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W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck
(Editors)

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R. ABELLAN, R. VENTURA, S. PICHINI, A.F. REMACHA, M.P. SARDA, S. DI CARLO, A. BACOSI, R. PACIFICI, J.A. PASCUAL, P. ZUCCARO, J. SEGURA:

Indirect Biomarkers of rhGH and rhEPO misuse. Factors affecting their Measurement in Serum Samples

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R. Abellan[1,2], R. Ventura[1,2], S. Pichini[3], A.F. Remacha[4], M.P. Sarda[4], S. Di Carlo[3], A. Bacosi[3], R. Pacifici[3], J.A. Pascual[1,2], P. Zuccaro[3], J. Segura[1,2].

Indirect biomarkers of rhGH and rhEPO misuse. Factors affecting their measurement in serum samples.

[1]Unitat de Recerca en Farmacologia, Institut Municipal d'Investigació Mèdica, Barcelona, Spain.
[2]Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain.
[3]Drug Research and Control Department, Istituto Superiore di Sanitá, Rome, Italy.
[4]Departament d'Hematologia, Hospital de Sant Pau, Barcelona, Spain.

Introduction

Serum concentrations of some macromolecules have been proposed as indirect biomarkers for the detection of recombinant human growth hormone (rhGH) [1] and erythropoietin (rhEPO) [2] misuse in sport. Two markers directly affected by rhGH administration, insulin-like growth factor-I (IGF-I) and procollagen type III peptide (P-III-P), were selected for rhGH misuse. Indirect detection of rEPO use is based on blood markers of altered erythropoiesis, and among them two serum biomarkers have been considered: erythropoietin (EPO) and soluble transferrin receptor (sTfR).

Serum concentrations of these biomarkers are usually measured using immunoassays. These immunoassay have been developed to be applied in the clinical field, where requirements are different from those of anti-doping control analyses. In the clinical field, the result of a sample is compared with a range defined for normal population, which has been defined using the same immunoassay and, frequently, in the same laboratory. In anti-doping control, the concentration of a biomarker is used to apply mathematical models and to obtain a "score" value that will be compared with a decision limit, and to define reference ranges for normal population that have to be applied by different laboratories around the world. In consequence,

accuracy and inter-laboratory precision have higher importance in anti-doping control field than in the clinical field [3-5]. Another important factor to take into consideration is that most immunoassays have been designed to be used in autoanalyzers (high-throughput instruments), not always available in anti-doping control laboratories.

In the present study, we aimed to study different immunoassays for the measurement of serum concentrations of indirect biomarkers of rhGH and rhEPO misuse, to evaluate the concordance of results between them, as well as to identify the most important variables that may affect the result for each biomarker.

Methodology

Immunoassays evaluated

A total of 11 immunoassays were evaluated for the different biomarkers. They are described in Tables I, III, V, and VII. They were selected among those commercially available for each biomarker. Whenever possible, the assays used in previous studies were selected.

Validation studies

The validation performed of those assays consisted on the following studies:

a. Intra-laboratory validation

Intra-laboratory validation for the techniques was performed during three subsequent days and by independent laboratories.

Five replicates of two quality control (QC) samples were analysed for the determination of intra-assay precision and accuracy; the inter-day precision and accuracy were determined taking into account all the values obtained along the three assays. Precision was expressed as the relative standard deviation (RSD%) of the measurements performed. Accuracy was expressed as the relative error (%) of the value obtained with respect to the assigned value for the QC samples or evaluated as “correct” or “incorrect” if the concentration obtained was inside or outside the acceptance concentration range defined by the manufacturer for the QC samples. The acceptance range was from 10 to 25% of the concentration, depending on the immunoassay.

The limit of quantitation (LOQ) was defined as the mean value obtained for the blank sample plus (or less, depending on the sign of the slope of the calibration curve) ten times the estimated value of the noise. The noise was measured as the standard deviation of the signal obtained after analysis of a blank sample (n=5).

b. Inter-laboratory

Inter-laboratory validation was performed by comparing results of human serum samples analyzed in two different laboratories. The intraclass correlation coefficient (ICC) using random effects mode was calculated to evaluate the concordance of results between laboratories [6]. To evaluate the dispersion of the results obtained between different laboratories the mean values of concentrations were represented against the relative differences between concentrations (modification of Bland-Altman plots). The 95% limits of agreement (95%LA) were calculated according to the following expression: relative difference mean±1.96 x standard deviation of relative differences.

c. Inter-technique validation

Inter-technique validation was performed by analysing human serum samples using two different techniques. Inter-technique concordance was evaluated using the Passing-Bablok method [7]. To evaluate the dispersion of the results obtained between different techniques, a modification of Bland-Altman plots was used, as described above.

Results

EPO Immunoassays validation

Among the great number of the existent immunoassays to measure serum EPO, the two immunoassays previously used in the development of the mathematical models to detect rhEPO misuse [2, 8, 9] were selected (Table I). The reference standards used in both of them were calibrated against WHO standards.

Better intra-laboratory precision and accuracy for CHEM were obtained (Table II), probably due to the use of autoanalysers. Inter-laboratory results were similar for both assays (ICC=0.980 for CHEM and ICC=0.920 for ELISA), but CHEM showed less dispersion.

Inter-technique validation showed that CHEM results were higher than ELISA results (Figure 1). Inter-technique comparison was consistent with previous studies [9].

Total IGF-I Immunoassays validation

Total IGF-I measurement needs a pre-treatment of the sample to separate IGF-I from the IGF-binding proteins. The selected immunoassays were chosen taking into account the different pre-treatments and the use of different techniques (Table III). The standards used were calibrated against the same WHO standard.

Precisions and accuracies obtained for RIA1 were worse than for RIA2 or ELISA (Table IV), probably due to the longer sample treatment. Moreover, for RIA1, concentrations of the 17% of actual non-users samples tested were below the LOQ.

For inter-laboratory validation , better ICC values were obtained for RIA1 (ICC= 0.794) and ELISA (0.733) than for RIA 2 (ICC=0.598), but the highest dispersion was obtained for RIA1. For inter-technique validation, Passing-Bablok equations showed that ELISA results were always lower than RIAs ([RIA2] = 0.74 [RIA1] + 56.28, N=71; [ELISA] = 0.55 [RIA1] - 16.12, N=50, and [ELISA] = 0.78 [RIA2] - 37.10, N=50). These lower results could be related to a lower efficiency of the dissociation pre-treatment.

P-III-P Immunoassays validation

The only two commercially available immunoassays for P-III-P were evaluated (Table V). The nature of the P-III-P reference standard used by the immunoassays was not described, and an international standard does not exist. Moreover, the units used are different and the equivalence to mass/volume was not described in the RIA1 assay.

For RIA1, concentrations in 30% of actual non-users samples tested were below the LOQ (Table VI), and most of the samples fall in the lowest part of the calibration curve, below 1 U/ml.

Better inter-laboratory results were obtained for RIA2 (ICC=0.943) than for RIA1 (ICC=0.666), and with lower dispersion. The Passing-Bablok comparison of both assays show the following equation: [RIA2] = 11.16 [RIA1] - 3.28 (N=54).

sTfR Immunoassays validation

The immunoassays evaluated were selected among those commercially available, taking into account the technique used (Table VII). The nature of the sTfR standard used was only described in ELISA test, and an international standard does not exist. Moreover, the units used are different between the assays.

Better intra-laboratory precision and accuracy results were obtained with the immunoassays using autoanalizers (Turbid and Nephel) (Table VIII). Inter-laboratory validation was only performed for ELISA1 and ELISA2. ICC values were similar for both assays (0.848 for ELISA1 and 0.973 for ELISA2), but ELISA2 showed less dispersion in results.

Important differences between immunoassays results were observed. Using ELISA2 as the reference immunoassay (units: nmol/l) (Figure 2), results for Nephel were lower than those of ELISA1 and Turbid, all of them using the same units (mg/l). These differences are most probably due to the different standard used for calibration of the techniques.

Conclusions

1. The results demonstrated the importance of an extensive validation of immunoassays used to measure indirect biomarkers of peptide hormones in serum before their eventual use in doping control.
2. The critical parameters identified were:
    a. Sample pre-treatment: IGF-1
    b. Calibration range: P-III-P, IGF-1
    c. Harmonization of the calibration standard: sTfR, IGF-1, P-III-P

   Use of autoanalysers improved significantly the intra-assay and inter-assay precision and reduced inter-laboratory variability, as expected.
3. The comparison of results of different immunoassays should be performed after appropriate transformation of the data. Inter-technique comparison is specially difficult in those cases where different units are used and the comparability of the standards is not available.
4. Any recommendation to use a specific immunoassay should take into consideration intra-laboratory as well as inter-laboratory and inter-technique validation results.

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Table I. EPO immunoassays evaluated.

	CHEM*	ELISA*
Name	Immulite (DPC)	Quantikine (R&D Systems)
Technique	Chemiluminescent	ELISA
Standard	rhEPO in non human serum matrix (calibrated against WHO EPO 67/343)	rhEPO in buffered protein base (calibrated against WHO EPO 67/343 and WHO EPO 87/684)
Units	mIU/ml	mIU/ml
Equipment	Immulite autoanalyser	-
Pre-treatment	No	No
1st Antibody	Ligand-labelled monoclonal anti-EPO	Monoclonal anti-EPO
Tracer	Polyclonal 2nd Ab anti-EPO conjugated to alkaline phosphatase	Polyclonal 2nd Ab anti-EPO conjugated to peroxidase
Separation	Bead coated with anti-ligand derived from streptavidin	Well coated with 1st Ab
Time	1.5 h	4 h

* Immunoassays used in previous studies [2, 8, 9].

Table II. EPO: summary of intra-laboratory validation.

		CHEM*		ELISA*	
		Laboratory 1	Laboratory 2	Laboratory 1	Laboratory 2
Intra-assay	Precision	< 6%	< 5%	< 7%	> 20%
	Accuracy	correct	correct	< 13%	> 20%
Inter-assay	Precision	< 5%	< 5%	< 5%	< 18%
	Accuracy	correct	correct	< 12%	< 12%
LOQ (mIU/ml)		0.5		2.0	
Calibration curve (mIU/ml)		Up to 200		2.5 – 200	
Actual non-users samples (mIU/ml)		3 – 25			

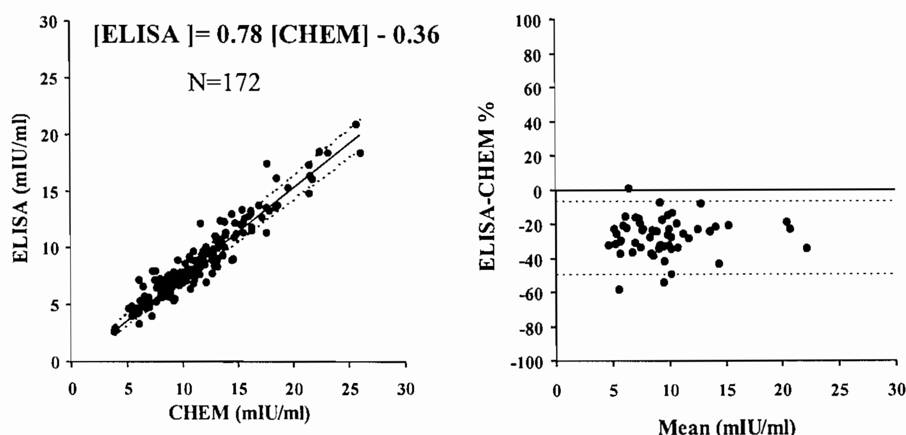

Figure 1. EPO inter-technique validation.

Table III. Total IGF-I immunoassays evaluated.

	RIA1	RIA2	ELISA
Name	Nichols Institute Diagnostics	Mediagnost	Quantikine (R&D Systems)
Technique	RIA competitive	RIA competitive	ELISA
Standard	rhIGF-I (calibrated against WHO IGF-I 87/518)	rhIGF-I (conversion factor: 1.66 against WHO IGF-I 87/518)	rhIGF-I (conversion factor: 0.816 against WHO IGF-I 87/518)
Units	ng/ml	ng/ml	Ng/ml
Equipment	-	-	-
Pre-treatment	Precipitation acid-ethanol	Acidic dissociation and IGF-II excess	Acidic dissociation
1st Antibody	Polyclonal rabbit anti-(IGF-I)	Polyclonal rabbit anti-(IGF-I)	Monoclonal anti-(IGF-I)
Tracer	[^{125}I]IGF-I	[^{125}I]IGF-I	Polyclonal 2nd Ab anti-(IGF-I) conjugated to peroxidase
Separation	Anti-rabbit precipitant and centrifugation	Capture 2nd Ab and tube coated with streptavidin	Well coated with 1st Ab
Time	32 h	16 h	6 h

Table IV. Total IGF-I: summary of intra-laboratory validation.

		RIA1		RIA2		ELISA	
		Laboratory 1	Laboratory 2	Laboratory 1	Laboratory 2	Laboratory 1	Laboratory 2
Intra-assay	Precision	< 20%	> 20%	< 10%	< 11%	< 12%	< 11%
	Accuracy	correct	not correct	correct	not correct	correct	correct
Inter-assay	Precision	< 20%	> 20%	< 10%	< 10%	< 10%	< 11%
	Accuracy	correct	correct	correct	correct	correct	correct
LOQ (ng/ml)		287.9	135.4	28.6	12.4	5.6	-
Calibration curve (ng/ml)		76.5 – 1170		9.6 - 780		9.4 - 600	
Actual non-users samples (ng/ml)		58.9 - 539.1		107.6 - 547.5		50.4 - 291.5	

Table V. P-III-P immunoassays evaluated.

	RIA1*	RIA2
Name	Cis-bioInternational	Orion Diagnostica
Technique	RIA sandwich	RIA competitive
Standard	P-III-P in buffer solution	Human serum P-III-P
Units	U/ml	ng/ml
Equipment	-	-
Pre-treatment	No	No
1st Antibody	Monoclonal mouse anti-(P-III-P)	Polyclonal rabbit anti-(P-III-P)
Tracer	Monoclonal mouse [^{125}I] anti-(P-III-P)	[^{125}I]P-III-P
Separation	Monoclonal mouse anti-(P-III-P) coated to the tube	2nd Ab covalently bound to solid particles and centrifugation
Time	7 h	6 h

* Immunoassays used in previous studies [1, 10, 11].

Table VI. P-III-P: summary of intra-laboratory validation.

		RIA1		RIA2	
		Laboratory 1	Laboratory 2	Laboratory 1	Laboratory 2
Intra-assay	Precision	< 5%	< 10%	< 12%	< 10%
	Accuracy	correct	correct	correct	correct
Inter-assay	Precision	< 5%	< 10%	< 10%	< 10%
	Accuracy	correct	correct	correct	correct
LOQ		0.8 U/ml	0.6 U/ml	2.5 ng/ml	1.6 ng/ml
Calibration curve		0.6 - 4.2 U/ml		1 - 50 ng/ml	
Actual non-users samples		0.4 - 1.9 U/ml		1.9 - 10.9 ng/ml	

Table VII. sTfR immunoassays evaluated.

	ELISA1	ELISA2*	Turbid	Nephel*
Name	Orion Diagnostica	Quantikine (R&D Systems)	Turbidimetric assay (Roche Diagnostics)	Nephelometric assay (Dade Behring)
Technique	ELISA	ELISA	Turbidometry	Nephelometry
Standard	Human serum sTfR	Human plasma sTfR (MW 74 kDa)	Human serum sTfR	Human serum sTfR
Units	mg/l	nmol/l	mg/l	mg/l
Equipment	-	-	Roche/Hitachi 911	Autoanalyser BNTH System
Pre-treatment	No	No	No	No
1st Antibody	Monoclonal anti-(sTfR)	Monoclonal anti-(sTfR)	Monoclonal anti-(sTfR)	Monoclonal anti-(sTfR)
Tracer	Monoclonal 2nd Ab anti-(sTfR) conjugated to alkaline phosphatase	Monoclonal 2nd Ab anti-(sTfR) conjugated to peroxidase	-	-
Separation	Well coated with 1st Ab	Well coated with 1st Ab	Latex particles coated with 1st Ab	Polyestirene particles coated with 1st Ab
Time	4 h	4 h	10 min	8 min

* Immunoassays used in previous studies [2, 8, 9].

Table VIII. sTfR: summary of intra-laboratory validation.

		ELISA1		ELISA2*		Turbid	Nephel*
		Laboratory 1	Laboratory 2	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 3
Intra-assay	Precision	< 11%	< 20%	< 8%	> 20%	< 1%	< 3%
	Accuracy	not correct	not correct	correct	not correct	correct	correct
Inter-assay	Precision	< 9%	> 20%	< 7%	> 20%	< 2%	< 3%
	Accuracy	correct	correct	correct	not correct	correct	correct
LOQ		0.30 mg/l		1.42 nmol/l		0.09 mg/l	0.22 mg/l
Calibration curve		0.4 - 9 mg/l		3 - 80 nmol/l		1.7 - 39.5 mg/l	0.15 - 4.7 mg/l
Actual non-users samples		0.2 - 24.6 mg/l		4.6 - 131.9 nmol/l		0.2 - 24.6 mg/l	0.4 - 9.9 mg/l

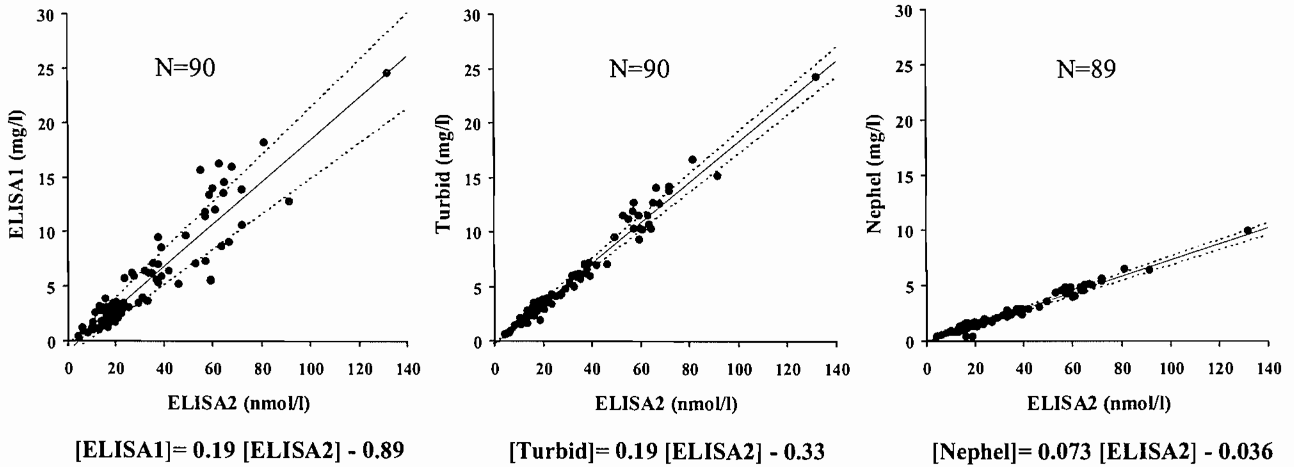

Figure 2. sTfR inter-technique validation.