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.

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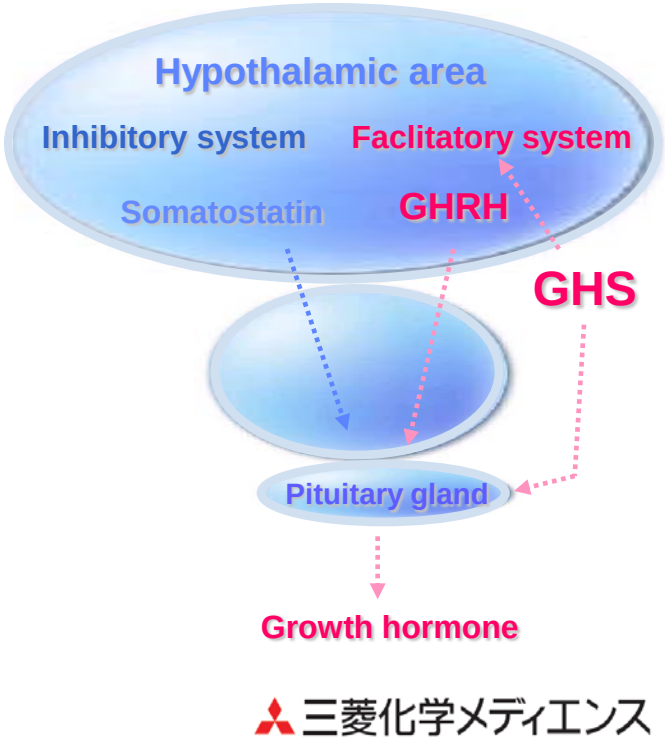
Growth Hormone Secretagogue Doping?

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.



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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・・・

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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

-2day	-1day	post 1day	2day	3day
-36hr Admission	-24hr Start of sampling Urine and Blood	0hr S.C. injection rGH *i.v.* injection GHRP-2	24hr	48hr Discharge

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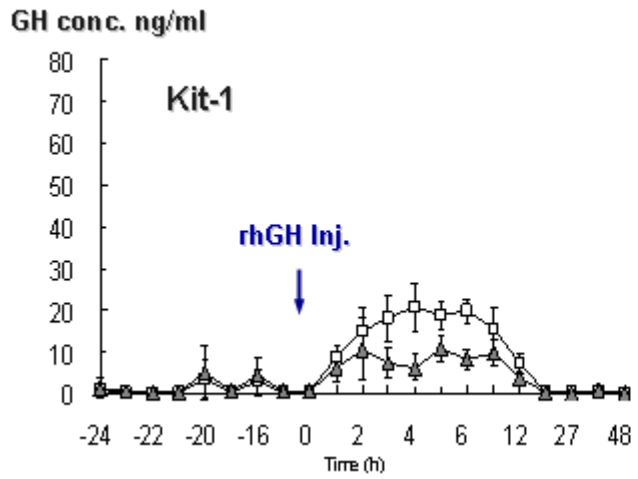
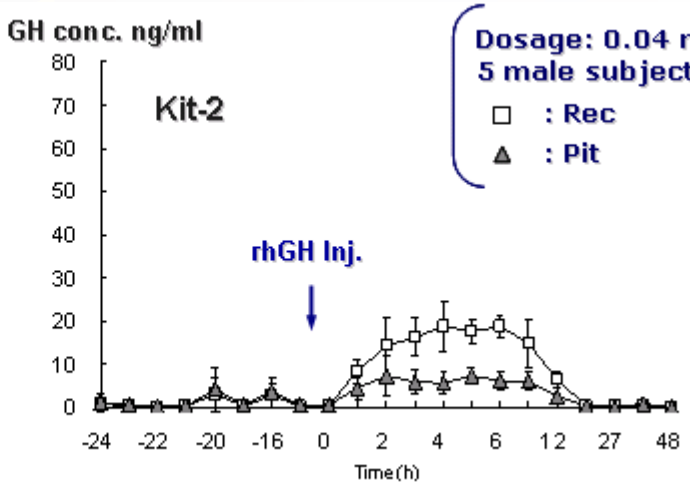
Analysis of GH by differential isoform immunoassay

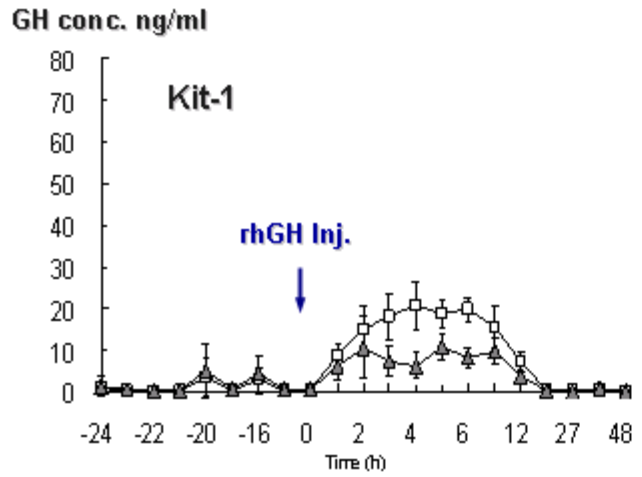


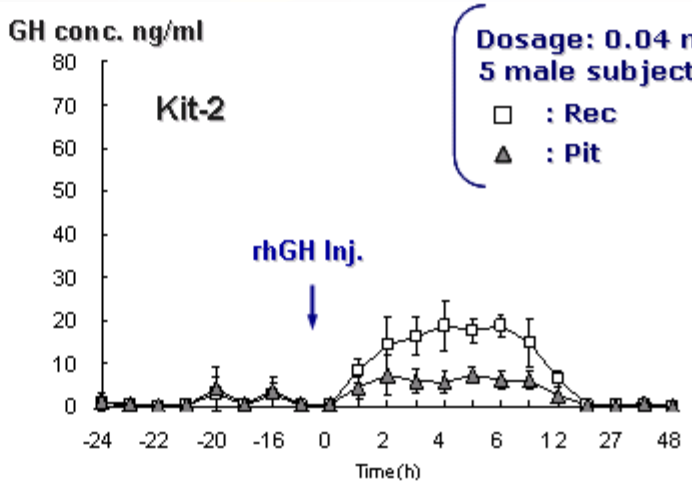


Kit1 : CMZ-Assay GmbH (Berlin, Germany)

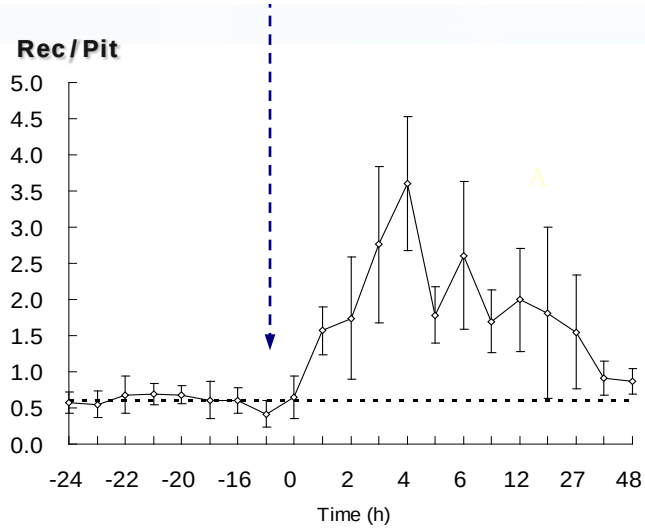
Ref.
Bidlingmaier M, Wu Z, Strasburger CJ, Test method: GH. *Bailliere's Clin Endocrinol Metab* 14:99-109 (2000).
Bidlingmaier M *et al,* High-Sensitivity Chemiluminescence Immunoassays for Detection of Growth Hormone Doping in Sports. *Clin Chem* 55: 3 (2009).

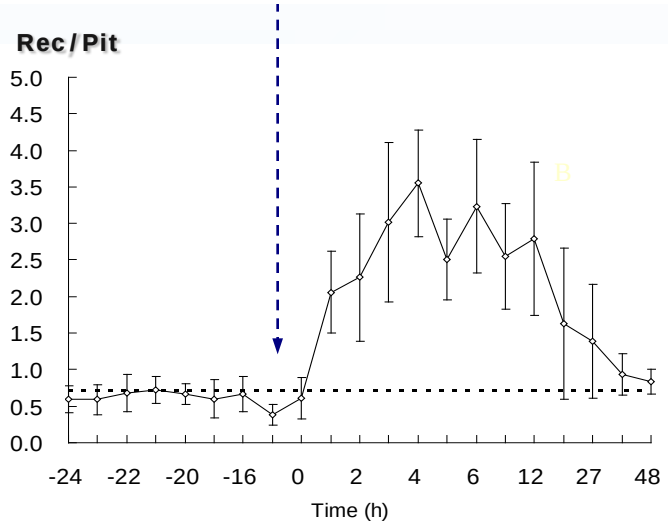
Analysis of GH by differential isoform immunoassay



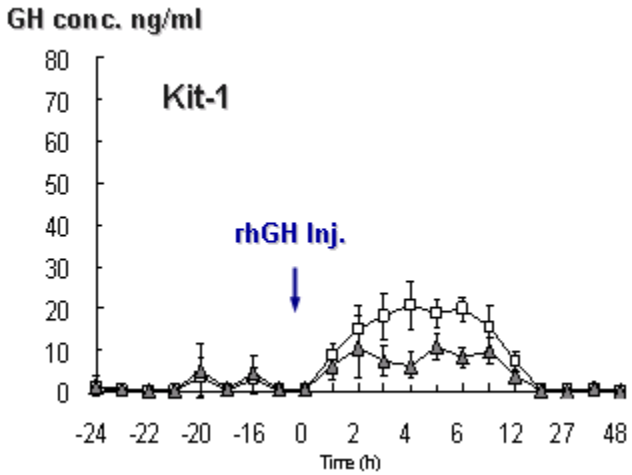


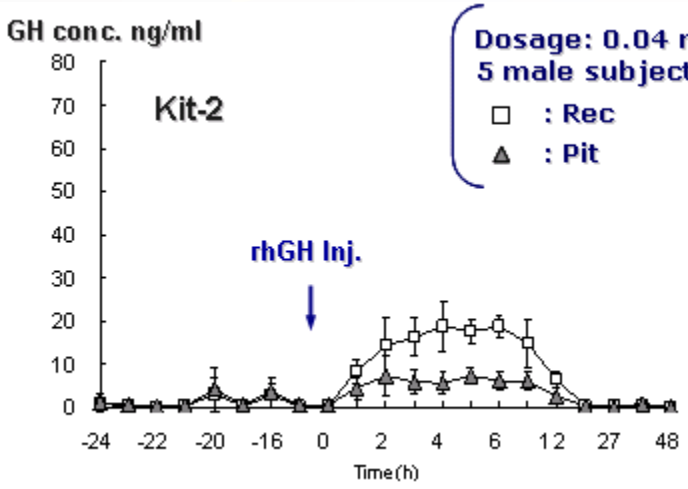
Dosage: 0.04 mg/kg S.C.
5 male subjects
□ : Rec
▲ : Pit

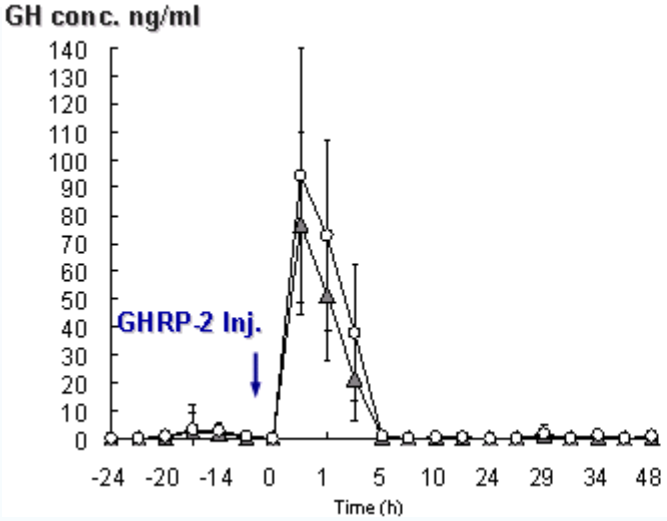


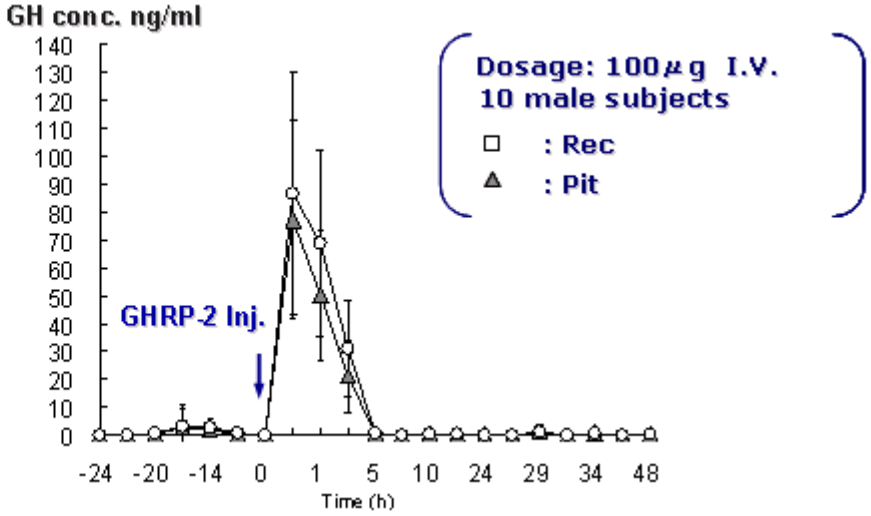


Analysis of GH by differential isoform immunoassay

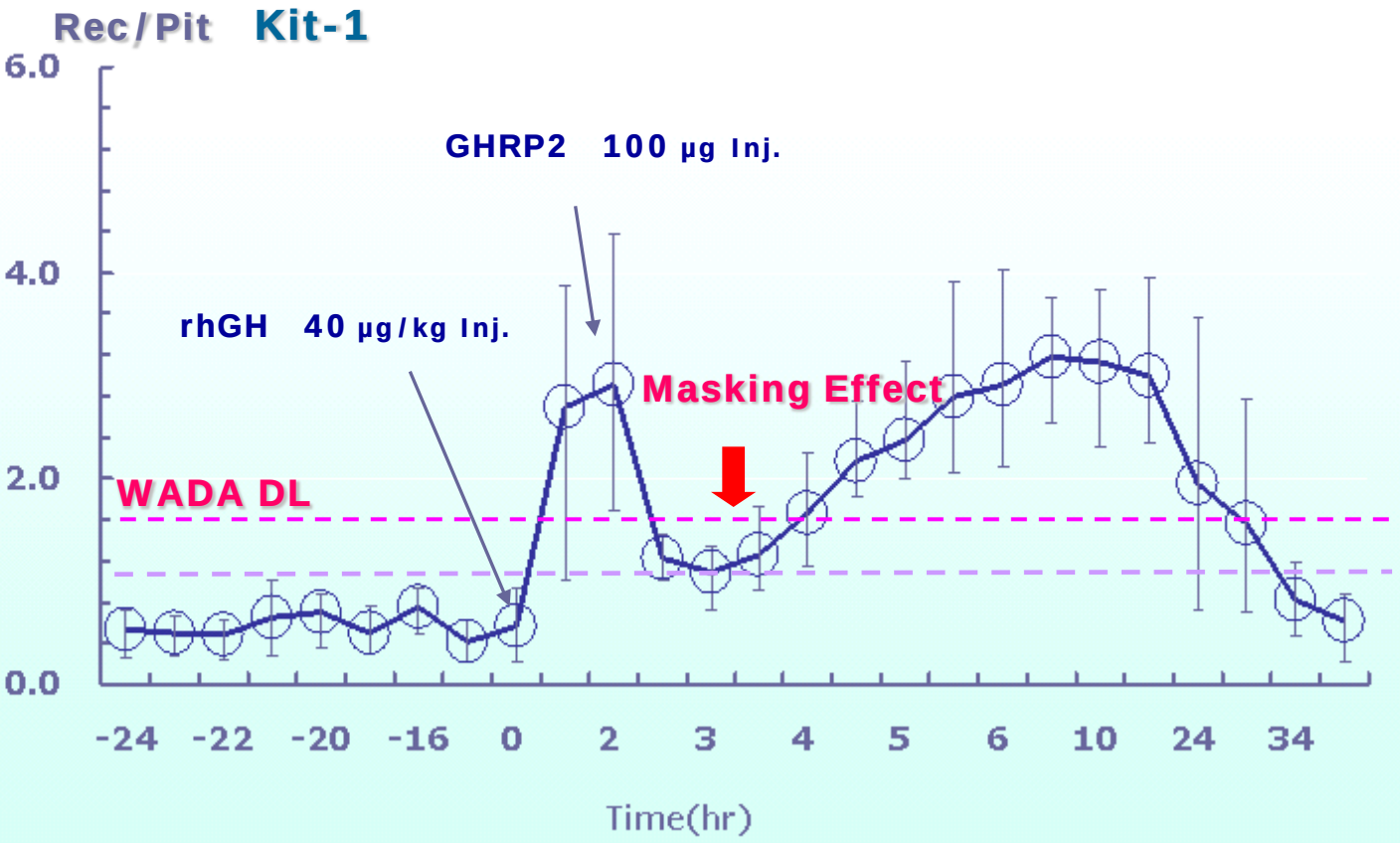








Combined Administration of GHRP-2 and GH



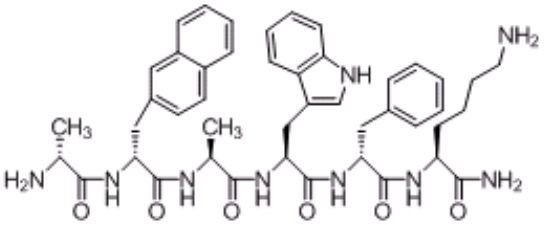
Therefore we have continued this research:

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

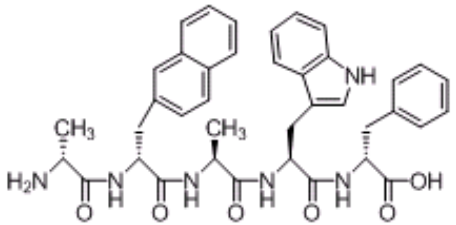
GHRP-2

Kaken Pharmaceutical Co., Ltd. Tokyo, Japan



D-Ala-D-(β-Naphthyl)-Ala-Ala-Trp-D-Phe-Lys-NH_2

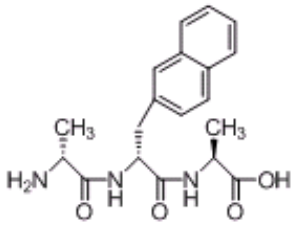
AF-6



D-Ala-D-(β-Naphthyl)-Ala-Ala-Trp-D-Phe-OH

AA-3

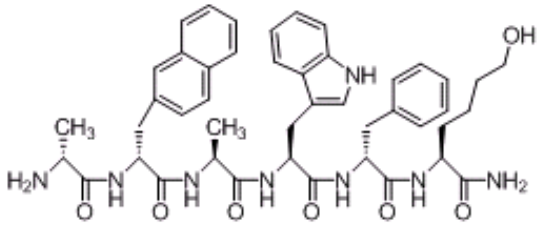
Major metabolite



D-Ala-D-(β-Naphthyl)-Ala-Ala-OH

AK-6

GL Biochem Ltd. , Shanghai, China



D-Ala-D-(β-Naphthyl)-Ala-Ala-Trp-D-Phe-Lys-OH

Reference
Kaken pharmaceutical co.,LTD.

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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_{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_3CN
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 μL

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

Peptide	Formura		Observed ion (*m/z*)	Theoretical ion (*m/z*)	Mass error (ppm)
GHRP-2	$C_{45}H_{56}N_9O_6$	$[M+H]^+$	818.4348	818.4348	0.00
		$[M+2H]^{2+}$	409.7208	409.7210	−0.49
AA-3	$C_{19}H_{24}N_3O_4$	$[M+H]^+$	358.1763	358.1761	0.56
Stable Isotope Labeled GHRP-2	$C_{36}H_{56}N_8O_6{}^{13}C_9{}^{15}N$	$[M+H]^+$	828.4610	828.4620	−1.21
		$[M+2H]^{2+}$	414.7360	414.7347	3.13

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 μm
Column oven tem.: 25 °C, Flow rate: 0.5 mL / min (Waters Corporation)
Mobile phases A: 0.1 % TFA, Mobile phases B: CH_3CN
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 μL

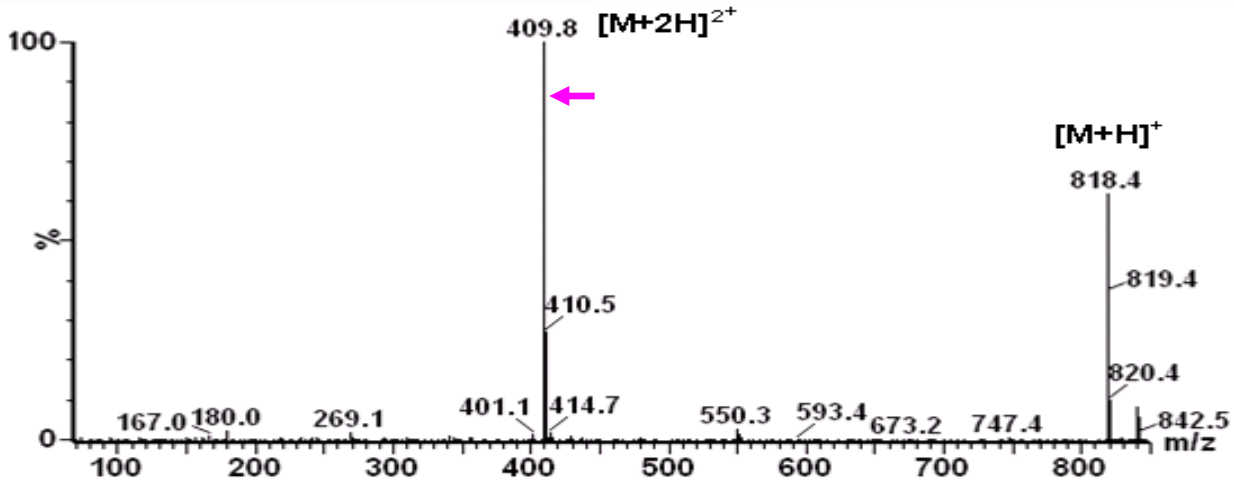
Equipment and condition

Ionization: ESI in positive mode
Ionspray temperature: 120 °C, Desolvation temperature: 400 °C
Capillary voltage: 3.5 kV, Cone voltage: 26 V, Cone N_2 gas: 50 L/hr
Desolvation N_2 gas: 600 L/hr, Collision Ar gas: 0.2 mL/min

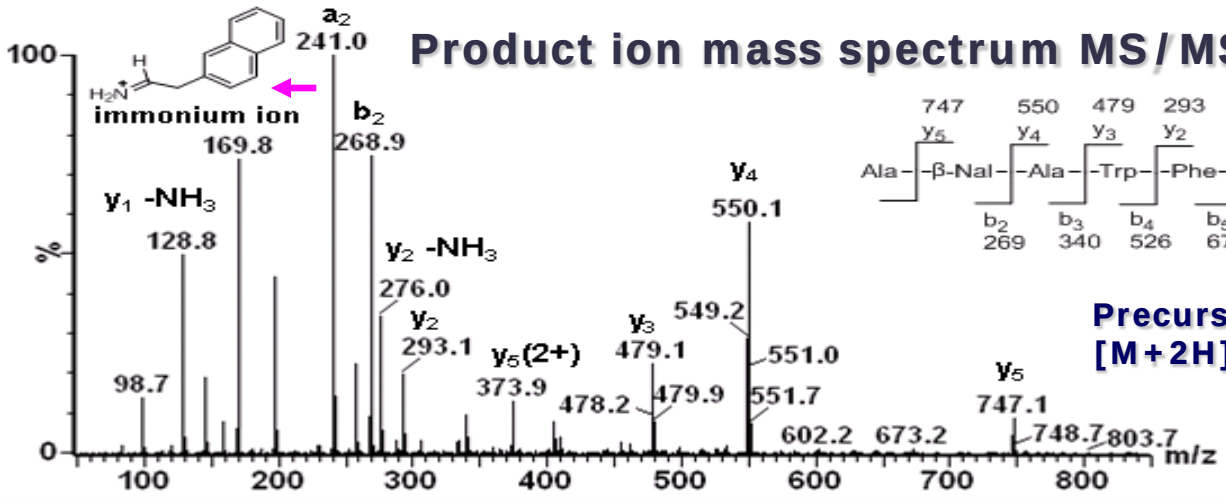
Collision energy: 15 eV

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

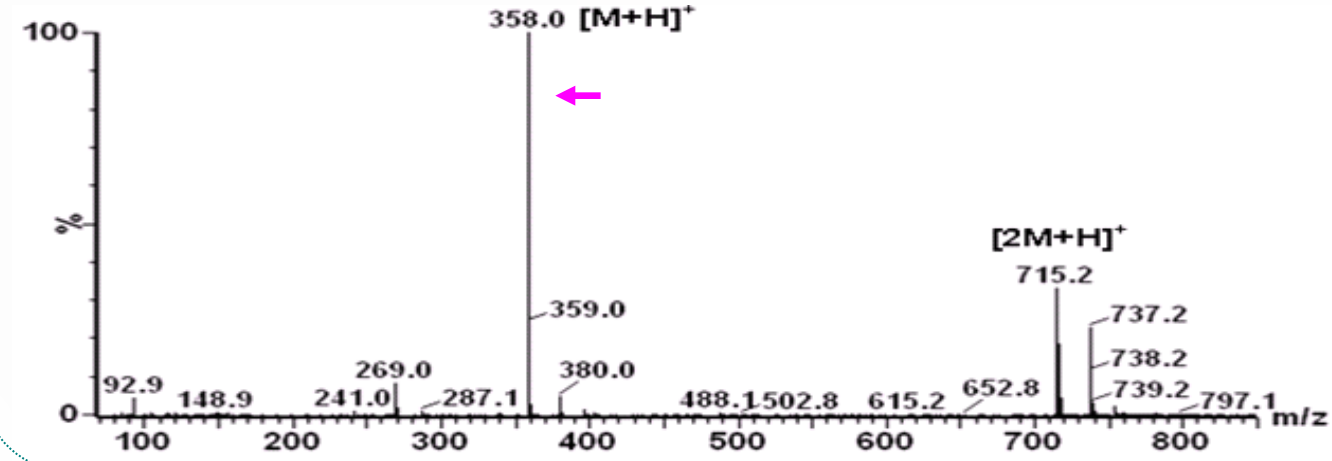


Product ion mass spectrum MS/MS

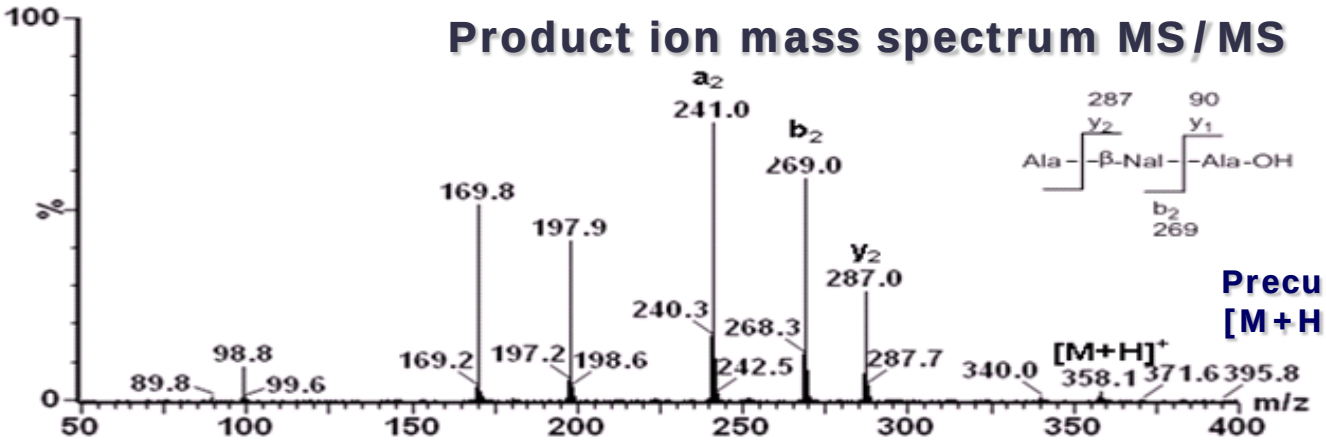


Precursor ion:
$[M+2H]^{2+}$ at *m/z* 410

AA-3 ESI mass spectrum in scan mode



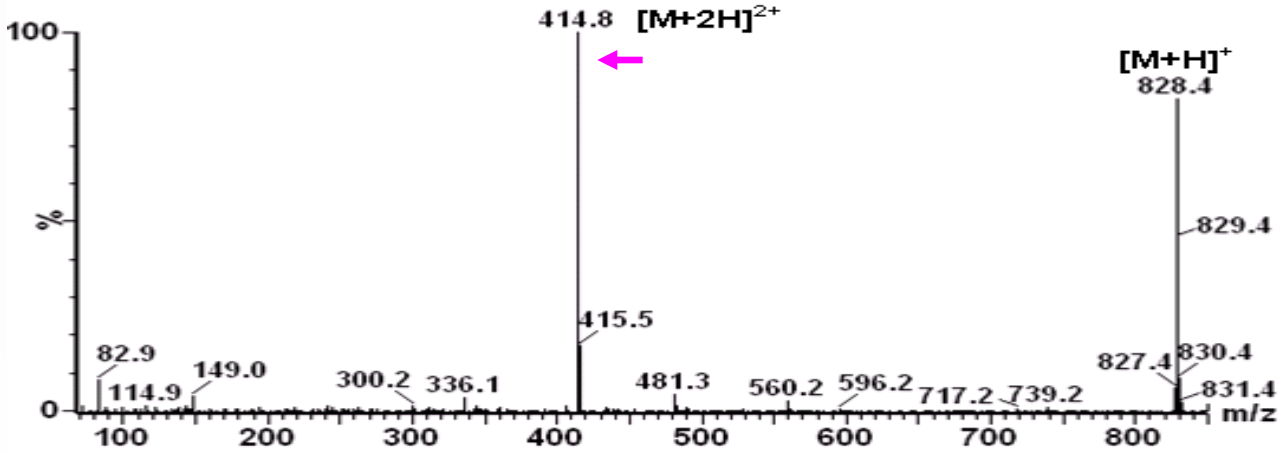
Product ion mass spectrum MS/MS



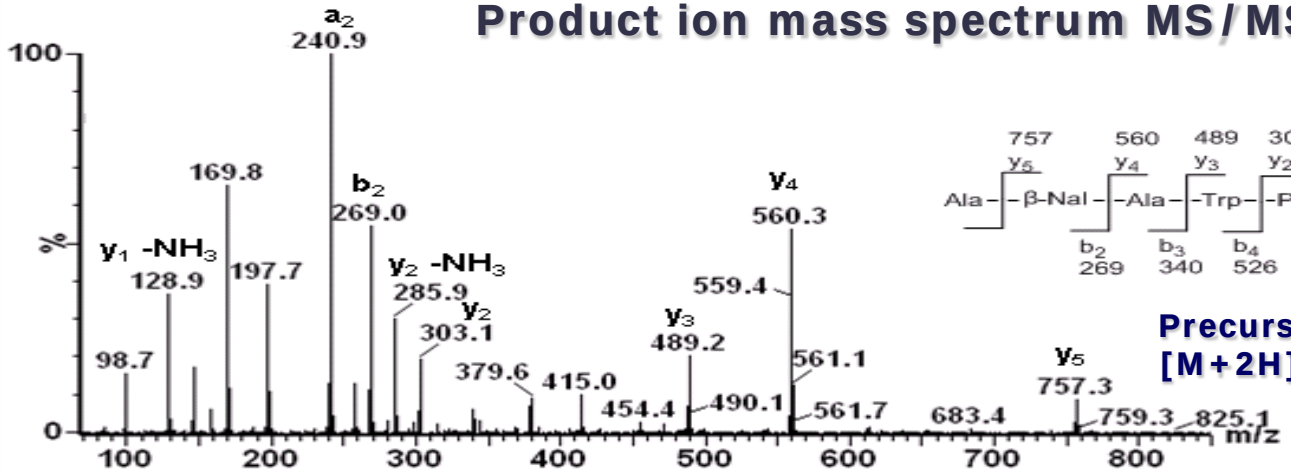
Precursor ion: $[M+H]^+$ at m/z 358.

Stable Isotope Labeled GHRP-2

ESI mass spectrum in scan mode

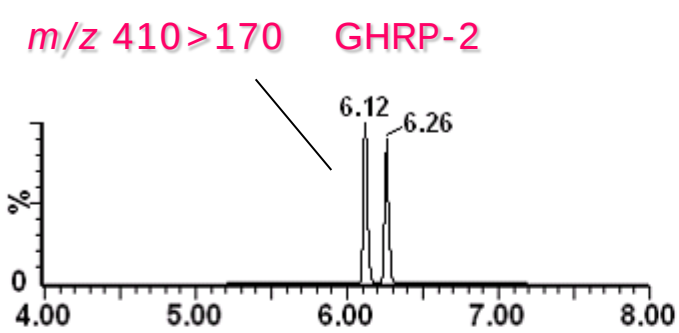


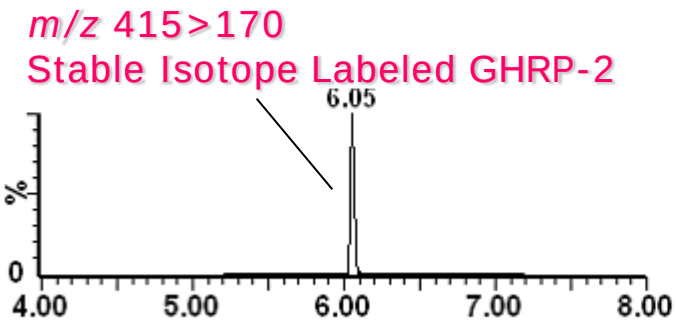
Product ion mass spectrum MS/MS

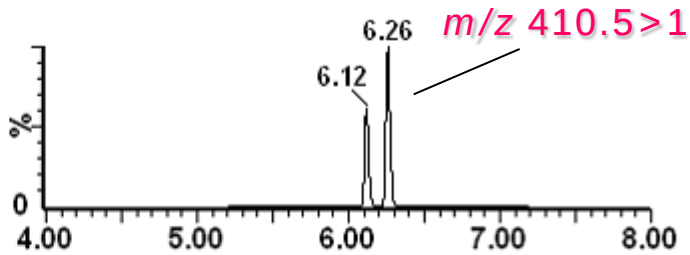


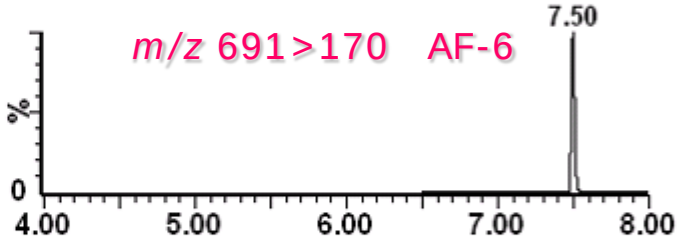
Precursor ion: $[M+2H]^{2+}$ at *m/z* 415.

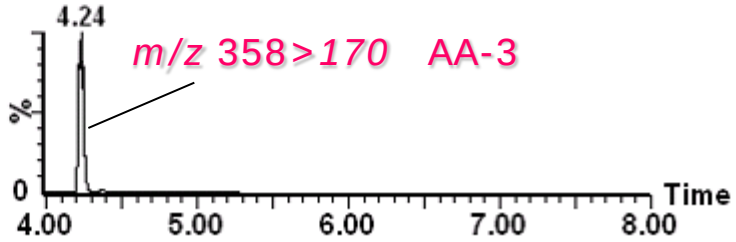
Selected ion chromatograms of GHRP-2 related peptide











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)

Soild-phase extraction

↓ wash 1 mL 0.1 % TFA

↓ elution 2 mL 0.5 % glycerol in CH_3OH

eluent

Under N_2 stream at 4 °C

residue

↓ 100 µL 0.1 % TFA / CH_3CN (90:10 v/v)

Centrififuge separation (1,000 g)

supernatant

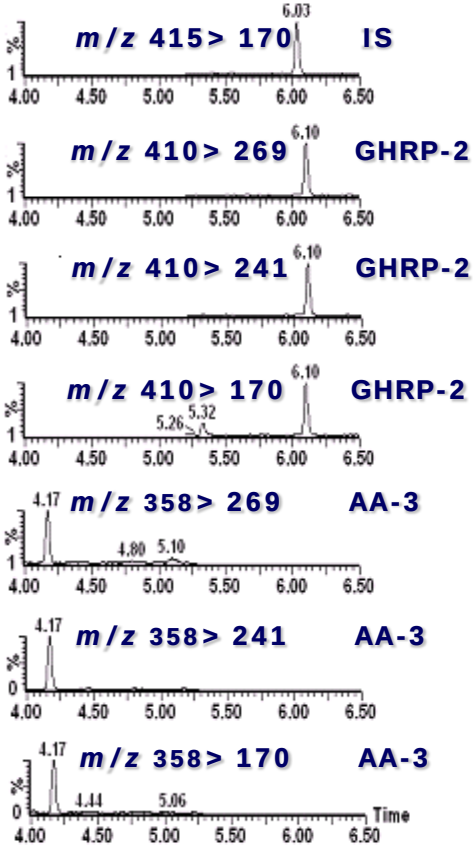
10 µL
Injection into the LC/MS/MS

Summary of assay validation for quantification analysis by means of UPLC®/ESI (+)-MS/MS in MRM mode

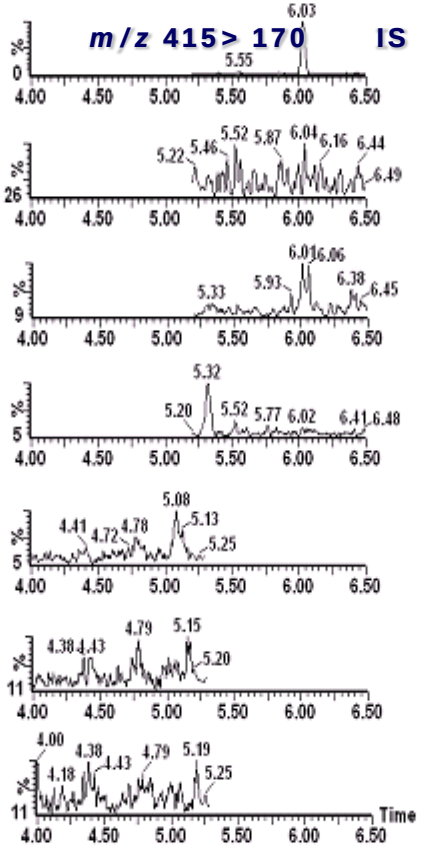
Compounds	Limit of detection	Correcttion coefficiency	Recovery rate	Conc.	Intra-day assay n=10		Inter-day assay 3days, n=30	
					Precision	Accuracy	Precision	Accuracy
	ng/ml	r	%	ng/ml	C.V.%	%	C.V.%	%
GHRP-2	0.05	0.9992	84	1	3.1	1.36	4.3	1.32
				4	2.3	0.82	2.2	0.02
				9	1.6	2.67	1.9	0.77
AA-3	0.02	0.9986	101	1	2.1	-2.38	3.2	-2.29
				4	3.0	-2.16	3.8	-2.80
				9	3.8	-2.79	3.9	-1.79

Selected ion chromatograms of spiked urine, Blank urine and Administrated urine

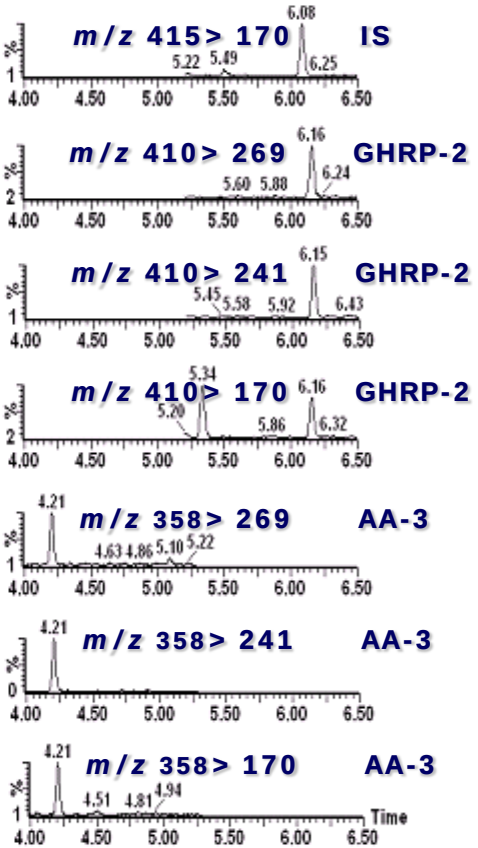
Spiked urine with 10 ng/mL of GHRP-2 and AA-3



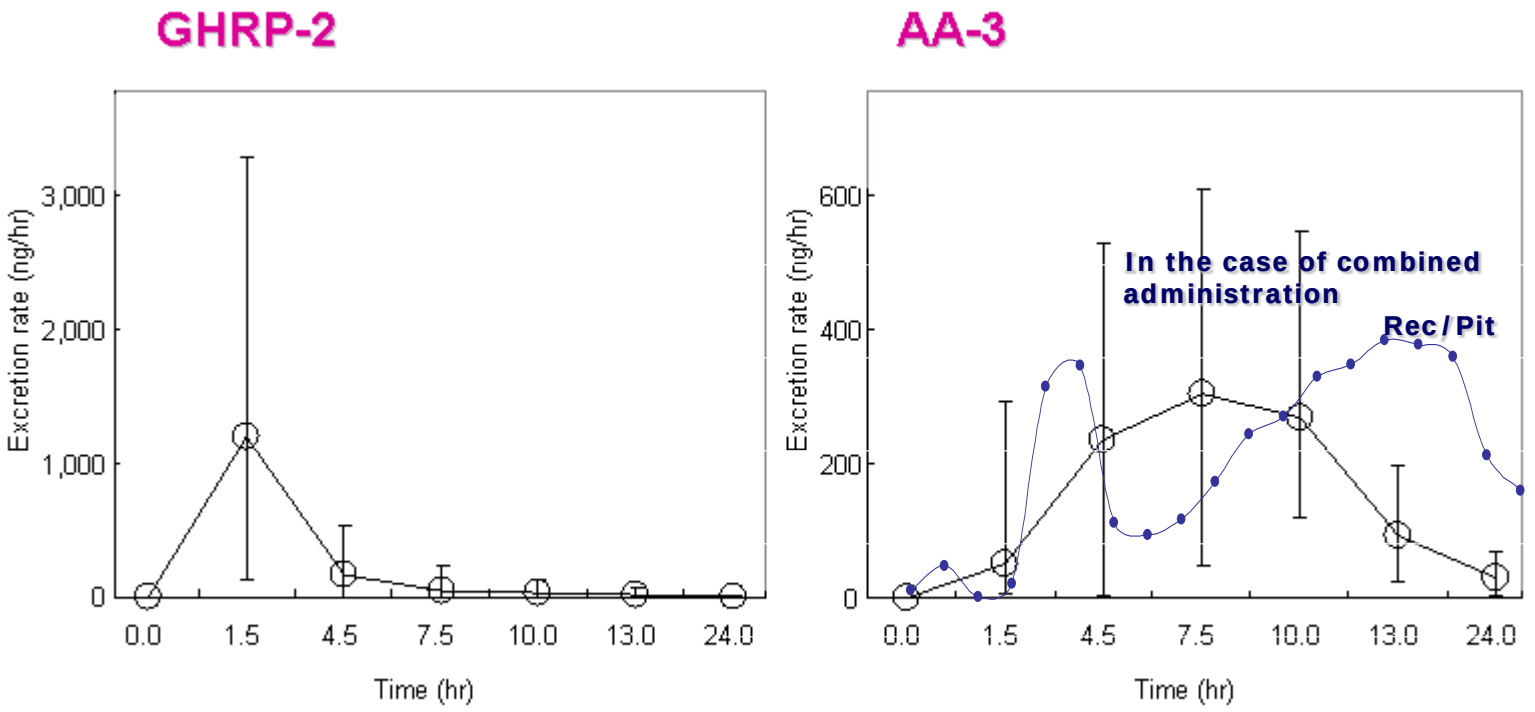
Blank urine



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



Excretion rates of GHRP-2 and AA-3 after I.V. administration of GHRP-2 (100 μg of pralmorelin dihydrochloride, n = 10).



Conclusion of our study

- Our LC/MS method is effective to detect GHPR-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. Rapid Commun. Mass Spectrom.* 2006
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.

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