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Analysis of growth hormone-releasing peptides for doping control

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Introduction

Growth hormone secretagogues (GHS) are being used as both diagnostic agent and treatment for growth hormone deficiency.[1,2] Also recently they're being used as health supplements for anti-aging. Growth hormone (GH) or GHS may be being used by some athletes to keep or elevate GH and IGF-1 blood levels. The use of GH or GHS by sports athletes is prohibited by the World Anti-Doping Agency.[3] Several testing methods of GH concentrations, as well as the potential misuse of recombinant GH, have been published. Wu *et al.* and Momoura *et al.* demonstrated the immunoassays of detecting GH doping using the ratio of 22KDa-/total-GH in serum and the ratio of 20KDa-/22KDa-GH respectively.[4-6] Recently, GH tests based upon the isoform differential immunoassay using commercial kits have advanced and are starting to be used for routine doping control. Pralmorelin hydrochloride (GHRP-2), a synthetic growth hormone releasing peptide, is being used diagnostically to detect a growth hormone deficiency in Japan. The new preparation for intranasal administration as both a diagnostic and a therapeutic agent have been developed to provide alternatives to diagnostic injection of pralmorelin by Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). Nasu *et al.* reported a pharmacokinetic model for pralmorelin hydrochloride in rats.[7] Pralmorelin methyl ester (HEMOGEX™, VPX Sports, USA) is available on the internet as a dietary supplement similar to designer steroids.[8] Thus, anti-doping control laboratories should immediately develop analytical methods for detecting GHS abuse. Thevis *et al.* already reported the detection method based on liquid chromatography/tandem mass spectrometry (LC/MS/MS) for some therapeutics with GHS activity such as acetylcholine esterase inhibitors (donepezil, galantamine and rivastigmine) and SM-130686.[9,10] Gallego *et al.* approached GHS doping detection with a new strategy based upon displacement of a labelled ligand bound to the receptor by non-labelled GHS present in a biological fluid.[11] The aim of this study is to establish adequate parameters for the screening of growth-hormone releasing peptide, namely, GHRP-2, GHRP-6, hexarelin, alexamorelin, sermorelin,

ipamorelin and tabimorelin. In this paper, a dietary supplement containing GHS was also discussed.

Materials and Methods

Chemicals

Pralmorelin hydrochloride was a kind gift from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). GHRP-2 for excretion study, hexarelin and alexamorelin were purchased from GL Biochem Ltd. (Shanghai, China). Tabimorelin hemisulfate was purchased from Tocris Cookson Inc. (MI, USA). GHRP-6 acetate and sermorelin acetate were purchased from ChemPep Inc. (FL, USA). Ipamorelin was purchased from Organic Specialty Chemicals (ME, USA). Caffeine-d_3 as internal standard was purchased from C/D/N Isotopes Inc. (Quebec, Canada). Acetonitrile (CH_3CN), methanol (CH_3OH), acetic acid (CH_3COOH), potassium dihydrogenphosphate (KH_2PO_4) and potassium hydroxide (KOH) were purchased from Kokusan Chemical Co., Ltd (Tokyo, Japan). Glycerol (99 %) was purchased from Sigma (St. Louis, MO, USA). Ultra-pure water was made using a Milli-Q Ultra pure system (Millipore, Bedford, MA, USA). ABS NEXUS-ELUT 650 mg/3 mL was purchased from Varian Inc. (CA, USA). The stock solutions and the working solutions of reference standards were prepared as aqueous solutions.

High performance liquid chromatography/Time-of-flight mass spectrometry (HPLC/TOFMS)

The HPLC/TOFMS system was an Agilent 1100 Series liquid chromatograph (Hachioji, Japan) coupled to a QSTAR XL MS/MS system (Applied Biosystems, CA, USA). The analytical column was a Zorbax Eclipse XDB-C8 (4.6 mm x 150 mm, 5 μm particle size), and the mobile phases used were 1 % acetic acid (mobile phase A) and acetonitrile (mobile phase B). The column oven temperature was 25 °C and the flow rate was 0.25 mL/min. A gradient elution was as follows: 65 % A for 3.0 min, linear to 35 % A in 15 min, followed by an increase to 65 % A in 10 min. Finally, the column was equilibrated for 2min, and then total runtime was 27 min. Ionization was accomplished using electrospray in positive mode. The ionspray temperature was at 450 °C and the ionspray voltage was set at 5,500 V. Declustering potential and focusing potential were set at 50 V and at 250 V respectively. Nitrogen gas was employed as nebulizer gas (2.85 L/min) and auxially gas (4.80 L/min). The mass range was set from *m/z* 140 to 1,000.

Ultra performance liquid chromatography/tandem mass spectrometry (UPLC™/MS/MS)
The UPLC™/MS/MS system was a Quattro Micro API with an ESI-Source (Z-spray) from Waters (Tokyo, Japan). The analytical column was an Acquity UPLC® BEH C18 (2.1 mm x 50 mm, 1.7 μm particle size), and the mobile phases used were 1 % acetic acid (mobile phase A) and acetonitrile (mobile phase B). The column oven temperature was 25 °C and the flow rate was 0.2 mL/min. A gradient elution was as follows: 90 % A for 0.5 min, linear to 20 % A in 7.5 min, followed by an increase to 90 % A in 0.1 min. Finally, the column was equilibrated for 2min, and then total runtime was 10 min. Ionization was accomplished using electrospray in positive mode. The ionspray temperature and the desolvation temperature were at 120 °C and 350 °C, respectively, and the capillary voltage was set at 3.0 kV. Nitrogen gas was employed as cone gas (50 L/hr). Argon gas was employed as collision gas (3.6×10^{-3} mbar) and collision voltages were as listed in Table 1. All target peptides were detected by means of product ions in the multiple reaction monitoring (MRM) mode as listed in Table 1.

Determination of GH and IGF-1 levels by Immunoradiometric assay (IRMA)
Serum GH concentration was determined using a commercially available radioimmunoassay kit: GH Kit Daiichi (TFB Inc., Tokyo, Japan). Serum IGF-1 concentration was determined using a commercially available radioimmunoassay kit: Somatomedin C·II Siemens (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan). Radioactivity was measured using an automated gamma counter (ARC-950, Aloka, Tokyo, Japan). The detection methods were validated and included in the scope of the ISO15189 accreditation in our facilities.

Urine Sample preparation
0.5 mL of 1 M phosphate buffer (pH 6.0) and internal standard solution (1 μg of caffeine-d_3) were added to 3 mL of urine sample. After centrifugation 3 mL of analyte was extracted by passage of the sample through a solid-phase extraction cartridge (ABS NEXUS-ELUT 650 mg/3 mL), followed by washing with 3 mL of distilled water and elution with 2 mL of 0.5 % glycerol in CH_3OH. The extract was evaporated to dryness under nitrogen stream at 40 °C and reconstituted in 130 μL of CH_3COOH (1 %) /CH_3CN (90:10, v/v). After centrifugation a volume of 5 μL of the supernatant was injected into the UPLC™/MS/MS system.

Excretion study
100mg of GHRP-2 (AK-6-NH_2) from GL Biochem Ltd. were self-administered sublingually

by a 50 year-old healthy male volunteer. The nutritional supplement with GHRP-2 Ester Z-11™ (D-Ala-D-β -Nal-Ala-Trp-D-Phe-Lys-OCH_3) used in the study was HEMOGEX™ from Vital Pharmaceuticals (VPX Sports, USA). One vial (5 mL softgel/vial) of HEMOGEX™ were self-administrated orally by three healthy male volunteers (A: 50 year-old, B: 39 year-old and C: 41 year-old) according to the manufacture's recommended use. All volunteers were informed in advance of the details of the study, and written consent was obtained from all participating volunteers. Blank urine samples were obtained before administration. Urine samples were collected for the first 24 hours after the administration. Urine samples were stored at –20 °C until analysis. Serum samples were collected before and after the administration of HEMOGEX™ to confirm GH and IGF-1 levels, and stored at –80 °C until analysis. The time courses of serum sample collection were -24 hours, -15 hours, -12 hours, -1 hours, 3 hours and 9 hours. The volunteers refrained from ingesting alcohol and caffeine for 24 hours prior to blood collection and not to perform any exercise.

Assay validation

The preliminary assay validation was performed. The limit of detection and recovery were estimated.

Table 1 Analytical parameters of UPLC™/MS/MS in MRM mode and validation results

Substance	Precursor ion	Ion transition	Retention time (min)	Cone (V)	Colliision (V)	Recovery (%)	LOD (ng/mL)
Hexarelin	$[M+2H]^{2+}$	444.2>129	3.05	24	22	53	12.0
Alexamorelin	$[M+3H]^{3+}$	320.2>144	3.02	14	18	64	2.0
Tabimorelin	$[M+H]^{+}$	529.2>280	4.63	14	14	89	0.1
GHRP-6	$[M+2H]^{2+}$	437.2>129	2.90	20	20	46	0.5
Ipamorelin	$[M+2H]^{2+}$	356.7>129	2.51	20	20	47	0.7
Sermorelin	$[M+5H]^{5+}$	672.5>136	3.75	28	30	30	40.0
Pralmorelin (GHRP-2)	$[M+2H]^{2+}$	409.7>170	3.84	16	28	63	0.3
Caffeine-d_3 (I.S.)	$[M+H]^{+}$	198>138	1.91	38	20	-	-

Results and Discussion

Molecular mass determination by Time-of-flight mass spectrometry

The elemental composition of seven peptides was confirmed employing high resolution and high accuracy mass spectrometry using a QSTSAR® XL System. Accurate masses and calculated elemental compositions of protonated ions were listed in Table 2.

The typical mass spectrum of pralmorelin was shown in Figure 1 (data of the other peptides were not shown).

Table 2 Accurate masses and calculated elemental compositions of protonated ions using HPLC/TOF/MS

Substance	**Molecular Formula**	**Protonated ion**				
		Elemental composition		**m/z (theor.)**	**m/z (exp.)**	**error (ppm)**
Hexarelin	$C_{47}H_{58}N_{12}O_6$	$C_{47}H_{60}N_{12}O_6$	**$[M+2H]^{2+}$**	444.2398	444.2397	-0.11
Alexamorelin	$C_{50}H_{63}N_{13}O_7$	$C_{50}H_{64}N_{13}O_7$	**$[M+H]^{+}$**	958.5052	958.5032	-2.09
		$C_{50}H_{65}N_{13}O_7$	**$[M+2H]^{2+}$**	479.7565	479.7564	-0.21
		$C_{50}H_{66}N_{13}O_7$	**$[M+3H]^{3+}$**	320.1736	320.1742	1.87
Tabimorelin	$C_{32}H_{40}N_4O_3$	$C_{32}H_{41}N_4O_3$	**$[M+H]^{+}$**	529.3179	529.3162	-3.21
GHRP-6	$C_{46}H_{56}N_{12}O_6$	$C_{46}H_{57}N_{12}O_6$	**$[M+H]^{+}$**	873.4524	873.4515	-1.03
		$C_{46}H_{58}N_{12}O_6$	**$[M+2H]^{2+}$**	437.2301	437.2301	0.00
Ipamorelin	$C_{38}H_{49}N_9O_5$	$C_{38}H_{50}N_9O_5$	**$[M+H]^{+}$**	712.3935	712.3913	-3.09
		$C_{38}H_{51}N_9O_5$	**$[M+2H]^{2+}$**	356.7007	356.7023	4.63
		$C_{38}H_{52}N_9O_5$	**$[M+3H]^{3+}$**	238.1364	238.1371	3.08
Sermorelin	$C_{149}H_{246}N_{44}O_{42}S$	$C_{149}H_{250}N_{44}O_{42}S$	**$[M+4H]^{4+}$**	839.9625	839.9605	-2.38
		$C_{149}H_{251}N_{44}O_{42}S$	**$[M+5H]^{5+}$**	672.1716	672.1704	-1.73
		$C_{149}H_{252}N_{44}O_{42}S$	**$[M+6H]^{6+}$**	560.3110	560.3107	-0.45
Pralmorelin (GHRP-2)	$C_{45}H_{55}N_9O_6$	$C_{45}H_{56}N_9O_6$	**$[M+H]^{+}$**	818.4354	818.4351	-0.37
		$C_{45}H_{57}N_9O_6$	**$[M+2H]^{2+}$**	409.7216	409.7214	-0.49

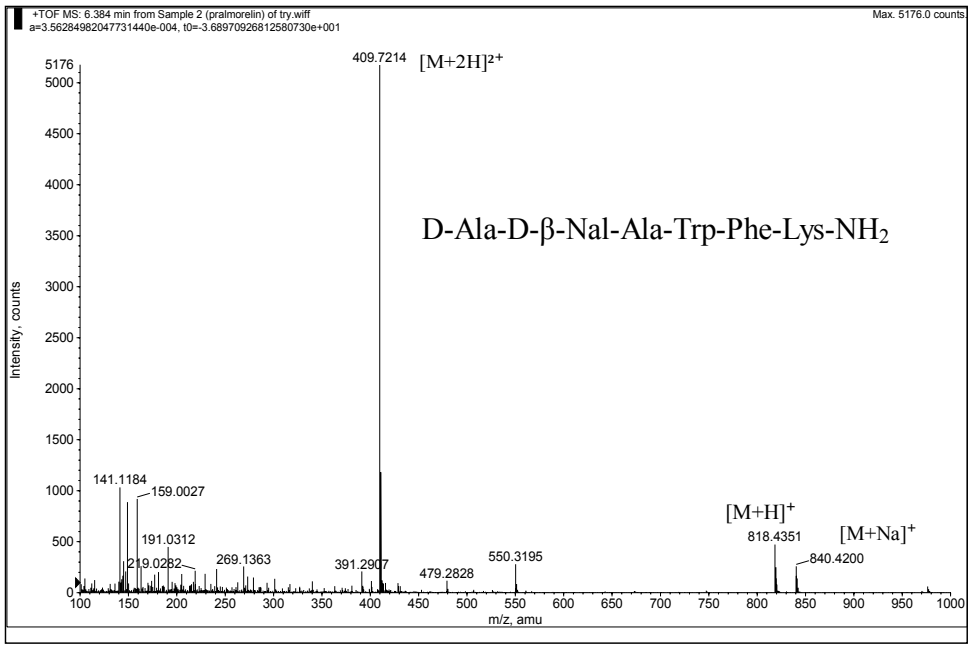

Figure 1 TOFMS spectrum of pralmorelin (GHRP-2)

Pralmorelin (GHRP-2), GHRP-6, hexarelin, alexamorelin, ipamorelin and tabimorelin were observed as $[M+H]^+$ and $[M+2H]^{2+}$ ions. Alexamorelin and ipamorelin were also observed as three-fold protonated ion. Sermorelin was determined as four-, five and six-fold protonated ions.

Screening for growth hormone releasing peptides

The UPLC™/MS/MS parameters of the target substances were described in Table 1. Product ion spectrum generated from $[M+2H]^{2+}$ of ipamorelin was shown in Figure 2. Product ion at *m/z* 129 of the peptides such as hexarelin, GHRP-6 and ipamorelin, having C-terminal lysinamide (Lys-NH_2) residue is suggested to originate from y_1-NH_3. For confirmation analysis, the collision voltages should be optimized to obtain minimum of three specific product ions generated from precursor ions of target peptides.[12]

The report format of screening for seven peptides by UPLC™/MS/MS in MRM mode was shown in Figure 3. The results of recovery and limit of detection were listed in Table 1.

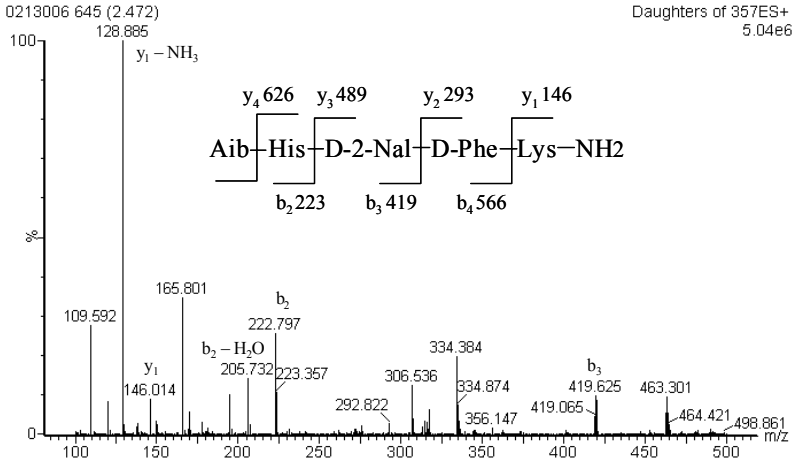

Figure 2 Product ion mass spectrum of ipamorelin ($[M+2H]^{2+}$ =356.7) by UPLC™/MS/MS

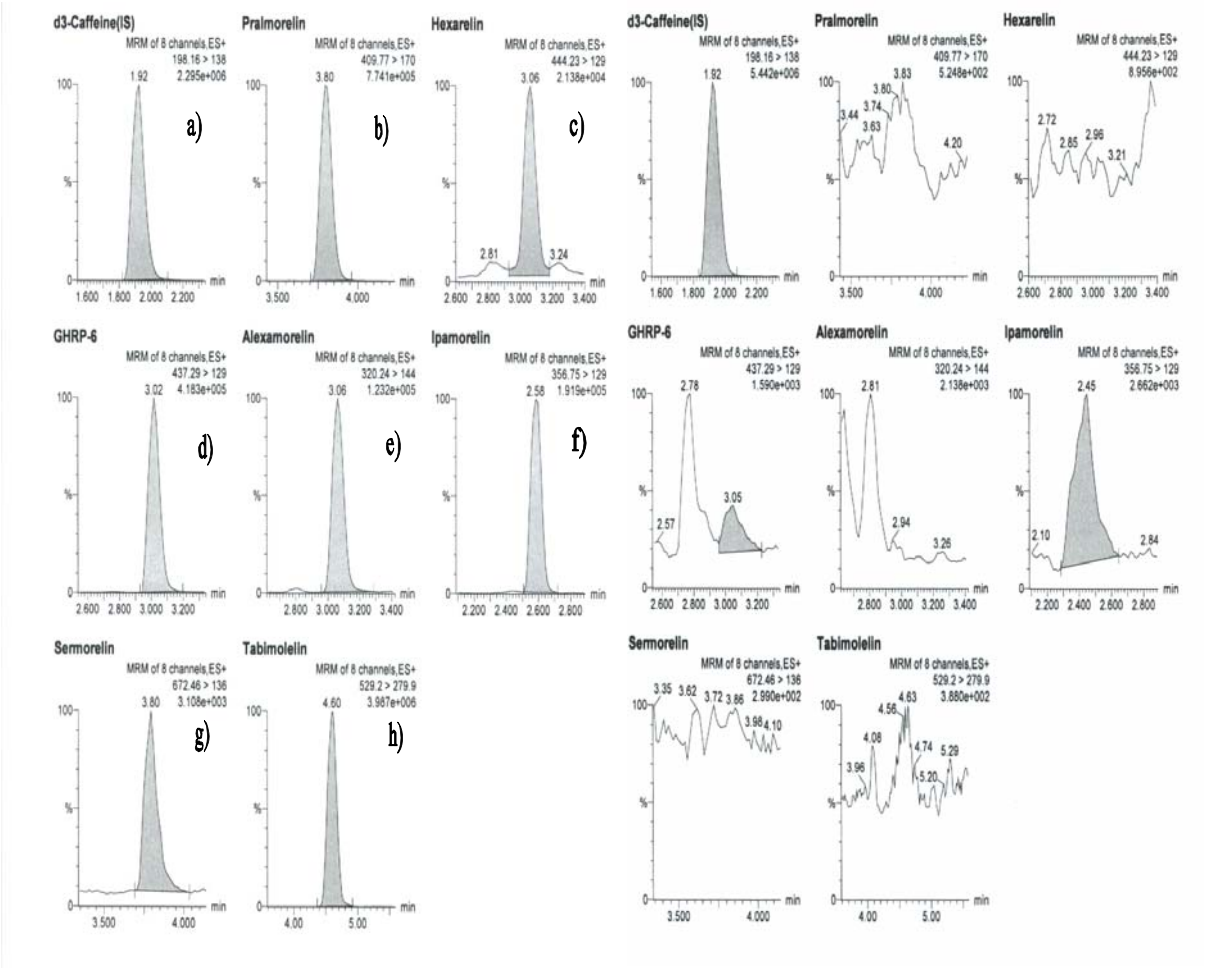

Figure 3 Screening report format by UPLC™/MS/MS (left: spiked urine 100 ng/mL, right: drug-free urine a) caffeine-d_3 b) pralomrelin c) hexarelin d) GHRP-6 e) alexamorelin f) ipamorelin g) sermorelin h) tabimorelin)

Excretion study of pralmorelin (GHRP-2)

After an administration of 100 mg of GHRP-2 obtained from GL Biochem Ltd. (Shanghai, China), pralmorelin was detectable up to 7.7 hours with a maximum concentration of 7 ng/mL (Figure 4). Pralmorelin is mainly excreted in feces, and its urinary excretion is at approximately 7 % of the administrated pralmorelin hydrochloride.[13]

In this administration study, pralmorelin was administrated sublingually. However, it may be suggested that the absorption via sublingual or oral administration is too low. Further excretion studies by intravenous administration must be performed.

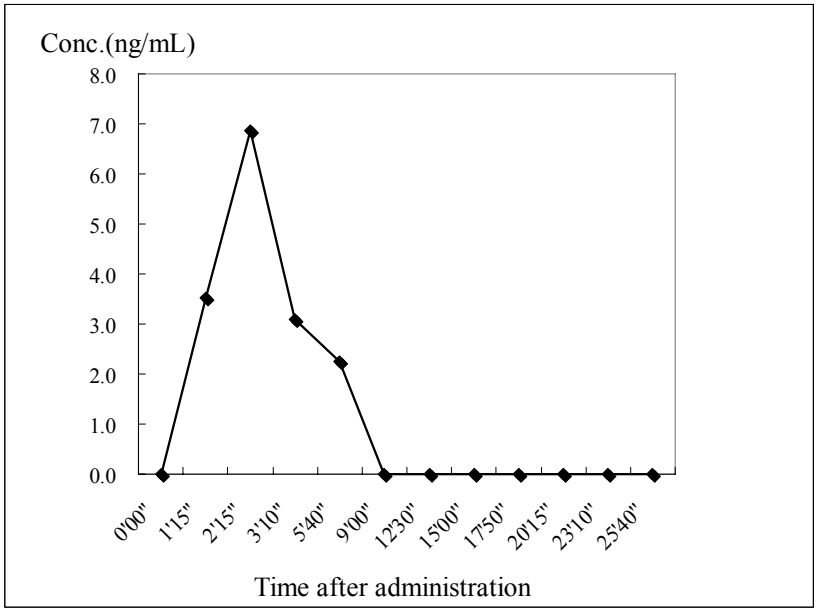

Figure 4 Urinary pralmorelin concentration after an administration of 100mg of GHRP-2.

Investigation of dietary supplement

HEMOGEX™ contains GHRP-2 Ester Z-11™ (D-Ala-D-β -Nal-Ala-Trp-D-Phe-Lys-OCH_3) if the label is correct. One vial (5 mL) was extracted according to the sample preparation above mentioned. The sample was analyzed by HPLC/TOF/MS. It could be detected small amount of GHRP-2, however, GHRP-2 Ester Z-11 is still in investigation as the lack of reference standard. Identification of GHRP-2 in HMOGEX™ was performed according to the published criteria.[12] The concentration was approximately 5 μg/ml.

Table 3 Serum GH and IGF-1 levels after an administration of HEMOGEX™

		Conc. (ng/mL)					
		-24 hours	**-15 hours**	**-12 hours**	**-1 hours**	**3 hors**	**9 hours**
GH	Volunteer A	0.09	0.08	2.69	0.2	0.06	0.26
	Volunteer B	0.06	<0.03	0.28	0.09	0.16	0.07
	Volunteer C	0.06	0.15	0.27	0.06	0.09	0.47
IGF-1	Volunteer A	337	271	268	367	360	387
	Volunteer B	108	102	96.9	91.5	96.7	104
	Volunteer C	574	505	653	595	645	500

Furthermore, an administration study of HEMOGEX™ was performed. GHRP-2 could not be detected in the urine sample during a time period of 24 hours, because of low amount of administration. The package of HEMOGEX™ illustrates an intake of HEMOGEX™ can increase serum GH levels up to 718 % above basal levels for a period of two hours, as well as it can increase serum IGF-1 levels. An administration study of HEMOGEX™ was performed for the investigation, and three volunteers' serum GH and IGF-1 levels were not significantly elevated for a period of 9 hours (Table 3).

Conclusions

Growth-hormone releasing peptides in urine could be detected using our diuretic screening by means of UPLC™/MS/MS in MRM mode. After an administration of recombinant GH (22 KDa isoform), it suppresses endogenous pituitary production of the various GH isoforms by negative feedback.[4-6] GH isoform tests based upon this principle may be difficult to detect the GH doping if both recombinant GH and GHS as masking agents will be simultaneous administrated by athletes. Therefore, the production of endogenous GH isoforms and the influence on the ratio of GH isoforms by GHS use should be further investigated.

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