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The effects of a freeze-thaw cycle and pre-analytical storage temperature on the stability of insulin-like growth factor-I and pro-collagen type III N-terminal propeptide concentrations: Implications for the detection of growth hormone misuse in athletes

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A method based on two serum biomarkers – insulin-like growth factor-I (IGF-I) and pro-collagen type III N-terminal propeptide (P-III-NP) – has been devised to detect growth hormone (GH) misuse. The aims of this study were to determine the stability of IGF-I and P-III-NP concentrations in serum stored at −20°C and to establish the effects of one freeze-thaw cycle.

Blood was collected from 20 healthy volunteers. Serum aliquots were analyzed after storage for one day at 4°C and one day, one week, five weeks, and three months at −20°C. IGF-I and P-III-NP results were combined to calculate a GH-2000 discriminant function score for each volunteer. Inter-assay precision was determined by analysing one quality control sample at each time-point.

A single freeze-thaw cycle, storage of serum at 4°C for one day and at −20°C for up to three months had no significant effect on IGF-I or P-III-NP concentration. Intra-sample variability for IGF-I was 6.8% (Immunotech assay) and 12.9% (DSL assay). Intra-sample variability for P-III-NP was 10.9% (Cisbio assay) and 13.7% (Orion assay). When IGF-I and P-III-NP results were combined, intra-sample variability of the GH-2000 score expressed as a standard deviation varied between 0.31 and 0.50 depending on the assay combination used. Variability in IGF-I and P-III-NP results of stored samples is largely determined by the characteristics of the assays. A single freeze-thaw cycle, storage of serum at 4°C for one day or at −20°C for up to 3 months does not result in a significant change in GH-2000 score. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: GH-abuse; IGF-I; P-III-NP; pre-analytical factors; doping

Introduction

Growth hormone (GH) is misused by athletes for its anabolic and lipolytic properties, and because athletes believe it assists their recovery from injury.[1] The GH-2000 research team proposed a method to detect GH misuse based on the measurement of the GH-sensitive serum biomarkers, insulin-like growth factor-I (IGF-I) and pro-collagen type III N-terminal propeptide (P-III-NP).[2] The stability of the concentrations of these GH-sensitive markers over time and under different storage conditions must be established before they can be used as part of an anti-doping test. The variability of IGF-I and P-III-NP concentrations in serum stored at −80°C and during transport at 4°C has already been established.[3] Anti-doping laboratories may not, however, have access to −80°C storage facilities. In addition, during major sporting events, blood samples are taken at sites where access to chilled storage or centrifugation facilities may not be available; blood samples may also be analyzed immediately without prior freezing. It is imperative that these sources of pre-analytical variability are thoroughly investigated to ensure the reliability of assay results.

The aims of this study were to investigate the apparent stability of IGF-I and P-III-NP concentrations in serum stored at −20°C and to establish the effects of a single freeze-thaw cycle on immunoassay results. The World Anti-Doping Agency (WADA) rules currently state that any analyte measured by immunoassays should be measured by two separate assays recognizing different epitopes[4] and

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therefore both IGF-I and P-III-NP were measured by two different assays in this study.

Methods

Subjects

Twenty healthy volunteers (12 men, 8 women; mean age 27.8 ± 0.8 years, range 22–34 years) were recruited to the study through personal contacts at UK Anti-Doping, the national body responsible for the implementation and management of the UK's anti-doping policy. Exclusion criteria included previous history of endocrine disorders and previous use of performance-enhancing drugs. Demographic data on gender, age, ethnic origin, physical activity, diet, injuries, medications, menstrual history, height (self-reported), and weight (self-reported) were recorded. The study received approval from the Southampton and South West Hampshire Research Ethics Committee and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Sample collection and storage

Blood samples were collected according to WADA guidelines.[5] Twenty ml of whole blood was collected from the antecubital fossa of each subject into 5 ml SSTII *Advance*™ Vacutainers (Becton Dickinson, Oxford, UK) and allowed to clot at room temperature for 15 min. All samples were coded and anonymized before analysis at the Drug Control Centre, King's College London. Samples were centrifuged for 15 min at 1300 *g* and all samples were centrifuged within 4 h of collection. After centrifugation, the serum was divided into 1 ml aliquots: 1 aliquot from 10 volunteers was analyzed immediately. There was insufficient time to analyze samples from all 20 volunteers on the day of sample collection but if these assays were used in an anti-doping context, it would be possible to measure larger numbers of samples on the day of collection because of greater capacity during major games. The remaining aliquots and those from the other 10 volunteers were stored overnight at 4°C and then frozen at −20°C. Aliquots from all 20 volunteers were analyzed after storage for 1 day at 4°C, 1 day at −20°C, 1 week at −20°C, 5 weeks at −20°C and 3 months at −20°C.

Analysis of GH-dependent markers (IGF-I and P-III-NP)

All serum samples were analyzed in duplicate. The 10 'fresh' samples assayed immediately after centrifugation were analyzed using the DSL 10–5600 IGF-I ELISA (Diagnostics Systems Laboratories Inc., Webster, TX, USA) and the UniQ™ P-III-NP RIA (Orion Diagnostica, Espoo, Finland). Samples from all remaining time-points were analyzed using the DSL 10–5600 IGF-I ELISA, the Orion UniQ™ P-III-NP RIA, the Immunotech A15729 IGF-I IRMA (Immunotech SAS, Marseille, France) and the RIA-gnost P-III-NP from Cisbio (Gif-sur-Yvette, France). The concentration of the highest calibrant provided by the manufacturer was used to define the reportable range of each assay.

The DSL 10–5600 IGF-I ELISA is a manual, enzymatically amplified, one-step sandwich immunoassay. Acid-ethanol extraction was used to separate IGF-I from its binding proteins. Eight replicates of two quality control (QC) samples were analyzed in our laboratory to determine intra-assay precision. The QC samples were provided by the manufacturer and consisted of IGF-I lyophilized in bovine serum albumin with a non-mercury preservative. Intra-assay coefficient of variation (CV) was 5.2% at 125 μg/l and 4.3% at 225 μg/l (n = 8 replicate samples). Inter-assay CV measured in our laboratory (n = 6 independent assays) was 10.7% at a concentration of 225 μg/l. The highest calibrant concentration was 600 μg/l.

The Immunotech A15729 IGF-I IRMA is a solid-phase, immunoradiometric assay (IRMA) using two monoclonal antibodies prepared against two different immunoreactive sites of the IGF-I molecule. The first antibody is coated on a solid phase and the second antibody is radiolabelled with ^{125}I. IGF-I is separated from IGF binding proteins (IGFBPs) by acidification. Excess IGF-II is added to prevent further interference with the assay from IGFBPs. Eight replicates of two QC samples were analyzed in our laboratory to determine intra-assay precision. The QC samples were provided by the manufacturer and consisted of IGF-I lyophilized in bovine serum albumin. Intra-assay CV was 1.6 and 2.2% at concentrations of 138 and 455 μg/l, respectively.[6] Inter-assay CV (n = 5 independent assays) was 8.8% at a concentration of 373 μg/l. The highest calibrant concentration was 1600 μg/l. Both IGF-I assays were calibrated against the World Health Organisation International Standard for IGF-I, 87/518.

The Orion UniQ™ P-III-NP RIA is a competitive radioimmunoassay (RIA) based on the formation of a complex between solid-phase anti-P-III-NP polyclonal rabbit antibodies and P-III-NP in the serum samples in competition with ^{125}I-labelled P-III-NP. The QC samples were provided by the manufacturer and consisted of lyophilized human serum. Intra-assay CV measured in our laboratory (n = 8 replicate samples) was 4.1 and 2.4% at a concentration of 4.27 and 56.1 μg/l, respectively.[6] Inter-assay CV (n = 6 independent assays) was 5.0% at a concentration of 6.61 μg/l. The highest calibrant concentration was 50 μg/l.

The RIA-gnost P-III-NP from Cisbio is a two-stage sandwich RIA based on the formation of a complex between solid-phase monoclonal anti-P-III-NP antibodies, P-III-NP in the serum samples, and ^{125}I-labelled anti-P-III-NP monoclonal antibodies. The QC samples were provided by the manufacturer and consisted of lyophilized human serum. Intra-assay CV measured in our laboratory (n = 8 replicate samples) at a concentration of 0.12 and 3.46 U/ml was 10.8 and 18.2%, respectively.[6] Inter-assay CV was 11.7% at a concentration of 2.77 U/ml (n = 5 independent assays). The highest calibrant concentration was 3.9 U/ml.

Calculation of GH-2000 discriminant function scores

The previously published GH-2000 discriminant function formulae[2] for detecting misuse with GH are shown below where log is the natural logarithm:

$$\text{Male score} = -6.586 + 2.905 * \log(\text{P-III-NP}) + 2.100 * \log(\text{IGF-I}) - 101.737/\text{age} \quad (1)$$

$$\text{Female score} = -8.459 + 2.454 * \log(\text{P-III-NP}) + 2.195 * \log(\text{IGF-I}) - 73.666/\text{age} \quad (2)$$

These discriminant formulae were derived from amateur athletes participating in a recombinant human GH administration study. They were calibrated (mean 0, SD 1) against the sample of elite athletes in the GH-2000 study.[2,7] If a cut-off point equivalent to 3.72 SDs above the mean were used, the false-positive rate would be approximately 1 in 10 000 tests, assuming a Normal distribution of scores in elite athletes.

For each formula, IGF-I represents the IGF-I concentration measured by the Nichols Institute Diagnostics IGF-I RIA (no longer available) and P-III-NP represents the P-III-NP concentration measured using the Cisbio RIA-gnost P-III-NP assay. To calculate equivalent discriminant function scores for the healthy volunteers in the current study, it was necessary to convert the results from the current assays into equivalent results on the GH-2000 scales using the methods that have been published previously.[8]

Statistical analysis

Intra-sample variability describes the variability in IGF-I, P-III-NP and GH-2000 scores from aliquots exposed to different storage conditions. This was estimated using analysis of variance. Samples at each time-point were assayed in a single batch on that day. Inter-assay (i.e. between assay) variability describes the variability in QC sample results measured on the same days as the volunteer samples. Further assessments of inter-assay variability have been performed in our laboratory, as previously reported.[6] Variability in analyte values is expressed as the Coefficient of Variation (CV, %), while variability in GH-2000 scores is expressed as standard deviations. Data were analyzed using SAS version 8 (SAS Institute Inc., Cary, NC, USA).

Results

IGF-I concentration

Figure 1 shows the individual changes in IGF-I concentration with time and storage conditions as measured by the DSL ELISA and Immunotech A15729 IRMA. A single freeze-thaw cycle, storage of serum at 4°C for one day and at −20°C for up to three months had no significant effect on IGF-I results. The estimated intra-sample variability for IGF-I concentration along with corresponding inter-assay variability is shown in Table 1.

P-III-NP concentration

A single freeze-thaw cycle, storage of serum at 4°C for one day and at −20°C for up to three months had no significant effect on P-III-NP results (Figure 2). The estimated intra-sample variability for P-III-NP concentration along with corresponding inter-assay variability is shown in Table 1.

GH-2000 discriminant function scores

GH-2000 discriminant function scores were calculated using combinations of IGF-I and P-III-NP assay results (Figure 3 and Table 2). The estimated intra-sample variability (expressed as standard deviations) for GH-2000 score was 0.50 (Orion P-III-NP

Table 1. The intra-sample variability (Coefficient of Variation, CV) for IGF-I and P-III-NP results and corresponding inter-assay CV. Inter-assay CV was estimated from quality control results

	Intra-sample CV	Inter-assay CV
IGF-I Assay		
DSL 10–5600 ELISA	12.9 %	10.7%
Immunotech A15729 IRMA	6.8 %	8.8%
P-III-NP Assay		
Orion UniQ™ RIA	13.7%	5.0%
Cisbio RIA-gnost P-III-NP	10.9%	11.7%

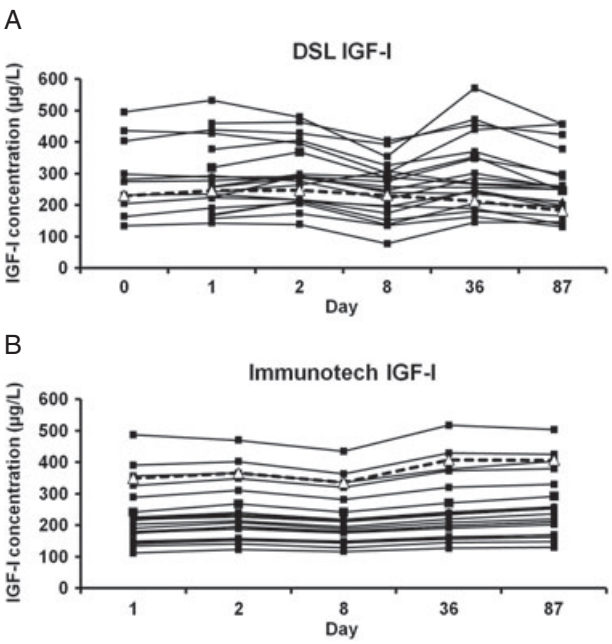

Figure 1. The effects of one freeze-thaw cycle and storage temperature on individual values of IGF-I. Samples were analyzed using the DSL 10–5600 ELISA (Figure 1A) immediately after centrifugation (Day 0) and after 1 day at 4°C (Day 1), after 1 day at −20°C (Day 2), after 7 days at −20°C (Day 8), after 35 days at −20°C (Day 36), and after 86 days at −20°C (Day 87). Samples were also analyzed using the Immunotech A15729 IRMA (Figure 1B) at all time-points except immediately after centrifugation. One quality control sample (Δ and dashed line) was analyzed at all time-points.

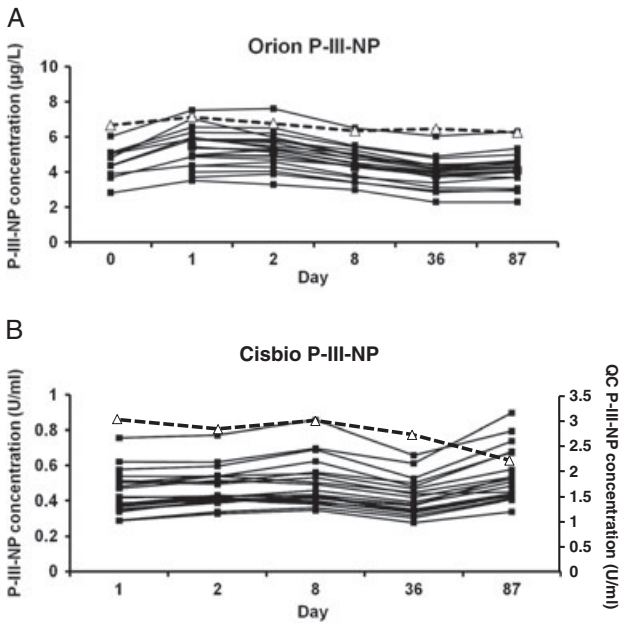

Figure 2. The effects of one freeze-thaw cycle and storage temperature on individual values of P-III-NP. Samples were analyzed using the Orion UniQ™ RIA (Figure 2A) immediately after centrifugation (Day 0), after 1 day at 4°C (Day 1), after 1 day at −20°C (Day 2), after 7 days at −20°C (Day 8), after 35 days at −20°C (Day 36), and after 86 days at −20°C (Day 87). Samples were also analyzed using the Cisbio RIA-gnost assay (Figure 2B) at all time-points except immediately after centrifugation. One quality control sample (Δ and dashed line) was analyzed at all time-points.

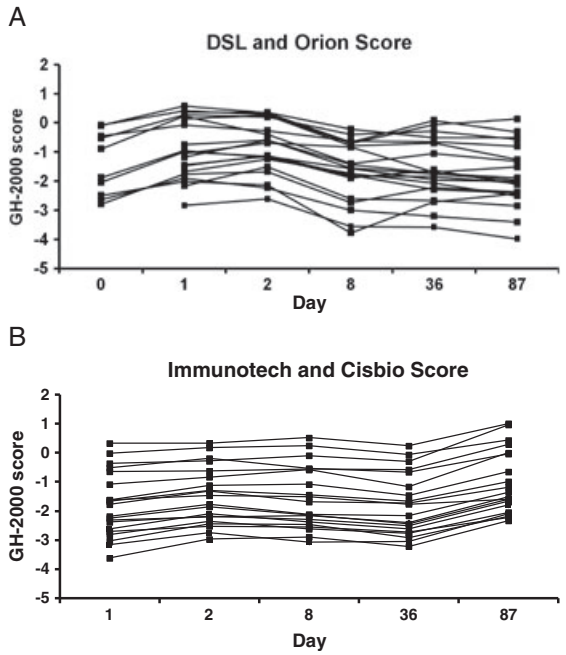

Figure 3. The effects of one freeze-thaw cycle and storage temperature on individual values of GH-2000 score. Scores were calculated by combining results from the DSL IGF-I assay and Orion P-III-NP assay (Figure 3A) and from the Immunotech IGF-I assay and Cisbio P-III-NP assay (Figure 3B) using the published GH-2000 discriminant function formulae.[2]

Table 2. Variability in individual volunteer GH-2000 scores. Scores were calculated by combining results from the DSL IGF-I assay and Orion P-III-NP assay and from the Immunotech IGF-I assay and Cisbio P-III-NP assay using the published GH-2000 discriminant function formulae[2]

Volunteer	DSL and Orion	Immunotech and Cisbio
	Median score (range)	Median score (range)
1	−2.46 (−3.78 to −2.01)	−2.98 (−3.63 to −2.33)
2	0.03 (−0.70 to 0.42)	0.18 (−0.06 to 0.44)
3	−2.82 (−3.41 to −1.91)	−2.47 (−3.03 to −2.18)
4	−2.30 (−2.79 to −1.67)	−2.55 (−2.73 to −2.22)
5	−1.75 (−2.12 to −1.00)	−2.29 (−2.50 to −1.67)
6	−0.97 (−1.40 to 0.23)	−0.83 (−1.17 to 0.00)
7	−0.01 (−0.67 to 0.58)	0.34 (0.24 to 1.01)
8	−0.34 (−0.57 to 0.27)	−0.58 (−0.64 to 0.30)
9	−1.84 (−2.07 to −0.96)	−2.20 (−2.40 to −1.50)
10	−0.54 (−0.81 to −0.07)	−0.28 (−0.36 to 0.97)
11	−0.70 (−1.26 to 0.26)	−1.12 (−1.62 to −0.65)
12	−1.56 (−1.97 to −0.60)	−1.45 (−1.67 to −0.99)
13	−3.56 (−3.99 to −2.61)	−3.05 (−3.17 to −2.10)
14	−1.79 (−2.40 to −1.12)	−1.59 (−1.76 to −1.47)
15	−0.22 (−0.51 to 0.36)	−0.52 (−0.66 to −0.03)
16	−1.43 (−1.89 to −0.59)	−1.69 (−1.78 to −1.18)
17	−1.73 (−2.51 to −1.24)	−2.62 (−2.81 to −2.05)
18	−1.56 (−2.02 to −1.14)	−2.38 (−2.62 to −1.79)
19	−2.61 (−2.85 to −1.51)	−2.14 (−2.45 to −1.61)
20	−1.05 (−1.69 to −0.69)	−2.12 (−2.19 to −1.35)

and DSL IGF-I combination), 0.35 (Orion P-III-NP and Immunotech IGF-I combination), 0.31 (Cisbio P-III-NP and DSL IGF-I combination) and 0.35 (Cisbio P-III-NP and Immunotech IGF-I combination). None of the GH-2000 scores were more than 3.72 SDs above the mean. If values above this cut-off point were used to indicate doping, the false-positive rate would be approximately 1 in 10 000 tests, at the time-points investigated in this study.

Discussion

The results of this study show that the variability in IGF-I and P-III-NP results of stored samples are largely determined by the precision of the assays. IGF-I and P-III-NP concentrations and GH-2000 discriminant function scores were unaffected by a single freeze-thaw cycle, by storage at 4°C for one day or by storage at −20°C for up to three months.

These results complement the findings of our previous study in which we showed that storage of serum or clotted blood samples at 4°C for up to five days did not result in any significant changes in IGF-I and P-III-NP concentrations.[3] Several other studies have investigated the effects of pre-analytical storage conditions on the stability of IGF-I results in blood samples.[9–11] Delays in centrifugation of whole blood samples stored at room temperature may lead to increased IGF-I concentrations as it may be released from lysed or dying blood cells, along with its dissociation from the IGF binding proteins.[9] Harris *et al.* showed that serum IGF-I increased significantly if whole blood was stored at room temperature for 24 h before centrifugation, but if the samples were centrifuged soon after blood collection and stored as serum aliquots, there was no significant change in results after 24 h.[10] By contrast, Kristal *et al.* showed a decrease in IGF-I concentrations in blood samples collected into tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA) after delays in centrifugation of between 32 and 144 h.[11] In our previous study, we showed that EDTA is not a suitable collection medium for GH-sensitive markers because it exerts a significant matrix effect on P-III-NP analysis.[3] Ideally delays in centrifugation should be avoided but if this test is used in an out-of-competition anti-doping setting with sample collection at the athlete's training venue or home, immediate access to a centrifuge will be difficult. Since this may result in delayed centrifugation, it is vital that the samples are kept chilled during transportation. This is reflected in the WADA guidelines for blood sample collection which state that samples should be transported to the laboratory in a refrigerated state. No sample should be allowed to freeze, and should ideally be kept at a temperature of approximately 4°C.[12]

IGF-I concentrations are highly stable in serum stored at −20°C for up to three months and therefore it appears these storage conditions are acceptable for serum samples collected for detection of GH doping. In comparison with −80°C storage facilities, -20°C freezers are widely available both in laboratories and also in other locations potentially closer to the site of blood collection, which could be an advantage for sample transport and storage.

None of the pre-analytical storage conditions investigated in the present study resulted in a significant change in P-III-NP concentration. This is in contrast with our previous study in which serum P-III-NP concentrations rose significantly if blood samples were stored at room temperature either as serum or clotted blood.[3] This emphasizes the need for anti-doping authorities to ensure that blood samples are kept chilled during transportation to the laboratory. The rise in P-III-NP at room temperature may result from cleavage of the P-III-NP molecule by collagenases to produce P-III-NP fragments and exposure of new antigenic sites.[3] Storage of samples at −20°C appears to inhibit these processes and the rise in P-III-NP.

Freezing and thawing serum can alter the measured concentrations of serum proteins[13] because of protein denaturation during the freeze-thaw cycle.[14] In our previous studies, all samples underwent at least one freeze-thaw cycle but in anti-doping testing at major sporting events, samples are often analyzed soon after collection without being frozen. This study has shown that one freeze-thaw cycle had no significant effect on IGF-I or P-III-NP results and that it is acceptable to measure fresh, unfrozen samples to detect GH doping.

The storage of serum at 4°C overnight or frozen at −20°C for up to three months had no significant effect on the GH-2000 score. The standard deviation of the GH-2000 score is dependent on the CVs of IGF-I and P-III-NP values. Score variability cannot be expressed as a percentage because it is impossible to calculate a percentage change from zero. The intra-sample standard deviation of GH-2000 score varied between 0.31 and 0.50, depending on the assay combination used. This indicates that the GH-2000 score of a sample may vary by up to 1.0 (two standard deviations) from the mean score for that sample, when exposed to these pre-analytical conditions. Although the variability in GH-2000 score appeared to be higher in some individuals than in others (Table 2), this score variability is largely determined by the inter-assay variability of the IGF-I and P-III-NP assays.

It is of considerable interest that the apparent variability in GH-2000 score in this study is very similar to that found in elite athletes when multiple blood samples were taken and analyzed over the course of a year.[15] It is likely that this apparent variability is entirely accounted for by assay variability and, in fact, marker concentrations may not vary at all. When more precise mass spectrometry assays become available for these protein analytes, this apparent variability will fall.

It is important to note that this study was performed using healthy volunteers rather than elite athletes. The IGF-I and P-III-NP concentrations and therefore GH-2000 scores in elite athletes are likely to be higher than in these healthy volunteers but this has been taken into account when setting the reporting thresholds. We have found no evidence, however, that the variability in results is dependent on analyte concentration and therefore we propose that these findings are applicable to samples taken from elite athletes.

Since this study was performed, the DSL 10–5600 IGF-I ELISA has been withdrawn. There was, however, no evidence to suggest that there was a difference in the effect of pre-analytical conditions between assays and, whilst it is possible that different results might be obtained if other assays are used, we believe that the findings from this study are likely to be applicable to other IGF-I and P-III-NP assays that may be available in the future.

In conclusion, this study shows that both IGF-I and P-III-NP concentrations are stable in serum stored at 4°C overnight and at −20°C for up to three months. Furthermore, a single freeze-thaw cycle has no significant effect on assay results. It therefore is acceptable for anti-doping laboratories to analyze samples immediately after centrifugation or for samples to be transported at 4°C and serum stored at −20°C for up to three months, prior to analysis.

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