Analysis of growth hormone releasing peptide GHRP-2 for doping control purposes

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Introduction

GHRP-2, which belongs to growth hormone secretagogues (GHS), has been used as diagnostic agent in Japan. Nasal GHRP-2 spray is on a clinical trial.

Out of Japan, several studies for oral products such as Capromorelin, Tabimorelin and others were going on. GHSs are expected to be used more widely for clinical purposes and promotion of health.

Currently, many kinds of GHS such as hexarelin, sermorelin (Geref), GHRP-2 (Pralmorelin), tesamoreline (Egrifta), GHRP-6 and many kinds of GHS-containing supplements are available.
Growth Hormone Secretagogue Doping?

Laboratories have been using Differential Isoform Immunoassay as an excellent method to detect GH Doping.

However, there are concerns about Doping violation by GHS, GHRH and their analogs.

Nowadays, there are numerous internet sites dealing with GHS.

We are afraid that GHS Doping becomes rampant.
Our Research for the detection of GHS Doping

2008  The study of GHS-screening analysis
2009  Administration study of GHRP-2 and hGH
2010  Development of analysis method of GHRP-2
2011・・・・
Administration of GH and GHRP-2 in 2009

Growth hormone

Generic name: Somatropin (22-kDa rhGH)
Trade name: Somatropin BS 5 mg SC Injection (Manufacturer: Sandoz K.K., Japan)
Dosage form: Subcutaneous injection during fasting
Dosage: 0.04 mg/kg single administration

GHRP-2

Generic name: Pralmorelin hydrochloride
Trade name: GHRP KAKEN 100 inj. (Manufacturer: Kaken Pharmaceutical Co., Ltd.)
Dosage form: Intravenous injection
Dosage: 100 µL single administration

Subjects

Japanese male (n=5): Age 20 – 37, body weight 54 kg - 63 kg, BMI 18.8-21.8

Time line of administration and sampling

<table>
<thead>
<tr>
<th>-2day</th>
<th>-1day</th>
<th>post 1day</th>
<th>2day</th>
<th>3day</th>
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<tbody>
<tr>
<td>36hr</td>
<td>24hr</td>
<td>0hr</td>
<td>24hr</td>
<td>48hr</td>
</tr>
<tr>
<td>Admission</td>
<td>Start of sampling</td>
<td>S.C. injection</td>
<td>Discharge</td>
<td></td>
</tr>
<tr>
<td>Urine and Blood</td>
<td></td>
<td>rGH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.v. injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GHRP-2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Analysis of GH by differential isoform immunoassay**

**Kit 1:** CMZ-Assay GmbH (Berlin, Germany)

**Ref.**
Analysis of GH by differential isoform immunoassay

**Kit-1**

**Kit-2**

**Rec/Pit**

**Dosage:** 0.04 mg/kg S.C. 5 male subjects

- □ : Rec
- △ : Pit

**Time (h)**

-24 -22 -20 -16 0 2 4 6 12 27 48

**GH conc. ng/ml**

-80 -70 -60 -50 -40 -30 -20 -10 0 10 20 30 40 50 60 70 80
Analysis of GH by differential isoform immunoassay

**GH conc. ng/ml**

**Kit-1**

- **rhGH Inj.**

**GH conc. ng/ml**

**Kit-2**

- **rhGH Inj.**

**GH conc. ng/ml**

**Dosage: 0.04 mg/kg S.C.**
- 5 male subjects
  - □ : Rec
  - △ : Pit

**GH conc. ng/ml**

**Dosage: 100 μg I.V.**
- 10 male subjects
  - □ : Rec
  - △ : Pit

Rome Italy 15/16 June 2011
Combined Administration of GHRP-2 and GH

- GHRP2 100 µg Inj.
- rhGH 40 µg/kg Inj.

Masking Effect

WADA DL
Therefore we have continued this research:

Determination of growth hormone secretagogue pralmorelin (GHRP-2) and its metabolite in human urine by liquid chromatography/electrospray ionization-tandem mass spectrometry
Target compounds for analysis

**GHRP-2**
Kaken Pharmaceutical Co., Ltd. Tokyo, Japan

![GHRP-2 structure]

Major metabolite

**AA-3**
D-Ala-D-(β-Naphthyl)-Ala-Ala-Trp-D-Phe-Lys-NH₂

**AF-6**

![AF-6 structure]

**AK-6**
GL Biochem Ltd., Shanghai, China

![AK-6 structure]
Confirmation of elemental compositions of GHRP-2, AA-3 and Stable isotope-labeled GHRP-2

Equipments

**HPLC/TOFMS system:**
- Applied Biosystems QSTAR XL MS/MS QTOF system (Life Technologies Corporation, Carlsbad, CA, USA)
- Agilent 1100 Series LC (Agilent Technologies, Palo Alto, CA, USA)

Analytical column: Supelco Discovery C\textsubscript{18} 4.0 x 50 mm (Sigma-Aldrich Co.)
Column oven temperature: 25°C, Flow rate: 0.25 mL/min

Mobile phase A: 0.1 % TFA, Mobile phase B: CH\textsubscript{3}CN
Gradient elution: 35 % B for 1.0 min, linear to 85 % B in 6.0 min (hold 2.0 min) followed by a decrease to 35 % B in 0.1 min (hold 2.0 min)
Injection volume: 10 \textmu L

Ionspray temperature: 450, Ion source voltage: 5,500 V
Declustering potential: 50 V, Focusing potential: 250 V
Nebulizer gas: Nitrogen gas (2.85 L/min)
Auxiliary gas: 4.80 L/min
Ionization: ESI in positive mode
Accurate masses and elemental compositions of target peptides by LC/ESI (+)-TOFMS

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Formula</th>
<th>Observed ion (m/z)</th>
<th>Theoretical ion (m/z)</th>
<th>Mass error (ppm)</th>
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</thead>
<tbody>
<tr>
<td>GHRP-2</td>
<td>C_{45}H_{56}N_{9}O_{6}</td>
<td>[M + H]^+</td>
<td>818.4348</td>
<td>818.4348</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[M + 2 H]^2+</td>
<td>409.7208</td>
<td>409.7210</td>
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<tr>
<td>AA-3</td>
<td>C_{19}H_{24}N_{3}O_{4}</td>
<td>[M + H]^+</td>
<td>358.1763</td>
<td>358.1761</td>
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<tr>
<td>Stable Isotope Labeled</td>
<td>C_{36}H_{56}N_{8}O_{6}{^{13}}C_{9}{^{15}}N</td>
<td>[M + H]^+</td>
<td>828.4610</td>
<td>828.4620</td>
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<tr>
<td>GHRP-2</td>
<td></td>
<td>[M + 2 H]^2+</td>
<td>414.7360</td>
<td>414.7347</td>
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</table>
Collision-induced dissociation experiments of GHRP-2, AA-3 and Stable isotope-labeled GHRP-2

Equipment and condition

Ultra-performance liquid chromatography/tandem mass spectrometry, UPLC®/MS/MS: Acquity UPLC®/tandem quadrupole mass spectrometer TQD with an ESI-Source Z-spray (Waters Corporation)

The mass range: \( m/z \) 70 to 850 in scan analysis

Analytical column: Acquity UPLC® BEH C\(_{18}\) 2.1 mm x 50 mm, 1.7 \( \mu \)m

Column oven tem.: 25 °C, Flow rate: 0.5 mL/min (Waters Corporation)

Mobile phases A: 0.1 % TFA, Mobile phases B: CH\(_3\)CN

Gradient elution: 10 % B for 1.0 min, linear to 35 % B in 7.0 min, linear to 80 % B in 8.0 min followed by a decrease to 10 % B in 0.1 min.

Injection volume: 10 \( \mu \)L
**Equipment and condition**

Ionization: ESI in positive mode
Ionspray temperature: 120 °C, Desolvation temperature: 400 °C
Capillary voltage: 3.5 kV, Cone voltage: 26 V, Cone N₂ gas: 50 L/hr
Desolvation N₂ gas: 600 L/hr, Collision Ar gas: 0.2 mL/min

Collision energy: 15 eV

Precursor ions: $m/z$ 410, 415 and 358
Product ion scan range for the CID experiment: $m/z$ 50 to 850
**GHRP-2 ESI mass spectrum in scan mode**

Product ion mass spectrum MS/MS

**Precursor ion:**

\[ [M+2H]^2+ \] at \( m/z \) 410
**AA-3**

**ESI mass spectrum in scan mode**

![ESI mass spectrum in scan mode](image)

**Product ion mass spectrum MS/MS**

![Product ion mass spectrum MS/MS](image)

**Precursor ion:**

\([M+H]^+\) at \(m/z\) 358.
Stable Isotope Labeled GHRP-2

ESI mass spectrum in scan mode

Product ion mass spectrum MS/MS

Precursor ion: 
\([M+2H]^2+\) at \(m/z\) 415.
Selected ion chromatograms of GHRP-2 related peptide

GHRP-2: $m/z$ 410$>$170 (CE: 26 eV)
AA-3: $m/z$ 358$>$170 (CE: 28 eV)
SI-labeled GHRP-2: $m/z$ 415$>$170 (CE: 24 eV)
**Sample preparation**

5 mL urine

- 1 mL 2M glycine pH 2.2
- 50 μL of stable isotope labeled GHRP-2 (2μg/mL)

**Solid-phase extraction**

- wash 1 mL 0.1 % TFA
- elution 2 mL 0.5 % glycerol in CH₃OH

**Eluent**

Under N₂ stream at 4 °C

**Residue**

- 100 μL 0.1 % TFA / CH₃CN (90:10 v/v)

**Centrifugation separation** (1,000 g)

**Supernatant**

10 μL
Injection into the LC/MS/MS
Summary of assay validation for quantification analysis by means of UPLC®/ESI (+)-MS/MS in MRM mode

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Limit of detection</th>
<th>Correction coefficient</th>
<th>Recovery rate</th>
<th>Conc.</th>
<th>Intra-day assay</th>
<th>Inter-day assay</th>
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<tr>
<td></td>
<td>ng/ml</td>
<td>r</td>
<td>%</td>
<td>ng/ml</td>
<td>Precision</td>
<td>C.V.%</td>
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<td></td>
<td>C.V.%</td>
<td>%</td>
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<tr>
<td>GHRP-2</td>
<td>0.05</td>
<td>0.9992</td>
<td>84</td>
<td>1</td>
<td>3.1</td>
<td>1.36</td>
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<td>4</td>
<td>2.3</td>
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<td></td>
<td>9</td>
<td>1.6</td>
<td>2.67</td>
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<td>AA-3</td>
<td>0.02</td>
<td>0.9986</td>
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<td>9</td>
<td>3.8</td>
<td>-2.79</td>
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</table>
Selected ion chromatograms of spiked urine, Blank urine and Administered urine

**Spiked urine with 10 ng/mL of GHRP-2 and AA-3**

- $m/z$ 415 > 170 IS
- $m/z$ 410 > 269 GHRP-2
- $m/z$ 410 > 241 GHRP-2
- $m/z$ 410 > 170 GHRP-2
- $m/z$ 358 > 269 AA-3
- $m/z$ 358 > 241 AA-3
- $m/z$ 358 > 170 AA-3

**Blank urine**

- $m/z$ 415 > 170 IS

**Administered urine 4.5hrs after I.V. injection of GHRP-2**

- $m/z$ 415 > 170 IS
- $m/z$ 410 > 269 GHRP-2
- $m/z$ 410 > 241 GHRP-2
- $m/z$ 410 > 170 GHRP-2
- $m/z$ 358 > 269 AA-3
- $m/z$ 358 > 241 AA-3
- $m/z$ 358 > 170 AA-3
Excretion rates of GHRP-2 and AA-3 after I.V. administration of GHRP-2 (100 µg of pralmorelin dihydrochloride, n = 10).

GHRP-2

AA-3

In the case of combined administration Rec/Pit
Conclusion of our study

- Our LC/MS method is effective to detect GHRP-2 and its metabolite AA-3 in human urine. Also, the AA-3 is better suited for detecting GHRP-2 doping.

- The differential isoform method could detect GH doping, even if in the case of low dose administration of rhGH.

- GHRP-2 doping couldn’t be detected by the method based on GH isoform-profile and GHRP-2 had masking effect against detecting rhGH doping. The analysis of GHS compensates the defect of the method based on GH isoform-profile.

Cologne: Thevis M et al. Anal Bioanal Chem. 2011
Barcelona: Gallego R et al. Anal Biochem. 2010
Acknowledgements

We conducted these study in cooperation with,

Ministry of Education, Culture, Sports, Science and Technology
Asian-Regional Office, WADA
Japan Anti-Doping Agency
Staff of Mitsubishi Chemical Medience Co.