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Influence of intravenous administration of growth hormone releasing peptide-2 (GHRP-2) on detection of growth hormone doping: growth hormone isoform profiles in Japanese male subjects

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Administration of exogenous 22 kDa recombinant human growth hormone (rhGH) suppresses the non-22 kDa pituitary growth hormone (GH) secretion by negative feedback; then, the elevated 22 kDa GH to non-22 kDa GH ratio (Rec/Pit ratio) can be utilized to detect doping with rhGH (isoform differential immunoassay). The influence of intravenous administration of growth hormone releasing peptide GHRP-2 on the isoform differential immunoassay for detecting rhGH doping has been investigated. In this study, a reference population (n=100) was used, with 0.04 mg/kg rhGH subcutaneous administration (n=5), 100 μg of GHRP-2 intravenous administration (n=10) and 0.04 mg/kg rhGH combined with 100 μg of GHRP-2 (n=10) in Japanese male subjects. The results indicated that the low dose (0.04 mg/kg) of rhGH led to significantly increased Rec/Pit ratio compared with the Japanese reference limit ($P < 0.001$). Because GHRP-2 dose led to increases in concentrations of both recombinant GH (rec GH) and pituitary GH (pit GH), no significant change in the Rec/Pit ratio was observed ($P > 0.05$). In a combined administration study, after GHRP-2 dose the Rec/Pit ratios decreased to 39.9–43.9% compared with the elevated ratio caused by the rhGH dose. The results indicated that GHRP-2 administration cannot only be detected by the isoform differential immunoassay but also masks rhGH doping. The analysis of GHRP-2 was found to be suitable for compensating for the disadvantages of the isoform differential immunoassay because GHRP-2 and its metabolite (AA-3) in urine could be detected during the periods of masking of the Rec/Pit ratio by means of liquid chromatography/tandem mass spectrometry. **Copyright © 2010 John Wiley & Sons, Ltd.**

Keywords: doping; growth hormone; growth hormone secretagogue; GHRP-2; GH isoform

Introduction

Ever since growth hormone (GH) was listed on the International Olympic Committee's (IOC's) prohibited list in 1989,[1] the misuse of GH by athletes has been prohibited by the World Anti-Doping Agency (WADA).[2]

Pituitary GH is composed of several different molecular isoforms: 22 kDa (48%), 20 kDa (9%), dimers/oligomers (30%), and less common fractions of modified and fragmented GH.[3]

In contrast, the isoform of recombinant human GH (rhGH) consists of the 22 kDa isoform only. Administration of exogenous 22 kDa rhGH suppresses the non-22 kDa hGH concentrations by negative feedback; then, the elevated 22 kDa hGH to non-22 kDa hGH ratio can be utilized to detect doping with rhGH.[3–6]

On the basis of this principle, a high-sensitivity chemiluminescence immunoassay[7] with potential utility for screening and confirmation for doping control purposes, was developed and is currently approved by WADA,[8] the so-called 'isoform differential immunoassay'.[8]

In 2010, exogenous GH was detected using this method by the WADA-accredited doping control laboratory in London (King's College London, UK) and it was the world's first analytical finding for exogenous GH.[9]

As another approach, the GH-2000 research team proposed a method to detect rhGH doping based on the measurement of GH-dependent markers, insulin-like growth factor-I (IGF-I) and type-III pro-collagen (P-III-P).[1,10]

Endogenous GH releasing substances, so-called GH-releasing peptide (GHRP) or GH secretagogue (GHS),[11] such as GHS/ghrelin receptor agonists,[11–13] or somatostatine inhibitors[14] have also been prohibited by WADA.[2]

For doping control purposes, several detection methods for GHSs have been developed.[14–17]

The effect of treatment with oral GHS (MK-677, ibutamoren) on GH isoforms was reported;[18] however, there is no evidence of how the isoform differential immunoassay would be affected by the use of GHS, and it is unclear whether rhGH abuse can be detected if used with GHSs. A synthetic hexapeptide GHRP-2 (pralmorelin, D-Ala-D-(β-naphthyl)-Ala-Ala-Trp-D-Phe-Lys-NH_2)

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representing GHSs was developed in the 1980s and has been used for diagnostic tests of GH deficiency.[19,20]

Moreover, several dietary supplements are available on the Internet, and GHRP-2 has been identified in the supplements.[16,21] This report describes statistical study of a reference population of Japanese male subjects, rhGH administration study, GHRP-2 administration study and study of combined administration of rhGH with GHRP-2. The present work is intended to investigate the influence of administration of GHRP-2 on detection of rhGH doping.

Experimental

Materials

All reagents were of analytical or high performance liquid chromatography (HPLC) grade. Distilled water was produced using a Milli-Q Ultrapure system (Millipore, Bedford, MA, USA). The Oasis® HLB cartridge (60 mg/3 mL) for solid-phase extraction (SPE) was purchased from Waters (Milford, MA, USA). GHRP-2, D-Ala-D-(β-naphthyl)-Ala-Ala-OH (AA-3) and stable-isotope-labelled GHRP-2 (D-Ala-D-(β-naphthyl)-Ala-Ala-Trp-Phe*-Lys-NH_2, $C_{36}H_{55}N_8O_6{}^{13}C_9{}^{15}N$) as an internal standard were purchased from GL Biochem Ltd. (Shanghai, China). For administration study, rhGH (22 kDa, Somatropin® BS 5 mg SC Injection) was purchased from Sandoz K.K. (Tokyo, Japan) and GHRP-2 (pralmorelin dihydrochloride, GHRP KAKEN100® 100 μg) was from Kaken Pharmaceutical Co., Ltd (Tokyo, Japan).

Determination of GH isoforms

The detection method was a quantitative analysis of recombinant GH (rec GH) and pituitary GH (pit GH) using commercial immunoassay kits provided by CMZ-Assays GmbH (Berlin, Germany) based on the isoform differential immunoassay,[7] and the rec GH to pit GH ratio (Rec/Pit ratio) was calculated.

The kits consist of two parts: kit 1 for initial screening test and kit 2 for confirmation test, and each kit consists of a test for rec GH (Rec1 for kit 1, Rec2 for kit 2) and a test for pit GH (Pit1 for kit 1, Pit2 for kit 2). The assay for rec GH uses a monoclonal capture antibody (AK566 for Rec1, AK568 for Rec2) that has a relatively high affinity for monomeric 22 kDa GH. The capture antibody used in the pit GH assay (AK567 for Pit1, AK565 for Pit2) has cross-reactivity with minor isoforms and 22 kDa dimers. All assays use the same detection antibody (AK569 labelled with Acridinium-NHS Ester). Berthold Auto Lumat Plus LB953 luminometer equipped with LBIS software ver. 3.3 was purchased from Berthold Technologies GmbH (Bad Wildbad, Germany). The sample preparation was performed as described in the instruction manual provided by the manufacturer. In brief, standards calibrated with pituitary-derived hGH (80/505; NIBSC) were reconstituted with 0.5 mL of sheep serum, and controls were reconstituted with 0.5 mL of human serum. 150 μL of incubation buffer (pH 9) was added to 50 μl of serum samples in duplicate. The mixed tubes were incubated for 2 h at ambient temperature. After washing, 200 μL of tracer antibody (AK569) was added to each tube. The mixed tubes were incubated for 2 h at ambient temperature protected from light. After washing, acidic H_2O_2 and NaOH solutions were added to tubes, which were placed in a luminometer within 1 h of the final wash. The inhouse method validation was conducted and the method was included in the scope of the ISO/IEC17025 accreditation. The lower limits of detection (LLOD) of Rec1, Rec2, Pit1 and Pit2 were 0.001, 0.001, 0.003 and 0.005 ng/mL, respectively. The limit of quantification (LOQ) of each kit was set at 0.05 ng/mL.

Determination of urinary GHRP-2 and its metabolite AA-3

Concentrations of urinary GHRP-2 and AA-3 were determined by ultra-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) in multiple reaction monitoring (MRM) mode in accordance with our recent literature.[15] In brief, 5 mL of human urine fortified with an internal standard (stable-isotope-labelled GHRP-2) was buffered to pH 2.5 with 2 M glycine buffer (pH 2.2). The target peptides were extracted by SPE using Oasis HLB and the methanol extract was evaporated to dryness. The residue was dissolved in 100 μL of 0.1% trifluoroacetic acid (TFA)/CH_3CN (90 : 10, v/v) and injected into the UPLC/MS/MS. The UPLC/MS/MS system was Acquity® UPLC/triple quadrupole TQD mass spectrometer from Waters. Ionization was accomplished using electrospray ionization (ESI) in positive mode. The instrument was calibrated using NaCsI prior to analysis. The analytical column was an Acquity UPLC BEH C_{18} (2.1 mm × 50 mm, 1.7 μm particle size), and the mobile phases used were 0.1% TFA (mobile phase A) and CH_3CN (mobile phase B). The column oven temperature was 25 °C and the flow rate was 0.5 mL/min. Gradient elution was as follows: 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. Finally, the column was equilibrated for 2 min. The injection volume was 10 μL. The mass transition and the collision energy (CE) of GHRP-2, AA-3 and stable isotope-labelled GHRP-2 were set at *m/z* 410>170 (CE: 26 eV), *m/z* 358>170 (CE: 28 eV) and *m/z* 415>170 (CE: 24 eV), respectively. The LLOD of GHRP-2 and AA-3 were 0.05 ng/mL and 0.02 ng/mL, respectively.

Determination of serum IGF-I and P-III-P by immunoradiometric assay (IRMA)

Serum IGF-I concentration was determined using a commercially available radioimmunoassay kit, Somatomedin C·II Siemens (Siemens Healthcare Diagnostics K.K., Tokyo, Japan). Serum P-III-P concentration was determined using a commercially available radioimmunoassay kit, RIA-gnost® PIIIP c.t. (CISbio International, Bagnols/Ceze, France). 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 for clinical testing.

Human subjects

The protocol was in accordance with the Helsinki Declaration and approved by the Ethics Review Board of Mitsubishi Chemical Medience Corporation (approval Nos. 2010-A008 and 2010-B005). All subjects were informed in advance of the details of the study and written consent was obtained from all participating volunteers. Serum samples were collected using BD Vacutainer® SST™-II (Becton Dickinson and Company, Franklin Lakes, NJ, USA) for determination of serum GH. Plain tube Benoject-II (Terumo Co., Tokyo, Japan) was used in blood collection for measuring of serum IGF-I and P-III-P. For safety, vital signs (blood pressure, body temperature, and pulse rate) were monitored during administration studies, heart rate was measured prior to administration, and laboratory clinical tests of hematology and blood chemistry were performed prior to and after administration at a clinical facility.

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Japanese male subjects (non-athletes)

Serum samples from 100 healthy male Japanese volunteers (Mongoloid, 20–66 years old, Body mass index (BMI): 15.7–32.4, body weight: 50.4–103.8 kg) were collected. No attempt was made to control diet intake. All samples were collected between 9:00 am and 12:30 pm. Serum samples were stored at −80 °C until analysis.

Administration study of rhGH

An injectable solution of rhGH (0.04 mg/kg, Somatropin®) was subcutaneously administered during fasting at 9:00 am. Five healthy male volunteers (subjects 1–5) were Japanese (Mongoloid, 20–37 years old, BMI: 18.8–21.8, body weight: 54–63 kg). The volunteers refrained from ingesting alcohol, caffeine, grapefruit, any supplements and medicines and did not perform any exercise during the studies. The time courses of serum sample collection were −24, −23, −22, −21, −20, −18, −16, −12, 0 (pre-administration), 1, 2, 3, 4, 5, 6, 8, 12, 24, 27, 34 and 48 h. Serum samples were stored at −80 °C until analysis.

Administration study of GHRP-2

An injectable solution of 100 μg of GHRP-2 (GHRP KAKEN100®, 10 μg/mL in saline) was intravenously (10 mL/2 min) administered after overnight fasting at 9:00 am. Ten healthy male volunteers (subjects 6–15) were Japanese (Mongoloid, 22–40 years old, BMI: 21.3 ± 1.8, body weight: 51.5–72.9 kg). The volunteers refrained from ingesting alcohol, caffeine, grapefruit, any supplements and medicines and did not perform any exercise during the studies. Urine samples were collected prior to administration (−24 h to 0 h) and during the first 72 h after administration. The time courses of serum sample collection were −24, −22.5, −19.5, −17, −14, −11, 0 (pre-administration), 0.5, 1, 1.5, 4.5, 7, 10, 13, 24, 25.5, 28.5, 31, 34, 37, 48, 49.5, 52.5, 55, 58, 61, 72 and 504 h. Samples were stored at −20 °C for urine and at −80 °C for serum until analysis.

Combined administration study of rhGH and GHRP-2

An injectable solution of rhGH (0.04 mg/kg, Somatropin®) was subcutaneously administered during fasting at 9:00 am. After 2 h, 100 μg of GHRP-2 (GHRP KAKEN100®) was intravenously administered. Ten healthy male volunteers (subjects 16–25) were Japanese (Mongoloid, 20–33 years old, BMI: 19.2–21.5, body weight: 56–66 kg). Urine samples were collected prior to administration (−24 h to 0 h) and during the first 48 h after administration. The time courses of serum sample collection were −24, −23, −22, −21, −20, −18, −16, −12, 0 (pre-administration of rhGH), 1, 2 (pre-administration of GHRP-2), 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 8, 10, 12, 24, 27, 34 and 48 h. Samples were stored at −20 °C for urine and at −80 °C for serum until analysis.

Statistics

Statistical analysis was conducted using the StatFlex 4.2 (Artech Inc., Osaka, Japan). Nonparametric Mann-Whitney U-test was used for between-group comparisons (non-administered and administered). $P < 0.01$ was considered significant. When a concentration of serum GH was less than 0.05 ng/mL of LOQ, the absolute value of 0.05 was used in ratio calculation.

Table 1. Statistical analysis of GH concentrations and Rec/Pit 1 ratios in Japanese healthy volunteers, rhGH treated subjects and GHRP-2 treated subjects

	Kit 1		
	GH (ng/mL)		Rec/Pit 1 ratio
	Rec1	Pit 1	
Japanese healthy male subjects, n = 100			
Mean	0.50	0.74	0.71
Maximum	7.92	13.54	1.55
Minimum	0.05	0.05	0.26
Median	0.15	0.25	0.71
75th percentile	0.27	0.42	0.82
25th percentile	0.09	0.13	0.55
95% Confidence Interval	0.27–0.74	0.39–1.08	0.66–0.75
rh GH treated male subjects, n = 45 (5 individuals × 9 data, 1-24h)			
Mean	12.82	5.07	2.62
Maximum	28.44	14.19	4.93
Minimum	0.14	0.09	0.71
Median	12.76	4.67	2.51
75th percentile	19.09	7.49	3.01
25th percentile	7.74	2.76	2.10
95% Confidence Interval	10.73–14.91	4.15–5.98	2.34–2.90
GHRP-2 treated male subjects, n = 80 (10 individuals × 8 data, 0.5-24h)			
Mean	18.74	23.52	0.68
Maximum	132.77	168.60	1.05
Minimum	0.05	0.05	0.22
Median	0.30	0.53	0.69
75th percentile	25.96	34.08	0.89
25th percentile	0.10	0.16	0.55
95% Confidence Interval	11.80–25.68	15.09–31.95	0.63–0.73

Results and Discussion

Determination of GH isoform profiles

Japanese male subjects

We investigated the distribution of GH concentrations and Rec/Pit ratios in normal subjects. The statistical results are shown in Tables 1 and 2. Concentrations of GH were measured using the Rec1, Rec2, Pit1 and Pit2 assays on 106 serum samples from male volunteers. Because GH concentrations (Rec1, Rec2, Pit1 or Pit2) obtained from six samples were below the LOQ of 0.05 ng/mL, the data from the remaining 100 serum samples were used in statistical analysis. Concentrations of Rec1, Rec2, Pit1 and Pit2 ranged from 0.05 to 7.92 ng/mL (median 0.15 ng/mL), 0.05 to 8.05 ng/mL (median 0.15 ng/mL), 0.05 to 13.54 ng/mL (median 0.25 ng/mL) and 0.06 to 12.80 ng/mL (median 0.29 ng/mL), respectively. The ratios of Rec/Pit1 and Rec/Pit2 were also determined. The ratios of Rec/Pit1 and Rec/Pit2 ranged from 0.26 to 1.55 (median 0.71) and 0.21 to 1.40 (median 0.61), respectively. On the basis of the mean + 2SD (n = 100), Japanese upper limits of Rec/Pit1 ratio and Rec/Pit2 ratio were set to 1.17 and 0.97, respectively.

Administration study of rhGH

The response to a low dose of rhGH (0.04 mg/kg) in Japanese male subjects for the isoform method was investigated. The trends in ratios for Rec/Pit and concentrations of rec GH and pit GH after subcutaneous injection of 0.04 mg/kg rhGH are

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Table 2. Statistical analysis of GH concentrations and Rec/Pit2 ratios in Japanese healthy volunteers, rhGH treated subjects and GHRP-2 treated subjects

	Kit 2		
	GH (ng/mL)		Rec/Pit 2 ratio
	Rec2	Pit2	
Japanese healthy male subjects, n = 100			
Mean	0.51	0.86	0.59
Maximum	8.05	12.80	1.40
Minimum	0.05	0.06	0.21
Median	0.15	0.29	0.61
75th percentile	0.30	0.49	0.69
25th percentile	0.08	0.16	0.46
95% Confidence Interval	0.27–0.76	0.49–1.24	0.56–0.63
rh GH treated male subjects, n = 45 (5 individuals × 9 data, 1-24h)			
Mean	13.81	7.03	2.17
Maximum	27.15	19.65	5.01
Minimum	0.16	0.08	0.74
Median	13.70	6.15	1.93
75th percentile	20.72	9.53	2.69
25th percentile	9.24	4.08	1.46
95% Confidence Interval	11.60–16.02	5.70–8.36	1.89–2.46
GHRP-2 treated male subjects, n = 80 (10 individuals × 8 data, 0.5-24h)			
Mean	18.85	25.86	0.61
Maximum	139.13	191.46	0.96
Minimum	0.05	0.05	0.20
Median	0.30	0.59	0.63
75th percentile	25.97	39.94	0.78
25th percentile	0.09	0.17	0.48
95% Confidence Interval	11.96–25.75	16.74–34.98	0.56–0.65

shown in Figures 1A, 1B, 2A, and 2B. The mean values of baseline concentration (−24 h to 0 h prior to administration) of Rec1 and Rec2 were 0.88 ng/mL (0.05–7.33 ng/mL) and 0.95 ng/mL (0.05–8.75 ng/mL), respectively. The concentrations of Rec1 and Rec2 increased to 18.70 ng/mL (13.49–28.44 ng/mL) and 20.73 ng/mL (13.67–27.15 ng/mL) at 4 h after administration, respectively, and then returned to the baseline at 24 h. The mean values of baseline Rec/Pit ratios (−24 h to 0 h prior to administration) of kit 1 and kit 2 were 0.61 (0.26–1.00) and 0.61 (0.27–1.00), respectively. The Rec/Pit1 and Rec/Pit2 ratios increased to 3.55 (2.73–4.47) and 3.60 (2.69–5.01) at 4 h after administration, respectively. The maximum ratios of Rec/Pit1 and Rec/Pit2 were 4.93 (subject-1, 3 h after administration) and 5.01 (subject-1, 4 h after administration), respectively (Tables 1 and 2). As expected, significant increases in ratios for Rec/Pit1 and Rec/Pit2 were observed with the administration of rhGH compared with those in Japanese male subjects ($P < 0.001$, Figure 3). The elevated Rec/Pit ratios peaked at 4 h in terms of the mean of five subjects and the ratios persisted above the Japanese upper limit for 12 h (subjects-1 and 2), 24 h (subject-3), 27 h (subject-5) and 34 h (subject-4) after the 0.04 mg/kg rhGH administration (Figures 1A and 2A).

Administration study of GHRP-2

We investigated whether GHS (GHRP-2) doping, which was expected to cause release of endogenous GH from pituitary gland, could be detected by the isoform method. The trends in ratios for Rec/Pit and concentrations of rec GH and pit GH after intravenous injection of 100 µg of GHRP-2 are shown in Figures 1C, 1D, 2C, and 2D. The mean values of baseline concentrations (−24 h to 0 h prior to administration) of Rec1, Rec2, Pit1 and Pit2 were 0.80 ng/mL (0.05–21.90 ng/mL), 0.81 ng/mL (0.05–22.00 ng/mL), 1.12 ng/mL (0.05–24.99 ng/mL) and 1.20 ng/mL (0.05–27.46 ng/mL), respectively. The elevated concentrations of rec GH and pit GH peaked at 0.5 h for all ten subjects after administration. The mean concentrations of Rec1, Pit1, Rec2 and Pit2 at 0.5 h after administration were 77.4 ng/mL (29.1–132.8 ng/mL), 86.6 ng/mL (33.1–168.6 ng/mL), 76.8 ng/mL (30.9–139.1 ng/mL) and 94.3 ng/mL (40.1–191.5 ng/mL), respectively (Figures 1D and 2D). However, it is recognized to be difficult to detect rhGH doping using the absolute concentration of GH because GH secretion is influenced by sleep, stress, exercise or nutrients, and the concentration of GH usually cycles through increases and decreases several times a day in normal individuals (i.e. subject-10). The mean values of baseline ratios of Rec/Pit1 and Rec/Pit2 were 0.72 (0.32–1.09) and 0.66 (0.27–1.11), respectively. The mean ratios of Rec/Pit1 and Rec/Pit2 were 0.91 (0.79–0.98) and 0.83 (0.73–0.96) at 0.5 h after administration (Figures 1C and 2C). The administration of GHRP-2, which has strong endogenous GH-releasing activity, led to increases in the concentrations of both rec GH and pit GH. As a result, the Rec/Pit ratios of ten subjects persisted below the Japanese upper limit prior to and after administration of GHRP-2 (Tables 1 and 2, Figures 1C and 2C). It seems that a slightly decreasing tendency of the ratios was observed in the elimination phase at 4.5 h after administration, and the means of Rec/Pit1 and Rec/Pit2 were 0.32 (0.22–0.59) and 0.27 (0.20–0.58), respectively. For reasons that are unknown, the acute endogenous hGH secretion by GHRP-2 administration through the GHS receptor, with subsequent transient depletion of endogenous GH, may alter the normal composition of different molecular isoforms of GH secreted into circulation. Nevertheless, no significant increases in ratios of Rec/Pit1 and Rec/Pit2 were observed during 72 h after the administration of GHRP-2, compared with those in Japanese male subjects ($P > 0.05$, Figure 3).

Combined administration study of rhGH and GHRP-2

The influence of GHS (GHRP-2) administration on the detection of rhGH doping by the isoform method was investigated. The trends in ratios for Rec/Pit and concentrations of rec GH and pit GH after combined subcutaneous injection of 0.04 mg/kg rhGH and intravenous injection of 100 µg GHRP-2 are shown in Figures 1E, 1F, 2E, and 2F. After administration of rhGH, the ratios of Rec/Pit1 and Rec/Pit2 increased significantly during 2 h in all ten subjects ($P < 0.001$), as did those with the sole administration of rhGH. Note that injection of GHRP-2 led to increases in the concentrations of Rec1, Pit1, Rec2 and Pit2; in contrast, the ratios of Rec/Pit1 and Rec/Pit2 rapidly decreased to the Japanese reference upper limit. The mean ratios for Rec/Pit1 and Rec/Pit2 decreased to 39.9% and 43.9%, respectively, compared with the highest value at 2 h. As shown in Figure 4, the Rec/Pit1 ratio in subject-17 increased from 0.78 (prior to administration) to 4.27 (2 h) after administration of rhGH, and after injection of GHRP-2, it decreased to 0.93 in 1.5 h and increased again to 3.84 in 6.5 h before finally returning to baseline in 24 h. The Rec/Pit1 ratio in subject-25 increased from 0.59 (prior to administration) to 3.92 (2 h) after administration of rhGH, and after injection of GHRP-2, it decreased to below the Japanese reference upper limit of 0.72 in 1 h and increased again to 3.76 in 21 h before finally returning to baseline in 24 h. This

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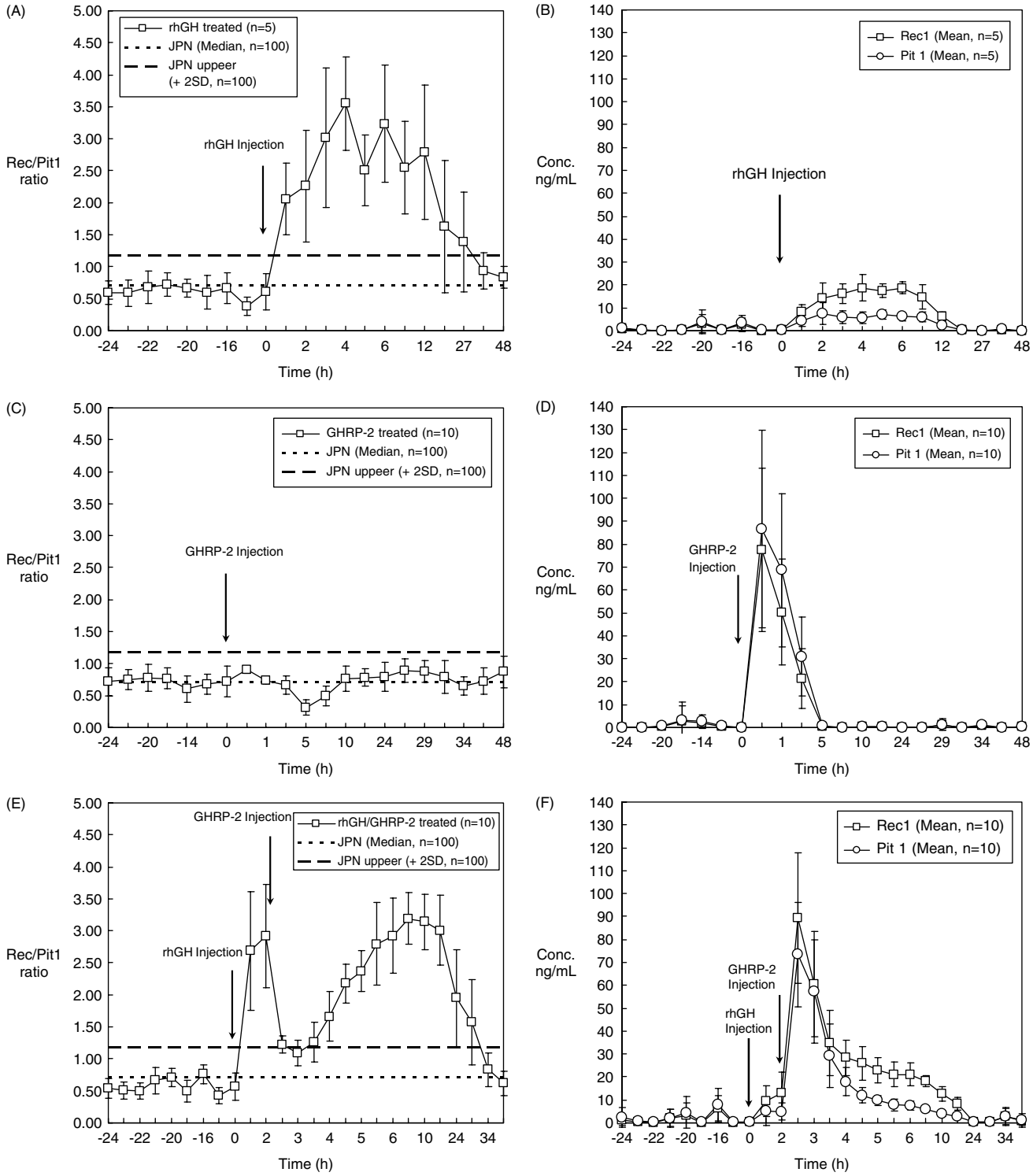

Figure 1. Change in ratios Rec/Pit1(left) and absolute hGH concentrations (right) of Rec1 and Pit1. The whiskers indicate mean ± standatd deviation. JPN means the median of Japanese male populations (n = 100). JPN upper means Japanese male reference upper limit. (A,B: rhGH 0.04 mg/kg S.C. Injection, C,D: GHRP-2 100 μ g I.V. Injection, E,F: Combined administration of rhGH and GHRP-2).

phenomenon can best be explained as follows. The first increase in the ratio is due to the presence of exogenous GH in circulation and negative suppression of endogenous GH. The presence of GHRP-2 after initial suppression of pit GH led to reproduction of the endogenous GH because the endogenous GH releasing activity through the GHS receptor is stronger than suppression of endogenous GH secretion by negative feedback; then, the Rec/Pit ratios decreased. After the disappearance of GHRP-2 from circulation, the release of rhGH from the subcutaneous depot occurred to increase the Rec/Pit ratios again, and finally the Rec/Pit ratios returned to the baseline level following elimination of rhGH. Thus, administration of GHRP-2 led to decrease of the elevated

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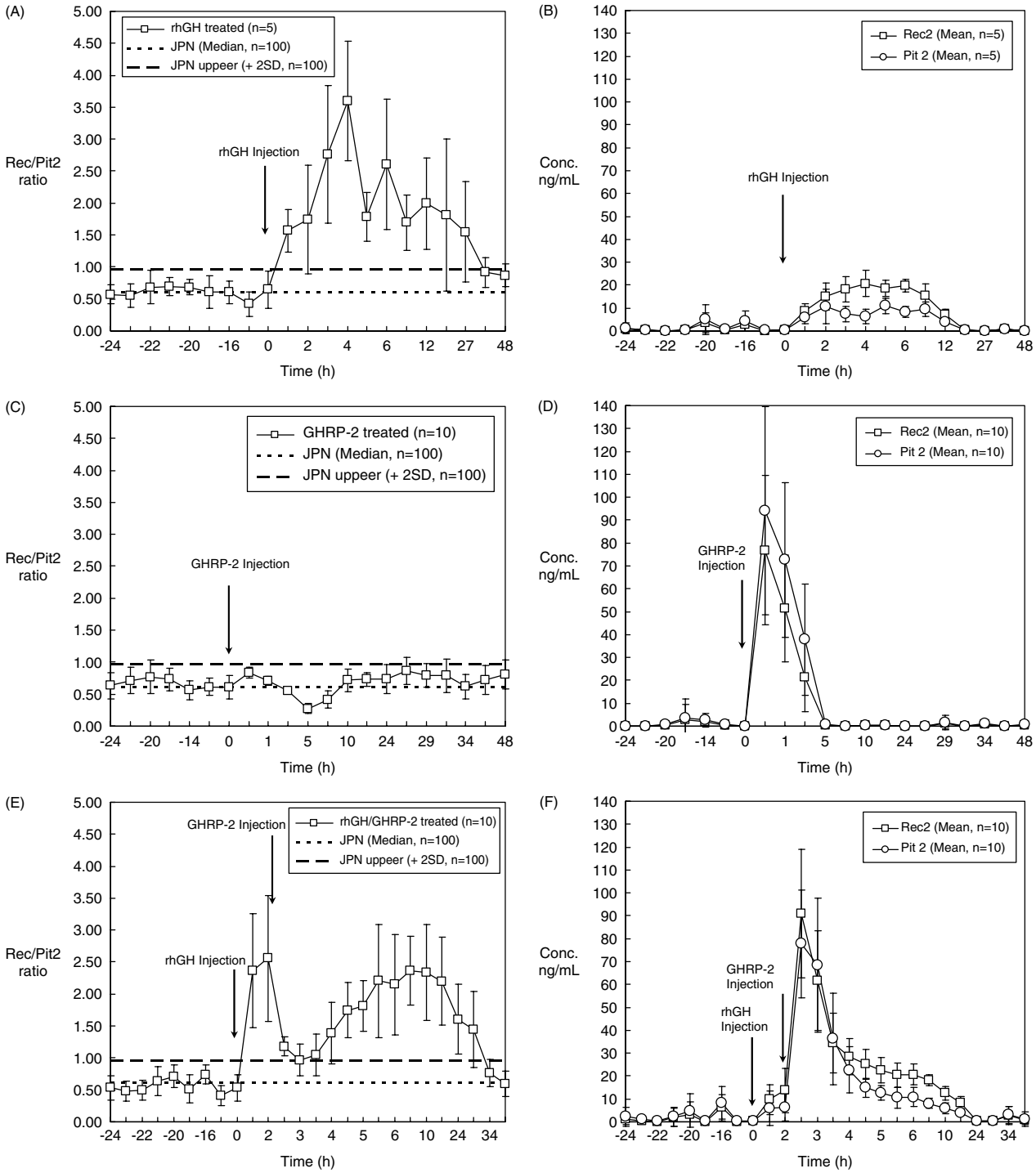

Figure 2. Change in ratios Rec/Pit2(left) and absolute hGH concentrations (right) of Rec2 and Pit2. The whiskers indicate mean ± standatd deviation. JPN means the median of Japanese male populations (n = 100). JPN upper means Japanese male reference upper limit. (A,B: rhGH 0.04 mg/kg S.C. Injection, C,D: GHRP-2 100 μ g I.V. Injection, E,F: Combined administration of rhGH and GHRP-2).

Rec/Pit ratios caused by administration of rhGH, considering the masking effect on detection of rhGH doping by the isoform method, similar to the way epitestosterone has a masking effect on the testosterone to epitestosterone ratio (T/E ratio).[22]

The masking term in this study was only approximately 2 h after intravenous administration of GHRP-2 because of the short half-life (25.2–41.4 min) of GHRP-2;[20] however, a longer masking effect may be expected with a multiple dose, subcutaneous dose, an oral administration or intranasal administration of GHRP-2 in real scenarios among athletes.

Determination of urinary GHRP-2 and its metabolite AA-3

Urinary concentrations of GHRP-2 and its metabolite AA-3 after combined administration of rhGH and GHRP-2 were determined in accordance with our literature.[15]

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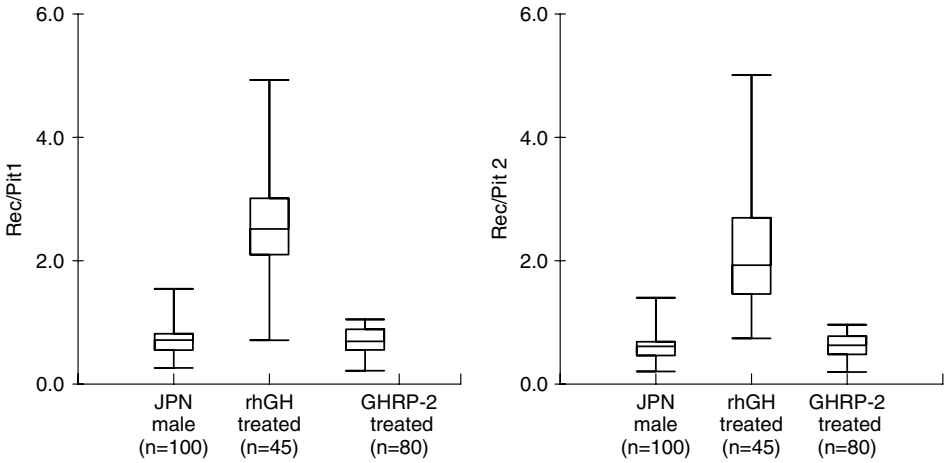

Figure 3. Box plots of Rec/Pit ratios from rhGH treated (5 individuals × 9 data, 1–24 h after administration) and GHRP-2 treated (10 individuals × 8 data, 0.5–24 h after administration) compared with Japanese male subjects (JPN male, n=100). The median value is shown as a line across the box. The upper edge of the box indicates 75th percentile of the data, and the lower edge indicates the 25th percentile of the data. The whiskers indicate the maximum and minimum including the outliers. (left: Kit 1, right: Kit 2) Nonparametric Mann-Whitney U-test : JPN vs rhGH treated ($P < 0.001$), JPN vs GHRP-2 treated ($P > 0.05$).

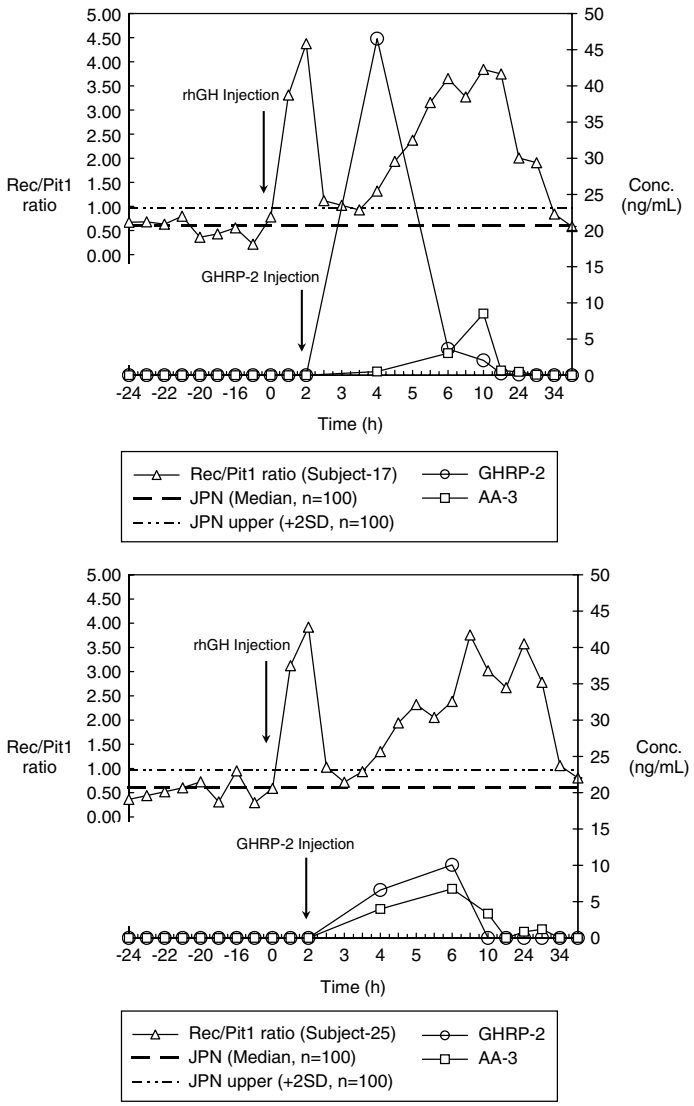

Figure 4. Change in ratios Rec/Pit1 and absolute concentrations of urinary GHRP-2 and metabolite AA-3 after combined administration of rhGH and GHRP-2. (top: Subject-17, bottom: Subject-25).

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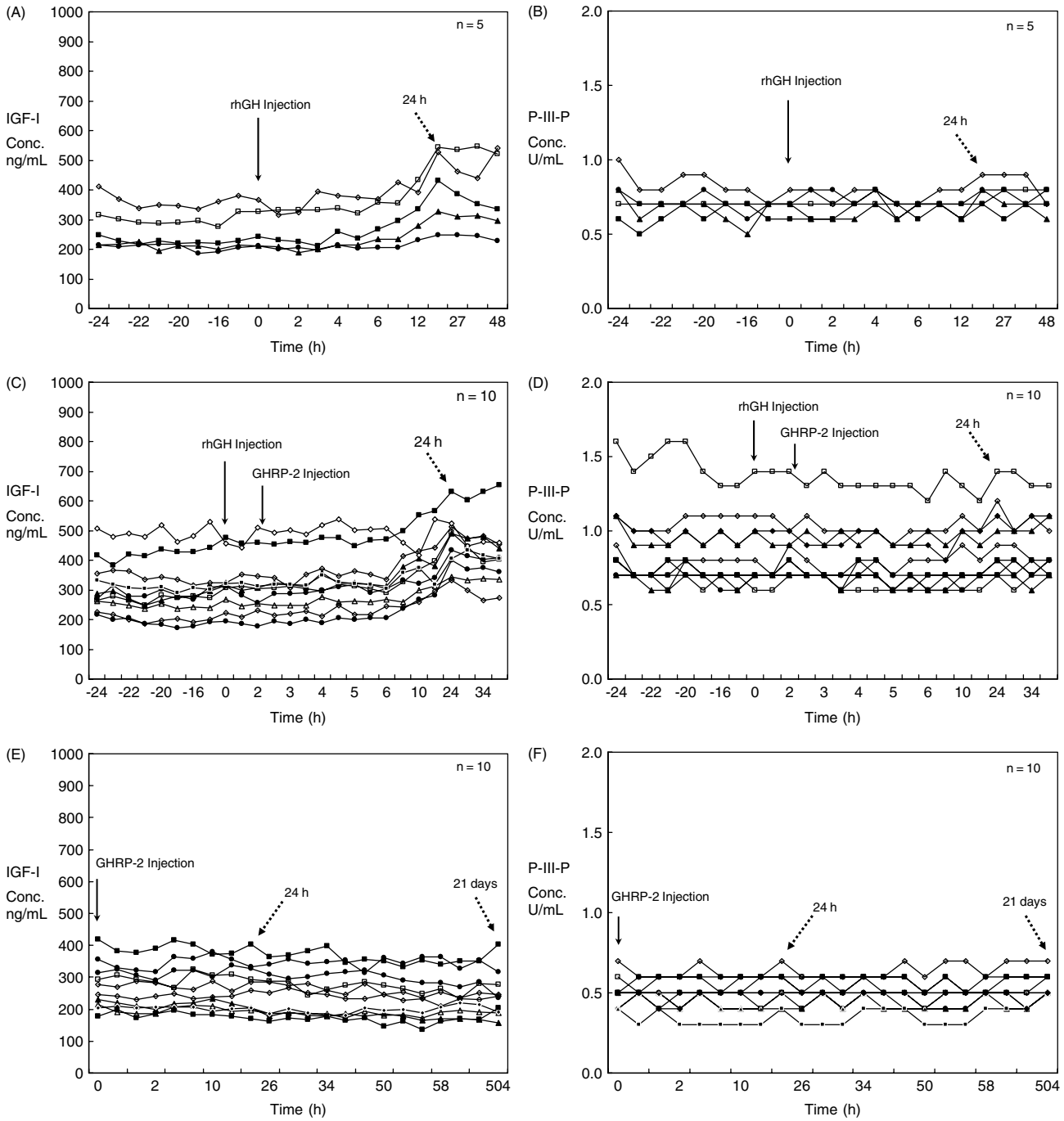

Figure 5. Change in absolute concentrations of IGF-I (left) and P-III-P (right). (A,B: rhGH 0.04 mg/kg S.C. Injection, C,D: Combined administration of rhGH and GHRP-2, E,F: GHRP-2 100 μ g I.V. Injection).

After combined administration of rhGH and GHRP-2, the mean values of urinary concentrations of GHRP-2 were 68.0 ng/mL at 2 h, 8.0 ng/mL at 4 h, 1.9 ng/mL at 8 h and 0.8 ng/mL at 11 h, then eliminated to less than the LLOD. Those of AA-3 were 2.0 ng/mL at 2 h, 5.6 ng/mL at 4 h, 4.6 ng/mL at 8 h, 3.5 ng/mL at 11 h and 0.7 ng/mL at 22 h. The trends in subjects 17 and 25 are shown in Figure 4. GHRP-2 or AA-3 could be successfully detected during the periods of masking of the Rec/Pit ratios. The results showed that the UPLC/MS/MS analysis for GHRP-2 and its metabolite AA-3 also compensates for the disadvantages of the method for detecting rhGH doping.

Serum IGF-I and P-III-P levels

In the GH-dependent marker method, the application of the developed discriminatory formulae to measure serum concentrations of IGF-I and P-III-P allows for identification of rhGH doping for several days after administration of a multiple dose of rhGH.[1] Despite a short period of sample collection and a single low dose (0.04 mg/kg) of rhGH, the maximum concentrations of serum IGF-I in serum samples collected at 24 h after administration of rhGH tended to be higher than those prior to administration (Figure 5A). In addition, that of combined administration of rhGH and GHRP-2

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tended to be higher than that prior to administration (Figure 5C), as well as that of sole administration of rhGH. However, values encountered with single intravenous administration of GHRP-2 did not show an upward tendency from those obtained prior to administration. In particular, spot serum samples collected 21 days after administration of GHRP-2 were also analyzed but the concentrations did not increase in all ten subjects (Figure 5E). It is important to mention here that these studies are of a single administration. It is assumed that intravenous single administration of GHRP-2 led to a transient increase in endogenous GH level; however, the GH releasing effect is very short because of the short half-life of GHRP-2. Therefore, it might not reflect the continuous generation of IGF-I through the GH receptor on the liver compared with subcutaneous administration of rhGH with a persistent residual effect of serum GH. It is also recognized that the rise of P-III-P level is slower than that of IGF-I;[1] therefore, serum P-III-P levels did not change between prior to and after administration (Figures 5B, 5D, and 5F). Because the main purpose of our study was not to investigate the influence of the GH-dependent marker method, long-term changes in IGF-I and P-III-P levels after multiple dosing of GHRP-2 (GHS) should be further investigated.

Conclusions

On the basis of these findings, we concluded that the isoform differential immunoassay is a highly sensitive and effective method for rhGH doping in Japanese subjects; however, it could not detect GHRP-2 doping because GRPP-2 does not alter the isoform ratio. It follows from these arguments that GHRP-2 has a masking effect on the detection of rhGH doping using the isoform method based on a principle similar to that of the isoform differential immunoassay. Moreover, the results showed that the illicit use of GHRP-2 (GHS) leads to false-negative results in rhGH abusers.

Clearly, these arguments point to a need for the development of an analytical method for GHS detection, which will be an effective strategy for doping control.

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