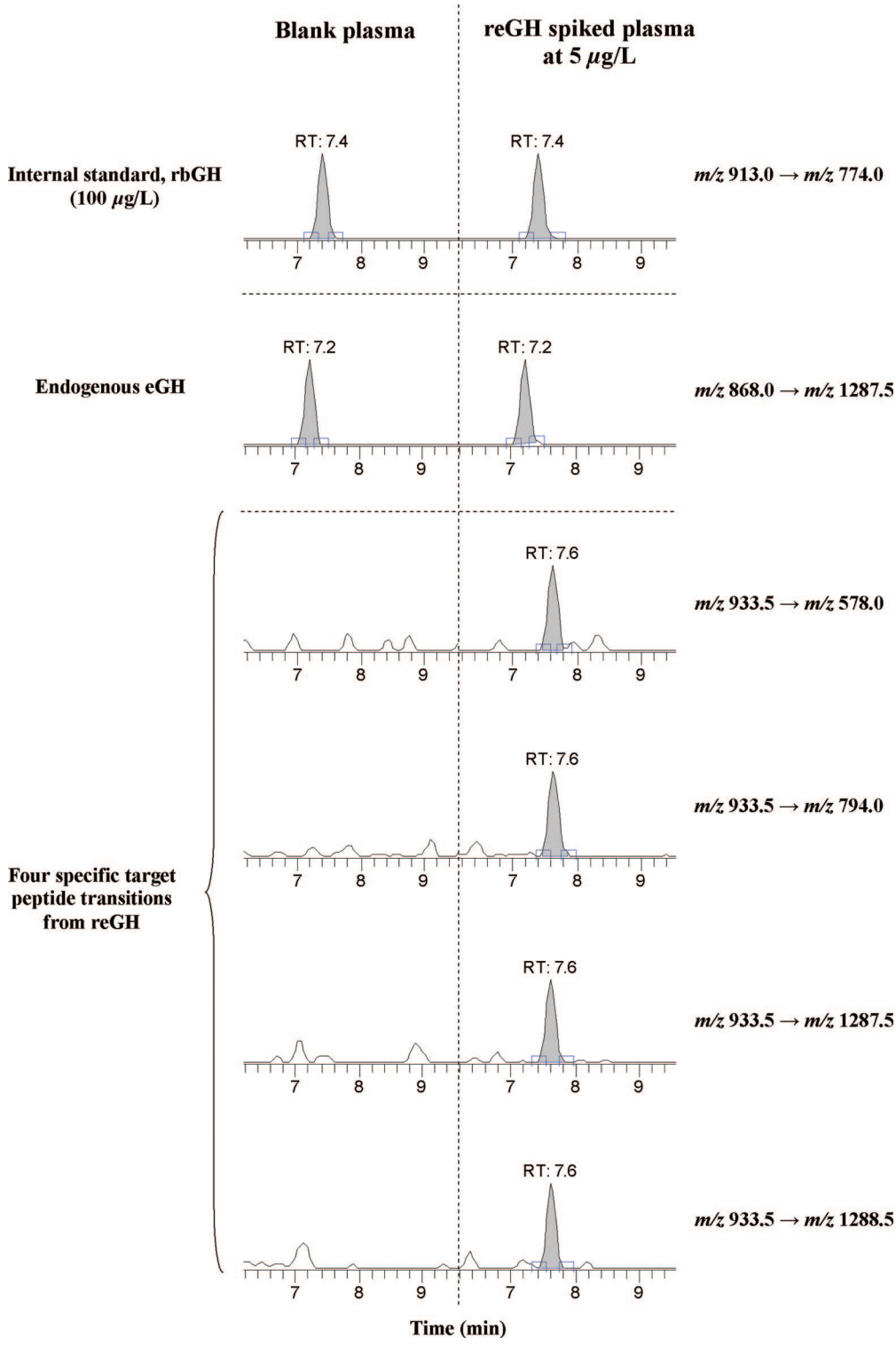

Figure 2. Ion chromatograms (blank plasma + IS and blank plasma spiked at 5 μg/L) of characteristic GH N-terminal peptides, after purification steps and LC−MS/MS ion product scan with linear ion trap. The characteristic rbGH N-terminal peptide ($[M + 2H]^{2+}$, *m/z* 913.0) spiked at 100 μg/L was the internal standard (IS), and eGH N-terminal characteristic peptide ($[M + 2H]^{2+}$, *m/z* 868.0 with retention time of 7.2 min) which is naturally present in plasma was presented with only one transition (*m/z* 868.0 → *m/z* 1287.5). In contrast, the specific target N-terminal peptide from reGH ($[M + 2H]^{2+}$, *m/z* 933.5, retention time of 7.6 min) was presented in both cases with its four specific ion transitions (*m/z* 933.5 → *m/z* 578.0, *m/z* 933.5 → *m/z* 794.0, *m/z* 933.5 → *m/z* 1287.5, *m/z* 933.5 → *m/z* 1288.5).

as a ring structure in the peptide backbone raising proton affinity and thus, enhancing cleavage at the N-terminal side of proline.

Method Development. The analytical challenge was to develop a method that would unambiguously discriminate pituitary GH from recombinant forms when present at low concentrations (1−10 μg/L) in equine plasma. The method comprised four optimized purification steps before analysis by LC−MS/MS.

Ammonium Sulfate Precipitation. To optimize a selective salt-induced precipitation, a pH of 6.9 corresponding to an isoelectric point (p*I*) of reGH, was evaluated in silico according to ProtParam

Table 4. Statistical Method Validation Using Two Transitions from reGH N-Terminal Characteristic Peptide Precursor Ion ([M + 2H]$^{2+}$, *m/z* 933.5)[a]

linearity (R^2)		repeatability				performances (μg/L)	
S1	S2	RT RSD	S1 intensity RSD	S2 intensity RSD	precision 5 μg/L	CCα	CCβ
0.9983	0.9912	0.4%	23%	16.1%	30% (3 μg/L)	0.3	0.7

[a] S, signal; RT, retention time; RSD, relative standard deviation. Signal 1 (S1), 933.5 → 1287.5 (y_{12}) and signal 2 (S2), 933.5 → 794.0 (y_{15}^{2+}) relative to rbGH N-terminal peptide precursor ion ([M + 2H]$^{2+}$, *m/z* 913.0) (internal standard, IS). IS transitions were 913.0 → 774.0, 913.0 → 960.0, and 913.0 → 1047.5. CCα was the decision limit, and CCβ was the detection capability.

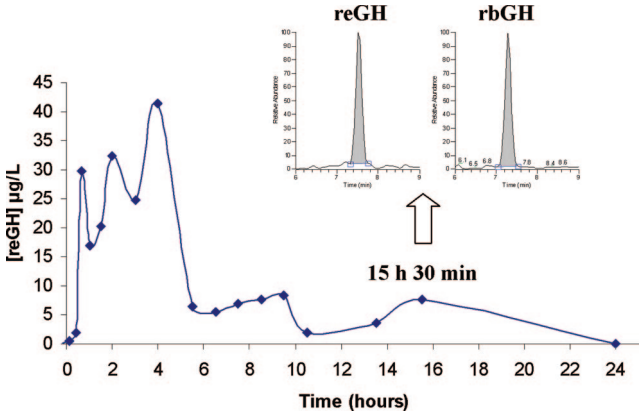

Figure 3. Plasma concentration of reGH at various time intervals after a single bolus subcutaneous injection of EquiGen-5, 14 mg to a horse. Predrug administration plasma samples were collected at 35 min and at 15 min prior to injection and at various time intervals up to 24 h postadministration. Characteristic N-terminal peptides from reGH and rbGH (IS) were identified by LC−MS/MS in the ion product scan mode by linear ion trap. The chromatograms shown were reconstructed using the product ions of *m/z* 578.0, 794.0, 1287.5, 1288.5 for reGH and of *m/z* 774.0, 790.0, 960.0, 1047.5, 1247.5 for rbGH. The last sample point that contained reGH was detected 15 h 30 min after reGH injection.

in ExPASy (http://www.expasy.ch/cgi-bin/protparam). At its p*I*, reGH exhibits no net charge leading to decreased solubility because the protein is unable to interact with the medium and thus precipitates. Therefore, various volumes of saturated ammonium sulfate (pH 6.9) were added to plasma spiked with reGH (50 μg/L) to yield a 20−90% saturation with salt. Maximum recovery of reGH was observed at 45% saturation which is consistent with data previously reported for GH precipitation: bGH precipitated at pH 7.2 and 45% ammonium sulfate from bovine pituitary extract,[27] hGH at 50% saturation in *Escherichia coli* extracts[37] or more recently rpGH was precipitated at 45% saturation from *Pichia pastoris* extracts.[38] Furthermore, in order to assess the impact of precipitation on protein content in the sample, protein was quantified by Lowry's method.[39] When the total amount of protein in plasma was 153 mg before precipitation, 50 mg was in the pellet and 103 mg in the supernatant with 45% ammonium sulfate saturation. Finally, this first step allowed 70% depletion of undesired proteins in the sample matrix.

Solid-Phase Extraction (SPE). For reGH enrichment, several SPE cartridges were evaluated. Optimal yields were obtained from C_4 cartridges. Evaluations were performed with or without TFA as an ion-pairing agent for the increase in protein hydrophobicity. Improved results were obtained when 0.1% TFA was present in the eluent (20/80; water/acetonitrile). Interaction of protein obtained after 45% ammonium sulfate precipitation, with a C_4 cartridge might be explained by the increase in hydrophobic interaction with the reversed phase in the presence of high salt content. The conditions reported here were close to those in hydrophobic interaction chromatography (HIC). Purification of GH by SPE is described for the first time, while HIC with phenylCL4B Sepharose was previously reported for eGH and hGH isolation from pituitary extracts.[37,40]

Methanol Precipitation. This step was introduced to improve the signal-to-noise ratio when peptides were analyzed by LC−MS/MS. Methanol (−20 °C) was used for dissolution of organic compounds, and simultaneous precipitation of proteins thereby enhance recovery and detection of N-terminal peptides. Effects of organic solvents on protein precipitation are similar to those observed in salting-out. A few examples of GH precipitation with organic solvents have been reported particularly with ethanol[25,41] or acetone.[42,43] These two organic solvents were also tested, but improved recovery was obtained from cold methanol.

Trypsin Digestion. Different quantities of the enzyme were tested; the optimal activity was by 2 μg in 4 mL of plasma. Trypsin concentration was experimentally determined in order to obtain the best yield of target reGH N-terminal peptide. Trypsin digestion was performed at 37 °C × 17 h in EPAB buffer (pH 7.9), containing EDTA to chelate metallic ions, and thereby inhibiting metalloprotease activity and to diminish analyte oxidation. Pepstatine A was used as an acid protease inhibitor to prevent undesirable cleavage. Parameters of hydrolysis presented here in terms of pH, temperature, and reaction time (pH 7.9, 37 °C, 17 h) are as previously reported to generate GH peptides.[17−21,43] In addition, similar trypsin concentration had already been reported for recombinant human erythropoietin identification in horse plasma by LC−MS/MS.[44]

LC-MS/MS Analysis. The analysis by LC−MS/MS of 20 different equine blank plasma extracts, purified as described above, allowed identification of the internal standard, rbGH (100

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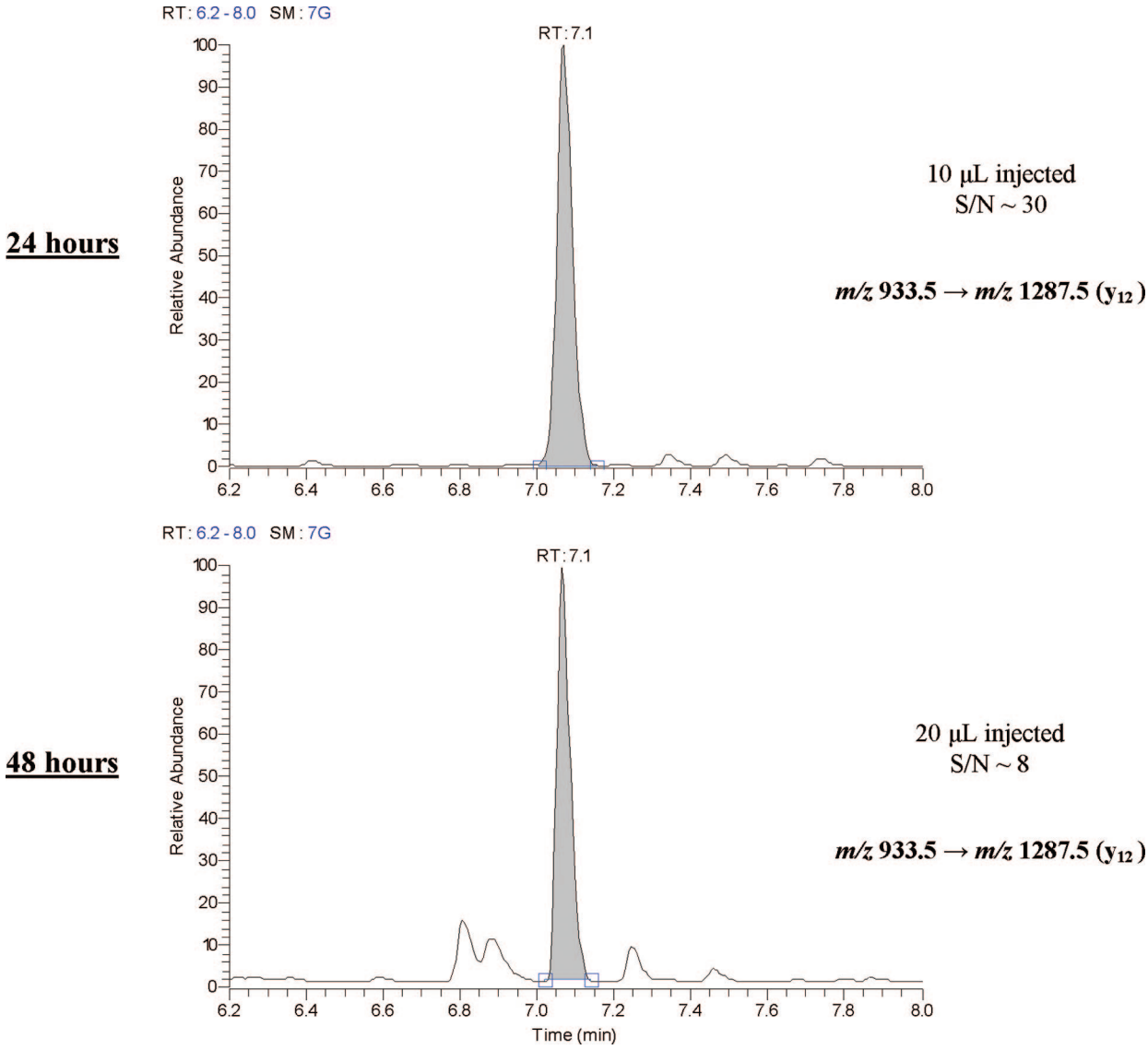

Figure 4. Detection of reGH in equine plasma (after one subcutaneous injection of EquiGen-5, 14 mg) at 24 and 48 h postadministration by recording the specific transition *m/z* 933.5 → 1287.5. Identification of the characteristic N-terminal reGH peptide was performed by LC−MS/MS in the selected reaction monitoring (SRM) mode by a triple quadrupole instrument.

μg/L), by its ion product scan after its $[M + 2H]^{2+}$ (m/z 913.0) fragmentation to m/z 774.0 at a retention time of 7.4 min, (Figure 2). Analysis of another aliquot of the same pool of equine plasma spiked with reGH at 5 μg/L allowed unambiguous identification of reGH by four specific ion transitions at the retention time of 7.6 min (Figure 2), after purification and LC−MS/MS detection of its characteristic N-terminal peptide ($[M + 2H]^{2+}$, m/z 933.5) fragment. Comparison chromatograms of blank and spiked plasma samples confirmed method specificity because no reGH signal was detected in blank plasma and thus, the ability to identify reGH in spiked equine plasma at the requested concentrations of 1−10 μg/L was successfully demonstrated. Moreover, for assessment of the detection limit at 1−10 μg/L, a search for pituitary GH, naturally present in plasma at the expected concentration of 1−10 μg/L was carried out in the same analysis for the detection of its characteristic N-terminal peptide ($[M + 2H]^{2+}$, m/z 868.0). The target peptide (m/z 868.0) was unambiguously identified at the retention time of 7.2 min (Figure 2), which was consistent with its hydrophobic nature compared with the other two peptides (recombinant equine and bovine). Identification of endogenous eGH in blank equine plasma indicated that the method had adequate sensitivity, consistent with RIA[2,7,9,40,45,46] and ELISA[47,48] in the detection of eGH. To the best of our knowledge, the present analytical procedure is the first to provide unambiguous identification of endogenous GH and its differentiation from the recombinant form in equine plasma. The only data so far reported were on the detection of rpGH in 10 mL of bovine plasma spiked at 10 μg/L.[30]

Method Validation. Validation of the protocol was performed by assessment of repeatability, selectivity, and robustness, according to the requirements of the European Union Decision

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2002/657/EC[33] and the AORC guidelines[34] for minimum criteria for analyte identification. New analytical considerations for identification of target peptides and proteins in doping control in sports were also taken into account.[49] First, the analysis of 20 blank samples was performed to evaluate selectivity. Specificity was satisfactory since no interferences with diagnostic ion chromatograms at the same retention time as that of reGH N-terminal peptide were observed. The calibration curve ($R^2 > 0.99$) for reGH concentrations of 0, 1, 5, 10, 20, and 40 μg/L in pooled equine blank plasma was created. Finally, 20 blank samples were spiked with reGH at 5 μg/L to determine detection capability and precision. The relative standard deviation (RSD) of the retention times (RT), with rbGH as internal standard, was below 2% (Table 4). RSD of signal intensity was slightly above 20% for S1 and below 20% for S2 (Table 4). Relative response of specific reGH N-terminal peptide versus its internal standard was acceptable with a precision of 30% in the low concentration range (Table 4), which is acceptable since in sports drug testing, only a qualitatively defensible method is needed to identify any illegal substance in plasma or urine of an athlete. Decision limit (CCα) and detection capability (CCβ) were calculated (Table 4). Detection capability (CCβ) is defined as the lowest concentration at which a method is able to detect a noncompliant sample with a statistical certainty of 1-β (error probability = 5%) was 0.7 μg/L, in accordance with results obtained from spiked plasma samples analyzed by a linear ion trap (0.8 μg/L; S/N = 3). Statistical analysis and identification of reGH in plasma spiked with less than 1 μg/L indicated that the method is capable of detecting and confirming the presence of reGH in equine plasma at low concentration.

Plasma Concentration of reGH. The method was successfully applied to plasma samples obtained from a horse that was subcutaneously administered with EquiGen-5 (14 mg). Figure 3 shows that no reGH was detected in the 35 and 15 min samples collected before administration. Plasma sample collected 8 min after EquiGen-5 administration contained reGH at an estimated concentration of 1 μg/L. Peak plasma concentration of reGH (42 μg/L) was 4 h after drug administration. Thereafter, reGH concentration decreased below 10 μg/L but was still detectable up to 15 h 30 min, and beyond that time no more reGH could be identified by the linear ion trap instrument. Consequently, we decided to evaluate the latest generation triple quadrupole mass analyzer in an attempt to improve detection of reGH in equine plasma beyond 15 h 30 min post drug administration. Figure 4 presents the chromatograms of reGH obtained from plasma samples collected 24 and 48 h after reGH administration (m/z 933.5 → m/z 1287.5). Low detection limits with a triple quadrupole instrument because of the high-scan rate frequency and high ion selectivity have been reported. In the 17 year-old horse used, no endogenous GH could be detected, which was consistent with the fact that the amplitude rather than the frequency of eGH secretory pulses decreases with advancing age and diminishing physiological activity.[6,8,50]

The present study is the first to provide plasma concentrations of reGH at various time intervals after reGH was subcutaneously administered to a horse. Moreover, reported values of GH plasma half-life (between 15−30 min),[51] obtained when GH was intravenously administered, cannot be compared with subcutaneous injection, since detection time attained lasted up to 48 h. In addition, the methionine which allows for the discrimination between endogenous and reGH played a key role in the detection time because the in vivo half-life of a protein is a function of its amino-terminal, following the "N-end Rule".[52,53] Consequently, the present method detected reGH several hours after its administration, either by linear ion trap or triple quadrupole mass analyzers.

CONCLUSION

The results were made possible by the first method that allowed unambiguous identification of reGH, eGH, and rbGH in horse plasma at trace plasma concentrations by monitoring their diagnostic tryptic N-terminal peptides either in product ion scans by a linear ion trap or in SRM by a triple quadrupole mass spectrometer. The present LC−MS/MS method was sufficiently specific and sensitive to confirm the presence of reGH in post-training or postrace plasma samples available to official racing laboratories. Moreover, this method can also be applied in the field of food safety in regulating the use and abuse of GH in farm animals and thus protect the consumer.

ACKNOWLEDGMENT

This work was funded by ANRT. The authors are indebted to Dr. Jean-Jacques Garin, veterinary surgeon at FNCF, to the horse farm manager in Coye la Forêt, and to the staff who participated in drug administration, sampling, and horse care, to the staff from the experimental breeding farm of Haras Nationaux for making available 20 blank plasma samples, and to Dr. Eric Génin from ThermoFisher Scientific (France) for encouraging the development of this method on a triple quadrupole instrument (TSQ Quantum Ultra). Finally, the authors thank Dr. Louis Dehennin for reviewing the manuscript.

Received for review June 17, 2008. Accepted August 25, 2008.

AC801234F

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