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Effectiveness of GH isoform differential immunoassay for detecting rhGH doping on application of various growth factors

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The analytical method for detecting growth hormone (GH) doping, the so-called GH isoform differential immunoassay, is currently approved by the World Anti-Doping Agency (WADA).

Anti-doping laboratories often face challenges by athletes' lawyers and need to have various types of scientific evidence against the claim that the adverse analytical finding (AAF) result was caused by excess ectopic or abnormal excretion. In this work, a population study of Japanese athletes (255 male and 256 female) and administration studies of recombinant human GH (rhGH) in Japanese females were conducted to confirm the applicability of GH isoform differential immunoassay. The present paper describes the effectiveness of the GH isoform differential immunoassay under abnormal excretion of endogenous GH as determined by administration studies of GH releasing hormone (GHRH(1–44)) and insulin-like growth factor-1 (IGF-1). No false positive findings were found in Japanese athletes. The GH isoform differential immunoassays could detect application of rhGH for approximately 12–24 h. The administration of GHRH(1–44) and IGF-1 as well as ghrelin receptor agonists did not affect the isoform ratio (no false positives). We conclude that the GH isoform differential immunoassay is a highly specific method for detecting rhGH doping. Subject-based profiling (i.e. athlete biological passport) very likely will represent a highly sensitive approach for detecting rhGH doping. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: doping; growth hormone; GH isoform; GHRH, IGF-1; WADA

Introduction

Growth hormone (GH) is an endogenous peptide hormone secreted from the anterior pituitary gland in a pulsatile fashion under the control of growth hormone releasing hormone (GHRH) and somatostatin (GH-inhibiting hormone). GHRH, a hypothalamic peptide hormone composed of 44 amino acids with C-terminal amidation (GHRH(1–44)), stimulates the transcription of GH gene by binding to the GHRH receptor (GHRHR) on somatotroph, followed by increasing cAMP in a cell.[1] Consequently, GH stimulates the secretion of insulin-like growth factor-1 (IGF-1) through the GH receptor on the hepatocyte membrane.[2] IGF-1 is a primary mediator of the growth-promoting effect of GH; it also regulates the secretion of blood GH level owing to the feedback inhibition.[3] There is another group of growth hormone secretagogues (GHS) as represented by endogenous ghrelin. This group does not act on GHRHR directly, however; it activates a G-protein-coupled GHS receptor (GHS-R1a).[4]

Although the varieties of pharmaceutical products that have been created through the accelerating pace of developments in biotechnology contribute to improving healthcare, they also convey a certain risk that some athletes will commit doping offences.

In Japan, there are not only therapeutic products for GH failure, i.e. recombinant human GH (rhGH) and recombinant IGF-1 (Mecasermin), but also diagnostic agents, namely, recombinant GHRH (1–44) and synthetic GHS (i.e. GHRP-2).

As shown in Table 1, the growth factors abused by illicit athletes can be mainly categorized into four groups by the mechanism of GH secretion: GH itself, GHRH analogues, ghrelin receptor agonists GHSs and GH mediators IGF-1 analogues.

In sports, the use of rhGH and these various growth factors is strictly prohibited by the World Anti-Doping Agency (WADA).[5] The analytical method for detecting rhGH doping, the so-called GH isoform differential immunoassay, was implemented for the first time at the Athens Olympic Games in 2004, and the method is currently approved by WADA.[6–8]

The method using serum samples is essentially based on the established principle that the composition of various GH isoforms in circulation remains constant at certain proportions. In contrast, rhGH only comprises the 22 kDa molecular form. Administration of exogenous 22 kDa rhGH suppresses the pituitary GH secretion by negative feedback; then, the elevation of 22 kDa GH relative to the pituitary GH ratio can be utilized to detect doping with rhGH.[6]

The first adverse analytical finding (AAF) for exogenous GH, in a rugby football player, and three further positive cases were reported in 2010.[9,10]

However, anti-doping laboratories often face challenges by athletes' lawyers in the Court of Arbitration in Sports (CAS) and need to have various types of scientific evidence against claims regarding the AAF result.[11]

Hence, the robust application of GH isoform differential immunoassay in doping control necessitates careful evaluation of any influences on the GH isoform composition that is in circulation. It should be noted that these factors have never led to a false positive result. It is also important to investigate the effects of ethnicity in terms of reference-population-based judgments,

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Table 1. List of growth factors abused by athletes

Mechanism	Substance		Other related substance
Exogenous GH	22 kDa rhGH	Somatropin	Genotropin
Release of endogenous GH (GHRH receptor agonist)	GHRH(1–44)	Somatorelin	GHRH(1–29) (Sermorelin), CJC-1295, Tesamorelin
Release of endogenous GH (Ghrelin receptor agonist)	GHRP-2	Pralmorelin	GHRP-6, Tabimorelin, Ibutamoren
Mediator of GH (Negative feedback of GH secretion)	IGF-1	Mecasermin	Long-R^3-IGF-1

and an ethnicity study of Caucasian and African athletes has been carried out.[12] For practical application, excretion studies with a low dose of rhGH in Japanese males and with two rhGH preparations produced in China and Switzerland were attempted.[13,14] Both the results clearly showed that the GH isoform differential immunoassay has high potential for detecting rhGH doping. As for the other growth factors, we demonstrated that administration of GHRP-2 led to increases in concentrations of endogenous GH and cannot only be detected by the GH isoform differential immunoassay but also masks rhGH doping.[13]

However, there have been few reports on the influence of other growth factors. Given this background, a population study of Japanese athletes and administration studies of rhGH in Japanese females were conducted to confirm the applicability of GH isoform differential immunoassay. Moreover, the present paper describes the effectiveness of the GH isoform differential immunoassay under abnormal excretion of endogenous GH as determined by administration studies of GHRH(1–44) and IGF-1.

Experimental

Materials

The immunoassay kit for determination of GH isoforms was purchased from CMZ-Assays GmbH (Berlin, Germany). The radioimmunoassay kit Somatomedin C·II Siemens for determination of serum IGF-1 levels was from Siemens Healthcare Diagnostics K.K. (Tokyo, Japan). Distilled water was produced using a Milli-Q Ultrapure system (Millipore, Bedford, MA, USA). For administration studies, Somatropin BS (22 kDa rhGH, 5 mg), Somazon® (recombinant IGF-1, Mecasermin 10 mg) and GRF Sumitomo (recombinant GHRH(1–44), Somatorelin acetate 100 μg) were purchased from Sandoz (Tokyo, Japan), Astellas Pharma Inc. (Tokyo, Japan) and Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan), respectively. All other reagents were of analytical grade.

Determination of serum GH isoforms

The detection method was a quantitative analysis using commercial immunoassay kits provided by CMZ-Assays GmbH using Berthold Auto Lumat Plus LB953 luminometer equipped with LBIS software ver. 3.3 (Berthold Technologies GmbH, Bad Wildbad, Germany).[7,8,13]

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 capture antibody for rec GH assay preferentially recognizes the monomeric 22 kDa GH, whereas that for pit GH assay preferentially recognizes pituitary-derived GH isoforms with lower preference for 22 kDa GH. The rec GH to pit GH ratios for Kit-1 (Rec/Pit1) and for Kit-2 (Rec/Pit2) were calculated. The serum sample preparation was performed as described in the instruction manual provided by the manufacturer.

The in-house method was fully validated and 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. All quality control samples were measured and evaluated in accordance with WADA guidelines.[8] Samples with CV% <10 were included for calculation.

Determination of serum IGF-1 by immunoradiometric assay

Serum IGF-1 concentration was determined using a commercially available radioimmunoassay kit, Somatomedin C·II Siemens. Radioactivity was measured using an automated gamma counter (ARC-950, Aloka, Tokyo, Japan). The detection method was validated and included in the scope of the ISO15189 accreditation for clinical testing.

Human subjects

The administration study was reviewed and approved by the Ethical Review Board of Mitsubishi Chemical Medience Corporation, with regard to ethical, scientific, and medical validity; the study was conducted on an open-label basis. Written consent was obtained from all participating volunteers. It is well known that the secretion of GH in response to exercise is dependent on body mass index (BMI).[15,16] The volunteers for administration studies refrained from ingesting alcohol, caffeine, grapefruit, any supplements or medicines and did not perform exercise during the studies. Serum samples were collected using BD Vacutainer® SST™-II (Becton Dickinson and Company, Franklin Lakes, NJ, USA) for determination of serum GH. Blood samples were collected and stored in accordance with WADA guidelines.[8] Plain tube Benoject-II (Terumo Co., Tokyo, Japan) was used in blood collection for measurement of serum IGF-1. Serum samples were stored at $-80\,^{\circ}C$ until analysis. 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.

Japanese male athletes

The samples of Japanese university student athletes included 255 males (20.3 ± 1.3 years old, BMI: 23.3 ± 2.1). The sports categories consisted of athletics, football, basketball, baseball, volleyball, and dance sports. The volunteers refrained from ingesting alcohol, any supplements or medicines three days before sample collection. No attempt was made to control diet intake. All samples were collected between 9:00 am and 12:30 pm.

Japanese female athletes

A total of 256 samples were collected from female Japanese university student athletes (19.8 ± 0.9 years old, BMI: 21.8 ± 2.2). The sports categories consisted of athletics, football, basketball, cheerleading, badminton, baseball, volleyball, skiing, lifesaving,

fencing, lacrosse, soft tennis, handball, kendo, golf, gymnastics, and dance sports. The volunteers refrained from ingesting alcohol, any supplements or medicines three days before sample collection. No attempt was made to control diet intake. All samples were collected between 9:00 am and 12:30 pm.

Administration study of rhGH

For female subjects, an injectable solution of rhGH (0.04 mg/kg, Somatropin) was subcutaneously administered at 9:00 am after overnight fasting. Three healthy female volunteers were Japanese (24.0 ± 3.6 years old, BMI: 19.5 ± 1.6). 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 and 24 h. For male subjects, an injectable solution of rhGH (0.04 mg/kg, Somatropin) was subcutaneously administered to five healthy male volunteers (24.6 ± 7.0 years old, BMI: 20.0 ± 1.2) as described in a previous report.[13]

Administration study of GHRH(1–44)

An injectable solution of 100 μg of Somatorelin was intravenously administered at 9:00 am after overnight fasting. Five healthy male volunteers were Japanese (24.8 ± 3.8 years old, BMI: 19.7 ± 1.8). The time courses of serum sample collection were 0 (pre-administration), 0.25, 0.5, 1, 1.5, 4.5, 7, 10, 13, 24, 25.5, 28.5, 31, 34, 37 and 48 h.

Administration study of IGF-1

An injectable solution of Mecasermin (0.05 mg/kg) was subcutaneously administered at 9:00 am after overnight fasting. Five healthy female volunteers were Japanese (24.2 ± 3.0 years old, BMI: 21.9 ± 2.5). 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 and 48 h.

Statistics and data evaluation

The statistical analysis was conducted using EXCEL Statistics 1.01 for Windows (Social Survey Research Information Co., Ltd., Tokyo, Japan). Nonparametric Mann–Whitney U-test and Wilcoxon signed-rank test were used for analyzing the significance of differences. $P < 0.0001$ or $P < 0.05$ was considered significant.

Japanese upper limits (JPN Ref) of Rec/Pit1 and Rec/Pit2 ratios were set on the basis of 'mean + standard deviation (SD_{n-1}) × 3'. The gender-specific decision limits set by WADA (WADA DL) of the Rec/Pit ratios for detecting rhGH doping were as follows:[8] Rec/Pit1: males (1.81), females (1.46); Rec/Pit2: males (1.68), females (1.55).

Each subject-based limit was defined as 'mean of basal values + SD_{n-1} × 3'. The results from each kit were compared with the corresponding JPN Ref, WADA DL and subject-based limits.

Results and discussion

GH isoform profiles in Japanese athletes

The distribution of GH concentrations and Rec/Pit ratios in Japanese athletes were investigated. After excluding the samples below the LOQ of 0.05 ng/ml, the remaining serum samples were from 211 males for Kit-1 and from 214 males for Kit-2, which were used in statistical analysis. For female athletes, all 256 samples were used in statistical analysis. The statistical data are summarized in Table 2 and Figure 1. The concentrations of rec GH and

Table 2. Statistical analysis of GH concentrations and Rec/Pit ratios in Japanese athletes

	GH (ng/ml)		Rec/Pit1 ratio	GH (ng/ml)		Rec/Pit2 ratio
	Rec1	Pit1		Rec2	Pit2	
Japanese healthy female athletes						
N	256	256	256	256	256	256
Mean	5.85	8.83	0.65	5.95	7.84	0.74
Maximum	28.34	48.37	2.19	29.02	37.66	1.54
Minimum	0.07	0.11	0.22	0.06	0.11	0.15
Median	4.36	6.79	0.65	4.62	6.13	0.74
75th percentile	8.68	12.72	0.75	8.98	11.15	0.87
25th percentile	1.58	2.67	0.53	1.69	2.27	0.62
95% Confidence Interval	5.19-6.51	7.84-9.82	0.62-0.67	5.29-6.62	6.96-8.72	0.72-0.77
SD_{n-1}	5.39	8.02	0.21	5.42	7.12	0.20
Japanese reference upper limit (JPN Ref)			1.27			1.35
WADA Descision Limit			1.46			1.55
Japanese healthy male athletes						
N	211	211	211	214	214	214
Mean	1.50	2.10	0.60	1.51	1.90	0.69
Maximum	33.42	45.36	1.38	29.90	39.57	1.31
Minimum	0.05	0.07	0.14	0.05	0.05	0.13
Median	0.19	0.38	0.58	0.19	0.34	0.67
75th percentile	0.71	1.11	0.76	0.79	1.02	0.89
25th percentile	0.09	0.19	0.40	0.10	0.17	0.50
95% Confidence Interval	0.95-2.06	1.37-2.83	0.56-0.63	0.98-2.05	1.24-2.55	0.65-0.72
SD_{n-1}	4.12	5.41	0.24	3.98	4.90	0.26
Japanese reference upper limit (JPN Ref)			1.33			1.45
WADA Descision Limit			1.81			1.68

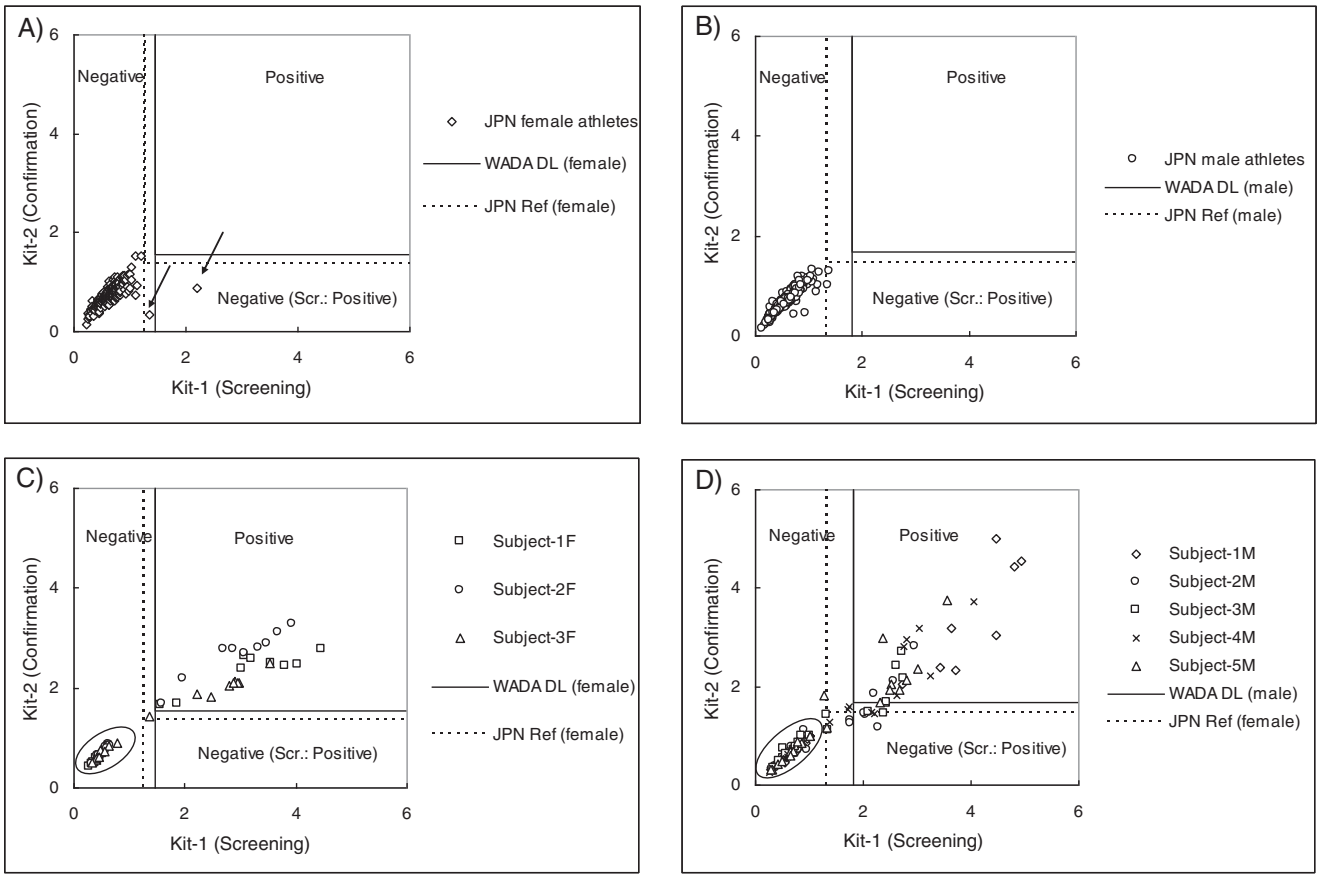

Figure 1. Correlation plots of Rec/Pit1 (Kit-1) and Rec/Pit2 (Kit-2). JPN Ref means Japanese reference upper limit. The data from pre-administration and post-administration (24 h for subjects-1 M and 2 M; 34 h for subjects-1 M, 2 M and 3 M; 48 h for subjects-2 M, 3 M and 4 M) are plotted in the circle. A) Japanese female athletes. B) Japanese male athletes. C) rhGH 0.04 mg/kg subcutaneous injection in female. D) rhGH 0.04 mg/kg subcutaneous injection in male.[13]

pit GH were significantly higher in females than in males ($P < 0.0001$). The mean ratio of the Rec/Pit was significantly higher in females than in males ($P < 0.0001$), whereas the WADA DL in males are higher than in females.[8]

Kohler *et al.* showed that the mean Rec/Pit1 ratios in Caucasians were 0.49 for males and 0.53 for females, respectively.[12] Also those in Africans were 0.41 (males) and 0.42 (females), respectively. These gender differences were not significant, but it seems that the ratios in females were slightly higher than in males. Our results indicated that the mean Rec/Pit1 ratios of males and females were 0.60 and 0.65, respectively. Based on these interesting findings, the current WADA DL may be adapted after more international population studies. On the basis of the mean + $SD_{n-1} \times 3$, the JPN Ref of Rec/Pit1 ratios were set to 1.33 (male) and 1.27 (female), and those of Rec/Pit2 were 1.45 (male) and 1.35 (female).

As shown in Figure 1B, the Rec/Pit1 ratios (1.38 and 1.35) in two males were outliers from the JPN Ref; however, those of the Rec/Pit2 ratios (1.29 and 1.00) were within the normal range. All the Rec/Pit ratios were less than the WADA DL and no false positives were found.

As indicated by arrows in Figure 1A, the Rec/Pit1 ratios (2.19 and 1.35) in two female subjects were outliers from the JPN Ref. Those of the Rec/Pit2 ratios (0.87 and 0.35) were within the normal range. It is noteworthy that one female individual was above the WADA DL of 1.46 in the screening (Kit-1). Although the concentrations of Rec1 and Rec2 were 5.50 and 5.38 ng/ml, those of Pit1 and Pit2 were 2.51 and 6.20 ng/ml. In other subject, although the concentrations of Rec1 and Rec2 were 0.55 and 0.51 ng/ml, those of Pit1 and Pit2 were 0.40 and 1.44 ng/ml, respectively. Thus, it appears that the concentrations of Pit1 were relatively low, compared with those of Pit2. It is recognized that the placental GH variant (hGH-V) showed cross reactivity from 14% to 28% with four antibodies.[7] Furthermore, Kaliszewski *et al.* showed the influence of the analytical results in serum samples collected from pregnant women (gestational week 7 to 37).[17] However, all female subjects were not pregnant. Although this finding cannot presently be explained, a similar observation for Kit-1 was previously reported by Jing *et al.*[14] Nevertheless, the results of confirmation analysis using Kit-2 clearly were negative and no false positive findings were found.

GH isoform profiles after administration of growth factors

rhGH

The response to a low dose of rhGH (0.04 mg/kg) in three Japanese female subjects was investigated. The trends in concentrations of Rec and Pit and the ratios for Rec/Pit after subcutaneous injection of 0.04 mg/kg rhGH are shown in Figures 2A, 2B, 3A, and 3B.

The mean values of baseline concentration (−24 h to 0 h prior to administration) of Rec1 and Rec2 were 1.38 ng/ml (0.32–2.73 ng/ml) and 1.49 ng/ml (0.35–2.90 ng/ml), respectively. The concentrations of Rec1 and Rec2 increased to 29.93 ng/ml (12.52–55.50 ng/ml) and 31.35 ng/ml (14.90–54.95 ng/ml) at 8 h after administration, respectively, and then decreased to the normal values 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

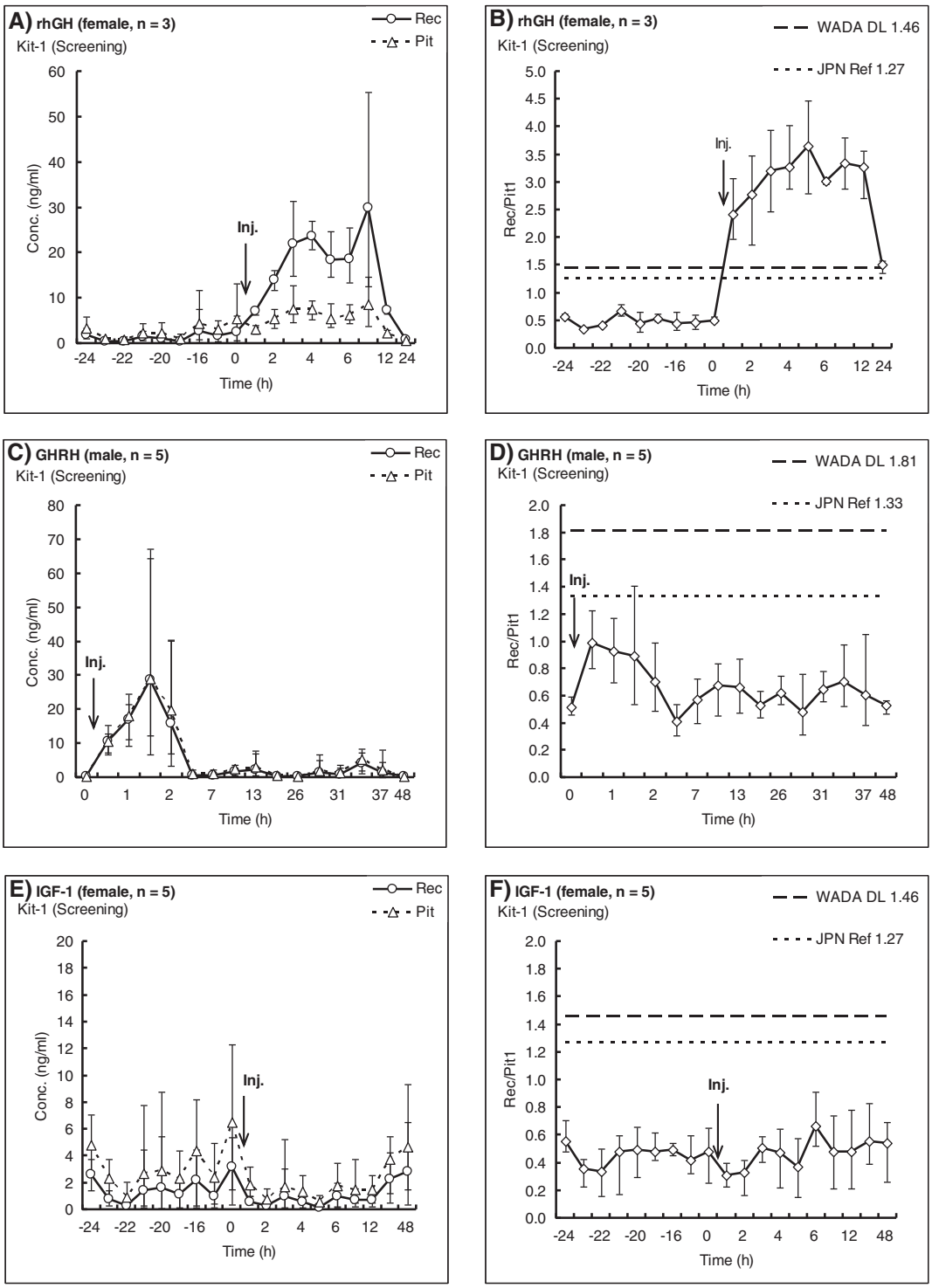

Figure 2. Change in ratio of Rec/Pit1 (right) and absolute GH concentrations (left) of Rec1 and Pit1. The whisker plots indicate maximums and minimums of measured values. JPN Ref means Japanese reference upper limit. A), B): rhGH 0.04 mg/kg subcutaneous injection in female. C), D): GHRH(1–44) 100 μg intravenous injection in male. E), F): IGF-1 0.05 mg/kg subcutaneous injection in female.

0.66 (0.49–0.85) and 0.61 (0.27–1.00), respectively. The Rec/Pit1 and Rec/Pit2 ratios increased to 3.65 (2.80–4.46) and 2.65 (2.06–3.11) at 5 h after administration, respectively. The maximum ratios of Rec/Pit1 and Rec/Pit2 were 4.46 and 3.29, respectively.

As expected, the ratios of Rec/Pit1 and Rec/Pit2 at post-administration were significantly higher than those prior to administration ($P < 0.0001$). The elevated Rec/Pit ratios persisted during 12 h after the 0.04 mg/kg rhGH administration (Figures 2B and 3B). Thus, the isoform differential immunoassay is an effective method for detecting rhGH doping in Japanese female subjects. In addition to the results of females in this study, the results of administration study of rhGH in males, which were reported previously,[13] were also re-evaluated (Figures 1C and 1D). When the JPN Ref threshold level was applied, the sensitivity increased and

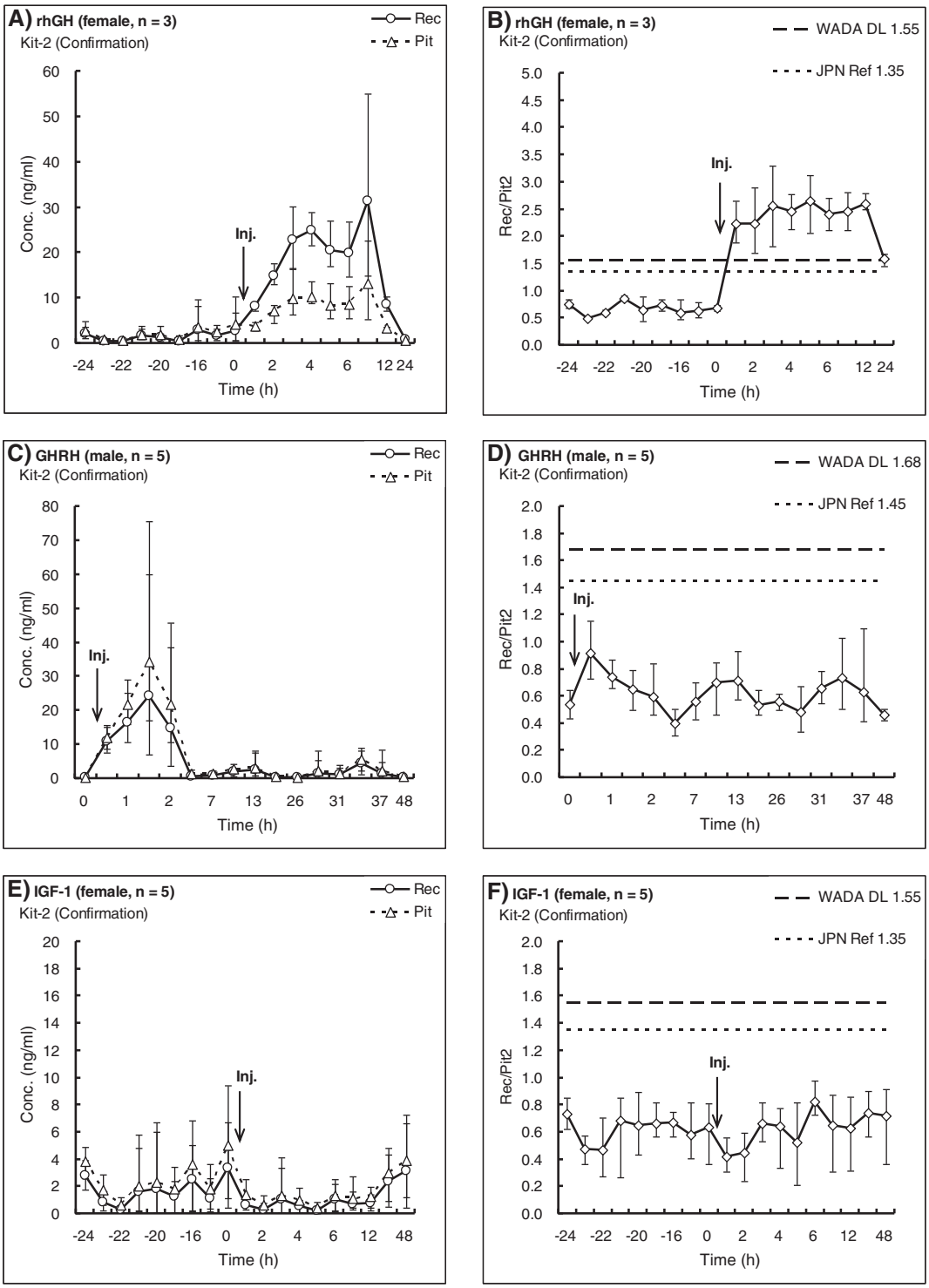

Figure 3. Change in ratio of Rec/Pit2 (right) and absolute GH concentrations (left) of Rec2 and Pit2. The whisker plots indicate maximums and minimums of measured values. JPN Ref means Japanese reference upper limit. A), B): rhGH 0.04 mg/kg subcutaneous injection in female. C), D): GHRH(1–44) 100 μg intravenous injection in male. E), F): IGF-1 0.05 mg/kg subcutaneous injection in female.

false negatives were somewhat eliminated (Table 3). As a tentative estimate, each subject-based reference limit defined as 'mean of basal values + $3SD_{n-1}$' was applied to the final determination of an AAF. The result showed that the subject-based isoform ratio profiling (i.e. athlete biological passport) dramatically improved the detection rates of rhGH doping.

GHRH(1–44)

The trends in concentrations of rec GH and pit GH and the ratios for Rec/Pit after intravenous injection of 100 μg of GHRH(1–44) are shown in Figures 2C, 2D, 3C and 3D. The mean values of pre-administration of Rec1, Rec2, Pit1 and Pit2 were 0.18 ng/mL

Table 3. Rate of adverse analytical finding (AAF) samples fulfilling the criteria based on the WADA decision limit (DL), Japanese upper limit (JPN Ref) and subject-based limit after subcutaneous administration of 0.04 mg/kg rhGH

		Rate (%) of AAF per sample ( ): *per individual*						
		Time course						
		Pre	2 h	4 h	6 h	12 h	24 h	48 h
Female (n = 3)								
WADA DL	Screening (Kit-1)	0.0%	100.0%	100.0%	100.0%	100.0%	66.7%	
	>1.46	*(0/3)*	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	*(2/3)*	
	Confirmation (Kit-2)	0.0%	100.0%	100.0%	100.0%	100.0%	66.7%	
	>1.55	*(0/3)*	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	*(2/3)*	
	Final Decision	0.0%	100.0%	100.0%	100.0%	100.0%	66.7%	
		0/3)	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	*(2/3)*	
JPN Ref	Screening (Kit-1)	0.0%	100.0%	100.0%	100.0%	100.0%	100.0%	
	>1.27	*(0/3)*	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	
	Confirmation (Kit-2)	0.0%	100.0%	100.0%	100.0%	100.0%	100.0%	
	>1.35	*(0/3)*	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	
	Final Decision	0.0%	100.0%	100.0%	100.0%	100.0%	100.0%	
		(0/3)	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	
subject-based limit	Screening (Kit-1)	0.0%	100.0%	100.0%	100.0%	100.0%	100.0%	
		(0/3)	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	
	Confirmation (Kit-2)	0.0%	100.0%	100.0%	100.0%	100.0%	100.0%	
		(0/3)	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	
	Final Decision	0.0%	100.0%	100.0%	100.0%	100.0%	100.0%	
		(0/3)	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	*(3/3)*	
Male (n = 5)								
WADA DL	Screening (Kit-1)	0.0%	60.0%	100.0%	100.0%	80.0%	40.0%	6.7%
	>1.81	*(0/5)*	*(4/5)*	*(5/5)*	*(5/5)*	*(4/5)*	*(2/5)*	*(1/5)*
	Confirmation (Kit-2)	0.0%	20.0%	100.0%	80.0%	50.0%	40.0%	13.3%
	>1.68	*(0/5)*	*(1/5)*	*(5/5)*	*(5/5)*	*(3/5)*	*(2/5)*	*(2/5)*
	Final Decision	0.0%	20.0%	100.0%	80.0%	50.0%	40.0%	6.7%
		(0/5)	*(1/5)*	*(5/5)*	*(5/5)*	*(3/5)*	*(2/5)*	*(1/5)*
JPN Ref	Screening (Kit-1)	0.0%	90.0%	100.0%	100.0%	100.0%	40.0%	13.3%
	>1.33	*(0/5)*	*(5/5)*	*(5/5)*	*(5/5)*	*(5/5)*	*(2/5)*	*(2/5)*
	Confirmation (Kit-2)	0.0%	60.0%	100.0%	90.0%	80.0%	40.0%	13.3%
	>1.45	*(0/5)*	*(4/5)*	*(5/5)*	*(5/5)*	*(4/5)*	*(2/5)*	*(2/5)*
	Final Decision	0.0%	60.0%	100.0%	90.0%	80.0%	40.0%	6.7%
		(0/5)	*(4/5)*	*(5/5)*	*(5/5)*	*(4/5)*	*(2/5)*	*(1/5)*
subject-based limit	Screening (Kit-1)	0.0%	100.0%	100.0%	100.0%	100.0%	60.0%	20.0%
		(0/5)	*(5/5)*	*(5/5)*	*(5/5)*	*(5/5)*	*(3/5)*	*(2/5)*
	Confirmation (Kit-2)	0.0%	100.0%	100.0%	100.0%	100.0%	60.0%	26.7%
		0/5)	*(5/5)*	*(5/5)*	*(5/5)*	*(5/5)*	*(3/5)*	*(3/5)*
	Final Decision	0.0%	100.0%	100.0%	100.0%	100.0%	60.0%	20.0%
		(0/5)	*(5/5)*	*(5/5)*	*(5/5)*	*(5/5)*	*(3/5)*	*(2/5)*

The data for males are from a previous report.[13] The subject-based limit is defined as 'mean of basal values + $3SD_{n-1}$'.

(0.07–0.35 ng/mL), 0.20 ng/ml (0.08–0.38 ng/ml), 0.36 ng/ml (0.20–0.77 ng/ml) and 0.39 ng/ml (0.16–0.89 ng/ml), respectively.

The elevated concentrations of rec GH and pit GH peaked at 1 h for all five subjects after administration. The mean concentrations of Rec1, Pit1, Rec2 and Pit2 at 1 h after administration were 28.8 ng/ml (6.6–67.3 ng/ml), 29.1 ng/ml (12.3–64.4 ng/ml), 24.2 ng/ml (6.9–59.9 ng/ml) and 34.3 ng/ml (13.8–75.6 ng/ml), respectively. The mean Rec/Pit ratios of Kit-1 and Kit-2 prior to administration were 0.52 (0.46–0.59) and 0.54 (0.43–0.64), respectively. The Rec/Pit1 and Rec/Pit2 ratios were 0.89 (0.53–1.41) and 0.65 (0.50–0.79) at 1 h after administration, respectively. After the administration of GHRH(1–44), which has strong endogenous GH releasing activity, no significant increases in ratios of Rec/Pit were observed ($P > 0.05$) and the ratios did not reach the WADA positivity criterion as shown in Figures 2D and 3D.

The trend agreed with the result of our previous study that the Rec/Pit ratios of ten subjects showed persistent basal levels of the ratio after the intravenous administration of GHRP-2, representing ghrelin receptor agonist, owing to increases in the concentrations of both rec GH and pit GH.[13]

Interestingly, there is most likely a slight decreasing tendency of the Rec/Pit ratios in the elimination phase at 4.5 h after administration (Figures 2D and 3D), in agreement with our GHRP-2 study.[13]

This is probably due to acute endogenous GH secretion by GHRH, with subsequent transient depletion of endogenous GH, which 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 the investigated period after the administration of GHRH(1–44) ($P > 0.05$).

IGF-1

Serum IGF-1 levels were measured to investigate the effect of the administration of recombinant IGF-1. The mean concentration of serum IGF-1 significantly increased to 614.6 ± 88.3 ng/ml at 5 h after administration of IGF-1 from the basal level of 257.7 ± 14.3 ng/ml ($P < 0.05$), and then returned to the normal range at 48 h.

In additions, the maximum concentrations of IGF-1 in serum samples collected after administration of recombinant IGF-1 was above the in-house reference range for clinical testing (healthy adult females: 121–436 ng/ml). Several reports have suggested that endogenous GH secretion is under the negative feedback control of IGF-1.[3]

It is important to mention here that this study is of a single administration of IGF-1 and the sample collection was performed as spot sampling. Therefore, the continuous suppression of GH might not be clearly determined (Figures 2E and 3E). The mean Rec/Pit ratios of Kit-1 and Kit-2 prior to administration were 0.48 (0.26–0.65) and 0.64 (0.36–0.81), respectively. The Rec/Pit1 and Rec/Pit2 ratios were 0.37 (0.15–0.57) and 0.53 (0.21–0.82) at 5 h after administration, respectively. Thus, no significant changes in ratios of Rec/Pit were observed during, prior to and after administration of IGF-1 ($P > 0.05$), whereas serum IGF-1 level significantly increased (Figures 2F and 3F). The results also suggested that the abnormal serum IGF-1 level in patients with acromegaly might not influence the GH isoform differential immunoassay.

Conclusions

It is concluded that the isoform differential immunoassay is a highly specific method for rhGH doping; however, the time window of detection is narrow (12–24 h). Unexpected false positive results could be eliminated using two kits with two different antibodies. The subject-based profiling (i.e. athlete biological passport (ABP)) very likely will represent a highly sensitive approach for detecting rhGH doping because it depends on an individual reference value. However, more longitudinal studies of the GH isoform ratio are required. Moreover, there are some tasks to be solved for the application of the ABP. The testing authorities have to bear the high shipping cost as well as cost of test. For laboratories, it is important to proceed with the inter-laboratory standardization of more high-precision assay.

The administration of GHRH(1–44) and IGF-1 as well as GHRP-2 did not affect the isoform ratio (no false positives), whereas rhGH administration could be successfully detected. Owing to the fact that administration of GHRH(1–44) led to the production of endogenous GH, it is suggested to mask the GH isoform ratio after rhGH use. Thus, these growth factors can be misused as masking agents or as doping substances themselves as well.

There is another method to detect rhGH doping based on the measurement of GH-dependent markers, IGF-1 and type-III pro-collagen (P-III-P).[18] Therefore, the influence of the administration of growth factors on the GH-dependent marker method should be further investigated.

In contrast, the GH isoform differential immunoassay could not detect growth factors except for rhGH itself. For doping control purposes, several detection methods for the growth factors (i.e. somatostatin inhibitors,[19] ghrelin receptor agonists[20–22]) have been developed. Moreover, several unapproved growth factors are available on the black market, and ghrelin receptor agonists (e.g. GHRP-2, GHRP-6),[23–26] GHRH analogues (e.g. CJC-1295, sermorelin),[26,27] IGF-1 analogue (e.g. long-R^3-IGF-1)[25] and fibroblast growth factor 1 (FGF-1) analogue[28] have been identified in the products, which is a deep concern for the abuse of growth factors by athletes. Obviously, these points indicate a need for the development of an analytical method for detecting growth factors.

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