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LC-MS/MS Method for Simultaneous Analysis of Growth Hormone-Releasing Peptides and Secretagogues in Human Urine

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Abstract : Growth hormone (GH)-releasing peptides (GHRPs) and GH secretagogues (GHSs) are listed in the World Anti-Doping Agency (WADA) Prohibited List. In the present study, we developed and validated a method for the simultaneous analysis of seven GHRPs (alexamorelin, GHRP-1, -2, -4, -5, -6, and hexarelin) and three GHSs (anamorelin, ibutamoren, and ipamorelin) in human urine. Method validation was performed at minimum required performance levels specified by WADA technical documents (2 ng/mL) for all substances, and the method was validated with regard to selectivity (no interference), linearity (R2 > 0.9986), matrix effects (50.0%-141.2%), recovery (10.4%-100.8%), and intra- (2.8%-16.5%) and inter-day (7.0%-22.6%) precisions. The limits of detection for screening and confirmation were 0.05-0.5 ng/mL and 0.05-1 ng/mL, respectively.

Keywords : Doping control analysis, Growth hormone, GHRP, GHS, Method validation

Introduction

Human growth hormone (hGH) is a powerful anabolic hormone that reduces body fat, increases muscle mass, and accelerates recovery from sports injuries.[1] For these reasons, hGH is listed on the Prohibited List for competitors “in” and “out” of athletic competitions by the World Anti-Doping Agency (WADA).[2] Despite its prohibition, athletes, particularly in power and endurance sports, often use hGH. However, since 2010, hGH has become detectable using two distinct tests, the hGH isoform immunoassay and hGH biomarker test.[3]

Since Bowers et al.[4] reported a GH-releasing peptide (GHRP) that stimulates hGH secretion, similar GHRPs (GHRP-1, GHRP-2, and hexarelin) have been developed.[5] In particular, GHRP-2 has been reported to be a stimulant in many studies and it is used in diagnostic tests for hGH deficiency. GHRP-2 can also reportedly hide signs of hGH abuse by quickly reducing the ratio of recombinant/pituitary hGH after its injection.[6] In addition, nonpeptidyl GH secretagogues (GHSs), such as anamorelin[7] and MK-677 (ibutamoren),[8] have been developed to stimulate hGH release. Thus, these hGH-releasing substances are on the WADA Prohibited List and must be assessed by antidoping laboratories.

To date, several methods have been developed for doping control analysis of GHRPs and GHSs using liquid chromatography (LC)-coupled mass spectrometry (MS),[9] and metabolites of certain GHRPs have been identified by an excretion study.[10] Interestingly, the use of GHRP-1 and alexamorelin can only be detected by their metabolites excreted in urine.[11] During the 2014 Sochi Winter Olympic Games, GHRPs and GHSs, except anamorelin, were analyzed in more than 1000 official samples by LC-MS.[12] However, each antidoping laboratory needs to have their evaluation method accredited by International Organization for Standardization (ISO)/International Electrotechnical Commission (IEC) 17025. The aim of the current study was to develop and validate a method for a simultaneous analysis of hGH-releasing substances and their metabolites in human urine samples for doping control testing.

Experimental

Chemicals and reagents

GHRP-2 and GHRP-6 were purchased from ProSpec (East Brunswick, NJ, USA). GHRP-1, -4, -5, hexarelin, and ipamorelin were purchased from Abbiotec (San Diego, CA,

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USA). Ibutamoren was purchased from Axon Medchem (Groningen, Netherlands). Alexamorelin and metabolites of all GHRPs were synthesized by Peptron (Daejeon, South Korea). Anamorelin was obtained from MedChem Express (Princeton, NJ, USA). Heavy isotope-labeled [(^{13}C, ^{15}N) Lys] GHRP-2 and -6 used as internal standards (ISTD) were synthesized by JPT Peptide Technologies (Berlin, Germany). High performance LC grade acetonitrile and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA). Formic acid was purchased from Wako Pure Chemicals (Osaka, Japan), and phosphoric acid was purchased from Yakuri Pure Chemicals (Kyoto, Japan). Ammonium hydroxide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was produced by Milli-Q water purification system (Millipore, Bedford, MA, USA).

LC-MS/MS conditions

The Shimadzu Prominence LC-20A series reverse-phase LC system comprised an LC-20AD XR binary pump system, SIL-20AC XR autosampler, CTO-20AC column oven, and a DUG-20AS degasser (Shimadzu, Kyoto, Japan). Chromatographic separation was performed on a Kinetex C18 column (100 mm × 2.1 mm, 2.6 μm; Phenomenex, Torrance, CA, USA). Mobile phase A comprised 0.1% formic acid in water, whereas mobile phase B contained 0.1% formic acid in acetonitrile. The column oven was set at 35°C, and the flow rate was 0.5 mL/min. Gradient elution was completed as follows: 0.5 min isocratic flow in 5% mobile phase B, 5% mobile phase B linear to 95% mobile phase B for 5 min, 1 min isocratic flow in 95% mobile phase B, followed by 0.1 min isocratic flow in 5% mobile phase B, and equilibration for 1 min (total run time, 8 min).

The LC system was coupled to a TSQ Ultra Triple Stage quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a heated electrospray ionization source. Ion spray voltages were set at 4.5 kV in positive mode. The capillary and vaporizer temperatures were set at 320°C and 320°C, respectively. The sheath gas flow rate was 60 arbitrary units, and the auxiliary gas flow rate was 20 arbitrary units. The tube lens voltage was automatically optimized by the instrument calibration procedure with a solution of polytyrosine-1,3,6 (CS Bio, Menlo Park, CA, USA) according to the manufacturer's instructions. Selective reaction monitoring (SRM) mode was applied for analysis using precursor/product ion information. All the data were acquired using Xcalibur™ software (Thermo Scientific), and data processing was performed using Skyline software (University of Washington).

Stock and working solution

Each stock solution was prepared in methanol at a concentration of 1 mg/mL and stored at -20°C until used. Working reference and ISTD solutions were freshly prepared at 1 ng/mL by dilution using methanol.

Sample preparation

Each urine sample (2 mL) was spiked with 100 μL of 4% phosphoric acid and 10 μL of ISTD solution (final concentration, 5 ng/mL of ISTDs) and then centrifuged for 5 min at 3000 ×*g*. Mixed-mode weak cation exchange (WCX) cartridges (60 mg; Waters, Milford, MA, USA) were conditioned with 2 mL of methanol and 2 mL of water before applying samples. After washing with 2 mL of water and 2 mL of methanol, target analytes were eluted with 1 mL of elution buffer comprising either 5% formic acid in methanol or 25% ammonia/10% formic acid/ methanol (8/12/80). Extracts were evaporated to dryness under an N2 stream at 50°C, reconstituted in 200 μL of the mobile phase mixture (95% A/5% B), and then injected (10 μL aliquot) into the LC-MS/MS system.

Method validation

The selectivity of the method was estimated by analysis of five positive control urine (PCU) and five negative control urine (NCU) samples. PCU samples were spiked with the working reference and ISTD solutions (final concentration, 2 ng/mL of substances and 5 ng/ mL of ISTDs), whereas NCU samples were spiked only with ISTD solution (final concentration, 5 ng/mL of ISTDs). Interference signals from endogenous substances were considered on the chromatograms at the expected retention times. If the highest signal obtained had interference, the second signal was considered as the qualifier ion. Calibration curves for linearity were analyzed at seven concentration points (1, 2, 5, 10, 25, 50, and 100 ng/ mL of substances); five urine samples were analyzed at each concentration point. For determining recovery, urine samples were prepared in two ways. Five samples were spiked before WCX extraction with the working reference solution in blank urine, whereas another five samples were spiked after extraction with the working reference solution. ISTD solution was added to both sample groups before extraction.

Intra- and Inter-assay precisions were determined by analyzing six replicates of each PCU sample per day on three independent days. The results were expressed as the coefficient of variation (%CV) of the peak areas. Limits of detection (LOD) were defined as the lowest concentration with a signal-to-noise ratio of >3 and %CV of <25%. For determination of the LOD, five PCU samples were analyzed at each of six concentration points (2, 1, 0.5, 0.2, 0.1, and 0.05 ng/mL). Confirmation analysis of GHRPs and GHSs was performed according to minimum criteria for chromatographic-MS confirmation of the identity of analytes for doping control purposes outlined in the WADA technical document TD2015IDCR.

Results and discussion

List of substances for the WADA 2016 External Quality Assessment Scheme included three GHSs (anamorelin, ibutamoren, and ipamorelin) and seven GHRPs

(alexamorelin, GHRP-1, -2, -4, -5, -6, and hexarelin). This is the first study to successfully develop and validate the method for simultaneous analysis of these 10 substances and their metabolites.

Optimization of LC-MS/MS and WCX extraction conditions

Prior to SRM assay, we evaluated possible product ions by direct infusion with each reference stock. From the results, we selected three product ions per target. Then, for higher sensitivity, the collision energy was optimized by acquiring replicate results ±2 V using five steps from the predicted optimal collision energy value determined by Skyline's default equation. Almost all target substances were shown to increase the peak area by 10%-30% (data not shown). The selected charge state of the precursor ion and three product ions with optimized collision energy are shown in Table 1.

Traditional WCX elution buffers contain 5% formic acid in methanol for strong acids and organic solvents. Therefore, almost all antidoping laboratories have reported the use of these elution conditions when analyzing GHRPs. However, Mazzarino et al.[13] recently reported that some GHRPs and their metabolites could not be detected under

Table 1. Mass spectrometry parameters and retention times for the 27 substances.

Name	Precursor charge state	Precursor ion (*m/z*)	Product ion (*m/z*)*	Collision energy (V)	Retention time (min)
GHRP-1	+2	478.25	84.0	34	3
			129.1	21	
			209.1	20	
GHRP-1 M1	+2	442.73	110.0	35	3
			129.0	21	
			335.2	29	
GHRP-1 M2	+2	374.20	153.0	35	3.2
			170.1	18	
			479.3	10	
GHRP-1 M3	+2	443.23	109.9	20	3.2
			146.9	16	
			306.9	23	
GHRP-1 M4	+1	620.29	158.9	33	3.8
			335.1	27	
			352.2	17	
GHRP-2	+2	409.72	170.1	28	3.3
			269.1	12	
			550.3	8	
GHRP-2 M1	+2	358.17	153.0	37	3.1
			170.1	22	
			269.1	10	
GHRP-2 M2	+2	410.21	152.8	32	3.4
			169.9	20	
			551.1	13	
GHRP-4	+1	608.30	159.1	35	3.5
			351.2	19	
			444.2	19	
GHRP-4 M1	+1	462.22	158.9	31	3.2
			230.0	18	
			258.0	10	
GHRP-5	+1	771.36	258.1	33	3.7
			350.1	34	
			421.2	27	
GHRP-5 M1	+1	439.18	190.9	22	3
			322.0	18	
			350.1	14	

Table 1. Continued.

Name	Precursor charge state	Precursor ion (*m/z*)	Product ion (*m/z*)*	Collision energy (V)	Retention time (min)
GHRP-5 M2	+1	625.28	321.9	28	3.4
			350.1	23	
			421.2	17	
GHRP-6	+2	437.23	129.1	21	2.8
			296.2	25	
			324.1	25	
GHRP-6 M1	+2	368.70	84.1	34	3.1
			129.1	20	
			345.8	10	
GHRP-6 M2	+1	609.28	159.1	39	3.8
			335.1	27	
			352.2	19	
Hexarelin	+2	444.24	110.1	29	2.9
			129.1	21	
			338.2	25	
Hexarelin M1	+2	427.21	110.1	32	2.4
			273.2	20	
			309.9	20	
Ipamorelin	+2	356.70	84.0	35	2.6
			129.1	16	
			223.1	16	
Ipamorelin M1	+1	585.28	166.0	30	3.1
			223.1	22	
			420.2	22	
Alexamorelin	+2	479.75	129.1	22	2.9
			209.1	22	
			813.3	14	
Alexamorelin M1	+2	444.73	109.9	27	3
			129.8	27	
			146.9	21	
Alexamorelin M2	+1	623.25	143.9	38	3.7
			334.9	24	
			352.2	16	
Anamorelin	+1	547.20	173.9	37	3.9
			202.1	33	
			275.9	22	
Ibutamoren	+1	529.20	234.9	24	3.7
			262.9	17	
			266.9	21	
ISTD 1 ($^{13}C^{15}N$ Lysine GHRP-2)	+2	413.90	136.9	25	3.3
			152.8	28	
			169.9	24	
ISTD 2 ($^{13}C^{15}N$ Lysine GHRP-6)	+2	441.40	109.9	28	2.8
			158.9	26	
			247.9	31	

GHRP, growth hormone-releasing protein; M1/M2, metabolite 1/2; ISTD, internal standards
*Qualifier ions are in bold.

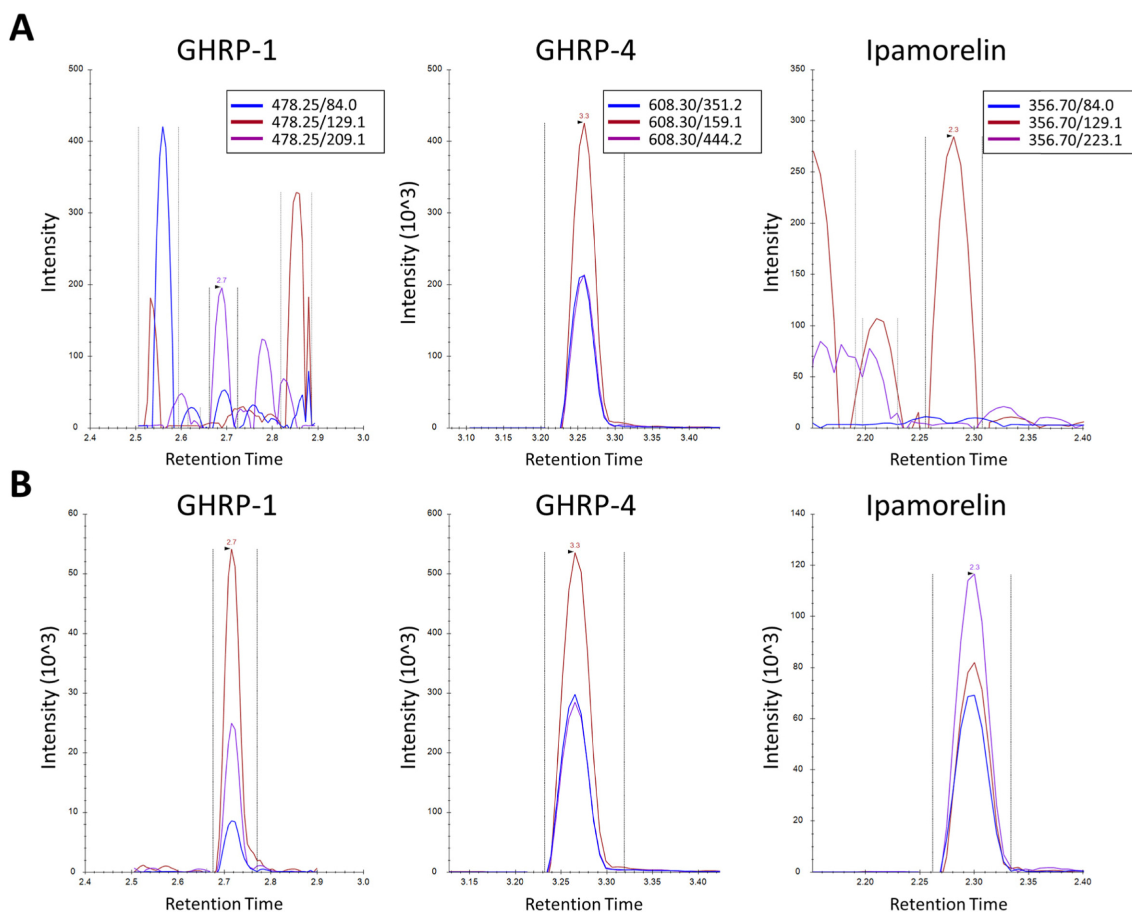

Figure 1. Extracted ion chromatograms of GHRP-1, GHRP-4, and ipamorelin using two elution buffer conditions. A) Elution with 5% formic acid in methanol. B) Elution with 2% ammonia and 1.2% formic acid in methanol.

these elution conditions. Here we compared the standard elution conditions (5% formic acid in methanol) and Mazzarino's condition (2% ammonia and 1.2% formic acid in methanol). Our results show that GHRP-1, GHRP-6, hexarelin, and ipamorelin could not be detected using the standard elution conditions but could be detected using other conditions (Figure 1); this was in agreement with a study conducted by Mazzarino et al..[13] We found that the standard elution condition sometimes eluted all substances within critical sample pH condition (about pH 5). Thus, when using the standard elution conditions, we should control the pH of the urine sample to be analyzed. Here we used Mazzarino's elution buffer conditions to validate our method for evaluating hGH-releasing substances in urine.

The representative SRM chromatograms at the minimum required performance level (MRPL, 2 ng/mL)[14] for the screening of 10 substances and their metabolites are shown in Fig. 2. All target analytes can be detected with sufficient peak intensities at the MRPL concentrations and achieve complete chromatographic separation to exclude false-positives/false-negatives or misinterpretation. These results indicated that the present method is acceptable for routine drug testing in doping control.

Method validation

Validation of our method was performed according to WADA and ISO/IEC 17025 guidelines. Qualitative determination of GHRPs and GHSs in human urine was assessed and validated with respect to selectivity, matrix effects, recovery, linearity, intra- and inter-assay precisions, and LOD. Concentration conditions were set using 2 ng/mL of substance to satisfy the minimum required performance level criteria outlined in the WADA technical document TD2015MRPL.[14] A summary of all method validation results is provided in Table 2.

Selectivity

To evaluate the interference signal of qualifier ions, five NCU and five PCU samples were prepared. The results of these experiments showed no observable interference signal from endogenous substances at the expected retention time.

Figure 2. Extract ion chromatograms of 25 substances and 2 ISTD in positive control urine (PCU). A) Chromatogram identifications: (1) Hexarelin M1, (2) Ipamorelin, (3) GHRP-6, (4) ISTD 2, (5) Alexamorelin, (6) Hexarelin, (7) GHRP-5 M1, (8) GHRP-1, (9) GHRP-1 M1, (10) GHRP-2 M1, (11) Alexamorelin M1, (12) Ipamorelin M1, (13) GHRP-6 M1, (14) GHRP-1 M2, (15) GHRP-1 M3 (16) GHRP-4 M1, (17) ISTD 1, (18) GHRP-2, (19) GHRP-2 M2, (20) GHRP-5 M2, (21) GHRP-4, (22) GHRP-6 M2, (23) Ibutamoren, (24) Alexaremolin M2, (25) GHRP-5, (26) GHRP-1 M4, (27) Anamorelin. B) Extract ion chromatograms of each substances.

Table 2. Method validation results: linearity, matrix effect, recovery, limit of detection (LOD), and precision.

Name	Linearity (R^2)	Matrix effect (%)	Recovery (%)	Screening LOD (ng/mL)	Confirmation LOD (ng/mL)	Intra-day precision CV (%)	Inter-day precision CV (%)
GHRP-1	0.9993	77.3	57.3	0.5	0.5	6.86	22.60
GHRP-1 M1	0.9999	102.7	48.0	0.5	0.5	11.04	16.85
GHRP-1 M2	0.9984	84.8	62.1	0.1	0.2	11.34	18.85
GHRP-1 M3	0.9995	110.7	82.4	0.2	0.5	6.23	18.24
GHRP-1 M4	0.9991	71.3	38.8	0.1	0.2	7.82	9.81
GHRP-2	0.9997	98.9	75.1	0.2	0.5	7.94	13.83
GHRP-2 M1	0.9995	50.0	20.5	0.1	0.2	9.36	18.60
GHRP-2 M2	0.9995	79.6	93.8	0.05	0.2	6.24	6.96
GHRP-4	0.9997	71.7	100.8	0.05	0.2	4.94	12.98
GHRP-4 M1	0.9996	73.6	60.6	0.1	0.5	10.14	14.60
GHRP-5	0.9997	101.1	100.3	0.2	0.5	14.09	19.08
GHRP-5 M1	0.9992	85.0	54.1	0.1	0.2	10.01	18.63
GHRP-5 M2	1	77.0	21.0	0.5	0.5	12.08	13.98
GHRP-6	0.9994	103.0	57.7	0.1	0.5	5.56	18.01
GHRP-6 M1	1	59.3	61.0	0.2	0.5	4.99	13.23
GHRP-6 M2	0.9999	64.9	10.4	0.5	0.5	16.51	18.79
Hexarelin	0.9991	86.7	65.5	0.1	0.2	7.07	15.74
Hexarelin M1	0.9999	65.9	89.1	0.05	0.2	6.17	20.52
Ipamorelin	0.9998	76.7	90.6	0.05	0.1	4.38	16.70
Ipamorelin M1	0.9997	68.4	95.8	0.05	0.1	7.22	14.83
Alexamorelin	0.9997	87.8	54.3	0.5	1	8.13	18.88
Alexamorelin M1	0.9999	89.1	93.2	0.2	0.5	5.97	16.48
Alexamorelin M2	1	84.9	23.0	0.1	0.2	11.32	12.21
Anamorelin	0.9994	141.2	99.7	0.05	0.05	7.88	9.31
Ibutamoren	0.9996	112.5	97.6	0.05	0.1	2.75	7.95

GHRP, growth hormone-releasing protein; M1/M2, metabolite 1/2; CV, coefficient of variation

Recovery

Almost all target substances were extracted with 50%-100% recovery. GHRP-4 and GHRP-5 were detected without loss of signal by extraction. However, six substances showed low recovery for preparation step. Specially, GHRP-2 M1 and GHRP-6 M2 showed only 20.5% and 10.4% recovery, respectively

Matrix effect

Five substances, GHRP-2 M1, GHRP-6 M1, GHRP-6 M2, hexarelin M1, and ipamorelin M1, showed ion suppression signal with less than < 70% in matrix compared with the signal in water, whereas 3 substance, GHRP-1 M3, anamorelin, and ibutamoren, were significantly increased by > 10%.

Linearity

Linearity was calculated using correlation coefficients (R^2) from calibration curves. Each substance was analyzed based on 35 runs (7 concentration points × 5 replicates). As shown in Table 2, R^2 values of each substance ranged from 0.9984 to 1.

Intra- and Inter-assay precision

For determination of intra- and inter-assay precision, we prepared a total of 18 PCU samples that were separated into three groups of six samples each. Each group was extracted and analyzed on different days for intra-assay precision. The relative retention times per day exhibited a %CV of <0.1% for each group (data is not shown), and the %CV for the peak area of qualifier ions was almost <15% (Table 2). For inter-assay precision, we calculated from relative retention time and peak area of 18 PCU samples from 3 days. The %CVs of all target substances were <25% (Table 2).

Limit of detection

For determination of LOD, we prepared a series of

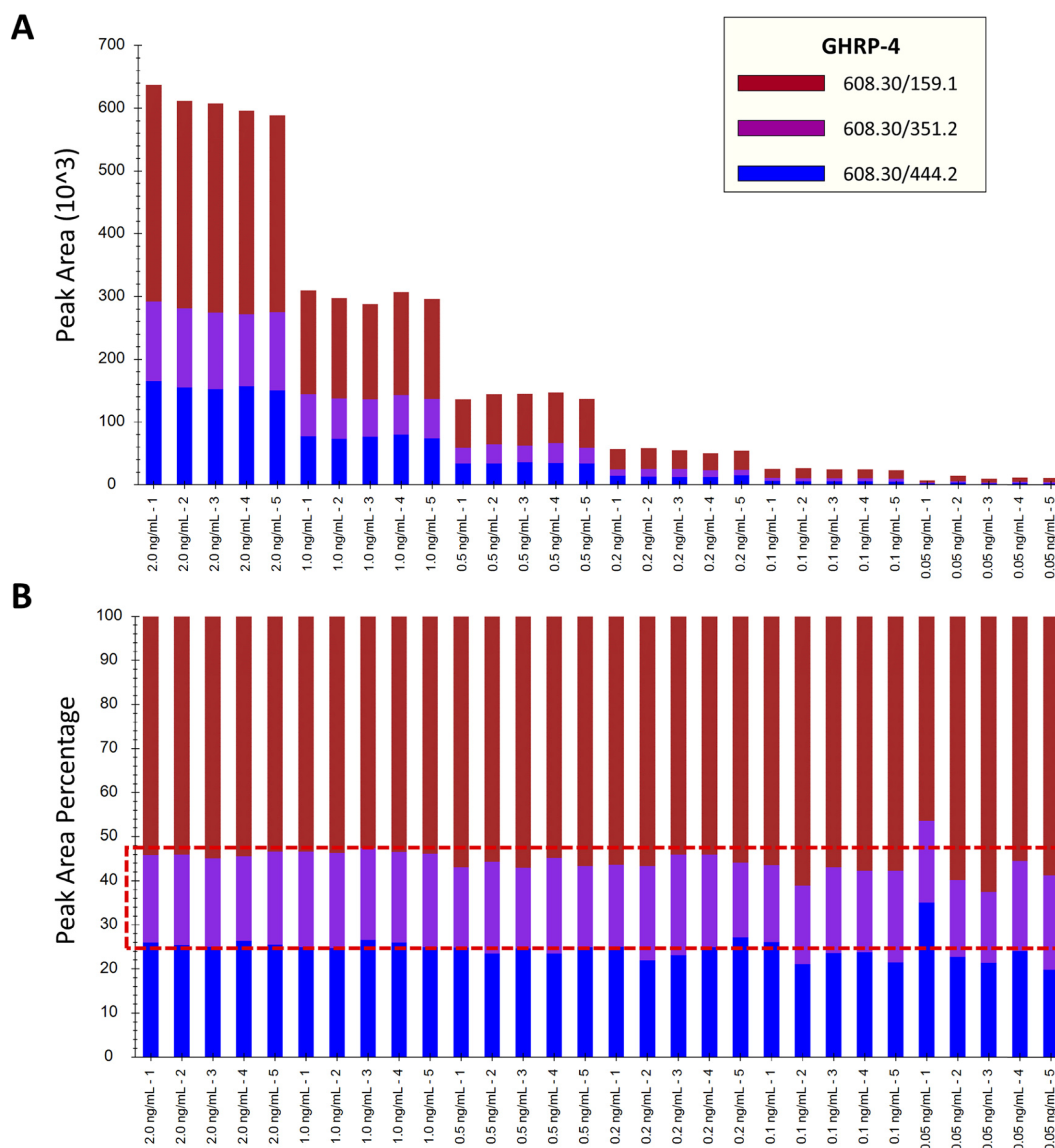

Figure 3. Peak area and peak area percentage of the three product ions. A) The peak areas of the three product ions at different concentrations. B) Peak area percentages of the three product ions at each concentration point. Red dot box, expected percentage of product ion depicted in purple.

diluted reference substances ranging from 0.05 to 2 ng/mL in PCU samples and then performed SRM analysis of five samples per concentration point. The LOD of all substances was <50% of the minimum required performance level (1 ng/mL). In particular, seven substances could be detected at the 0.05 ng/mL concentration point with a %CV of <25% and signal-to-noise ratio of >3.

Confirmation test

For confirmation of substances, each substance had to show a regular peak area ratio or peak area percentage of three product ions. Three product ions per target were analyzed in the SRM mode. All LC-MS/MS conditions were used as described above for the screening test, but product ions were added with the qualifying ion. For example, the LOD of GHRP-4 was determined to be 0.05 ng/mL. Therefore, we tested whether the ratio of the three product ions of GHRP-4 was fixed at any concentration above the LOD (Figure 3). Percentages of the three product ions were similar at concentration points ranging from 0.2 to 2 ng/mL. However, the percentages of the three product ions differed at low concentration points (0.05 and 0.1 ng/mL). From these results, the presence of

GHRP-4 could be confirmed at concentrations above 0.2 ng/mL but not at LOD. This likely resulted from the qualifier ion being selected as the highest signal, whereas the other two product ions having a lower signal, thereby making the concentration limits for confirmation higher than LOD.

Conclusions

For doping control purposes, LC-MS/MS method based on ISO/IEC 17025 and WADA guidelines was developed and validated for simultaneous analysis of three GHSs (anamorelin, ibutamoren, and ipamorelin), seven GHRPs (alexamorelin, GHRP-1, -2, -4, -5, -6, and hexarelin) and their metabolites in human urine. This method was successfully applied to analysis of urine samples and seems to be useful for the simultaneous qualitative determination of GHRPs and GHSs.

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References

1. Saugy, M.; Robinson, N.; Saudan, C.; Baume, N.; Avois, L.; Mangin, P. *Brit J. Sport Med.* **2006**, 40, Suppl 1, i35.
2. Saugy, M.; Cardis, C.; Schweizer, C.; Veuthey, J. L.; Rivier, L. *J. Chromatogr. B.* **1996**, 687, 201.
3. Baumann, G. P. *Endocr. Rev.* **2012**, 33, 155.
4. Bowers, C. Y.; Sartor, A. O.; Reynolds, G. A.; Badger, T. M. *Endocrinology* **1991**, 128, 2027.
5. Arvat, E.; Maccario, M.; Di Vito, L.; Broglio, F.; Benso, A.; Gottero, C.; Papotti, M.; Muccioli, G.; Dieguez, C.; Casanueva, F. F.; Deghenghi, R.; Camanni, F.; Ghigo, E. *J. Clin. Endocr. Metab.* **2001**, 86, 1169.
6. Okano, M.; Nishitani, Y.; Sato, M.; Ikekita, A.; Kageyama, S. *Drug Test Anal.* **2010**, 2, 548.
7. Garcia, J. M.; Polvino, W. J. *Growth Horm. Igf. Res.* **2009**, 19, 267.
8. Svensson, J.; Boguszewski, C. L.; Shibata, F.; Carlsson, B.; Carlsson, L. M.; Bengtsson, B. A. *Growth Horm Igf. Res.* **2003**, 13, 1.
9. Thomas, A.; Hoppner, S.; Geyer, H.; Schanzer, W.; Petrou, M.; Kwiatkowska, D.; Pokrywka, A.; Thevis, M. *Anal. Bioanal. Chem.* **2011**, 401, 507.
10. Okano, M.; Sato, M.; Ikekita, A.; Kageyama, S. *Rapid Commun. Mass Spectrom.* **2010**, 24, 2046.
11. Thomas, A.; Delahaut, P.; Krug, O.; Schanzer, W.; Thevis, M. *Anal. Chem.* **2012,** 84, 10252.
12. Sobolevsky, T.; Krotov, G.; Dikunets, M.; Nikitina, M.; Mochalova, E.; Rodchenkov, G. *Drug Test Anal.* **2014**, 6, 1087.
13. Mazzarino, M.; Calvaresi, V.; de la Torre, X.; Parrotta, G.; Sebastianelli, C.; Botre, F. *Forensic Toxicol.* **2015,** 33, 321.
14. World Anti-Doping Agency. **2015**, Technical Document for minimum required performance levels. https://www.wada-ama.org/en/resources/science-medicine/td2015-mrpl. Accessed 22 May 2015.