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A high-throughput test to detect C.E.R.A. doping in blood

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ABSTRACT

C.E.R.A., a continuous erythropoietin receptor activator, is a new third-generation erythropoiesis-stimulating agent (ESA) that has recently been linked with abuse in endurance sports. In order to combat this new form of doping, we examined an enzyme-linked immunosorbent assay (ELISA) designed to detect the presence of C.E.R.A. in serum samples. The performance of the assay was evaluated using a pilot excretion study that involved six subjects receiving C.E.R.A. Validation data demonstrated an excellent reproducibility and ensured the applicability of the assay for anti-doping purposes. To maximize the chances of detecting the drug in serum samples, we propose the use of this specific ELISA test as a high-throughput screening method, combined with a classic isoelectric focusing test as a confirmatory assay. This strategy should make C.E.R.A. abuse relatively easy to detect, thereby preventing the future use of this drug as a doping agent.

1. Introduction

Erythropoietin (EPO) is a 30.4 kDa human glycoprotein hormone produced mainly in the kidney [1]. Its chief physiologic effect is the stimulation of erythropoiesis [2], which results in the formation of red blood cells and the consequent improvement of blood oxygen-carrying capacity.

It is well established that patients suffering from chronic renal failure, as well as other chronic diseases, frequently develop anemia, the primary cause of which is EPO deficiency [3]. Over the last two decades, synthetic EPO analogs, such as recombinant human EPO (rhEPO, epoetins) and darbepoetin alfa – collectively known as erythropoiesis-stimulating agents (ESAs) – have been developed by the pharmaceutical industry. These compounds are able to substitute for endogenous EPO by the activation of EPO receptors in a manner identical to that of the native hormone.

C.E.R.A., a continuous erythropoietin receptor activator, is the active ingredient of a new drug for the management of anemia in patients with chronic kidney disease (MIRCERA®, Roche Pharma AG, Reinach, Switzerland), and a third-generation ESA. C.E.R.A. is synthesized by integration of a single large polyethylene glycol (PEG) chain into the epoetin molecule, thus increasing the molecular weight to twice that of epoetin (~60 kDa) [4]. It has been reported that integration of PEG molecules may maintain *in vivo* biologic activity of some pharmaceutically active molecules [5]. For C.E.R.A., integration of the PEG moiety has resulted in a prolonged half-life and increased biologic activity *in vivo* when compared with epoetin. Patients treated with short-acting and frequently administered ESAs can be switched directly to once-monthly C.E.R.A. administration without compromising efficacy or safety [6].

In endurance sports, an increase in the number of erythrocytes is known to enhance athletic performance [7]. The availability of synthetic forms of EPO means that this ergogenic hormone could be used illicitly in sport [8]. As a result, the International Olympic Committee (IOC) Medical Commission banned these drugs in 1990. While the current urinary anti-doping test [9–11], based on isoelectric focusing (IEF) separation, differentiates between the various ESAs, such as endogenous EPO, rhEPO and darbepoetin alfa, preliminary results indicate that detection of C.E.R.A. misuse in urine is problematical [12]. For the time being, far less blood samples are collected compared to urine samples; this probably led to its abuse by some athletes.

Abbreviations: C.E.R.A., continuous erythropoietin receptor activator; DIG, digoxigenin; ELISA, enzyme-linked immunosorbent assay; EPO, erythropoietin; ESA, erythropoiesis-stimulating agent; IEF, isoelectric focusing; LLOQ, lower limit of quantification; LLQC, lower limit of quality control; PEG, polyethylene glycol; QC, quality control; rhEPO, recombinant human EPO; ROC, receiver operating characteristic.

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To combat illegal abuse of this new agent in sport, we have validated an enzyme-linked immunosorbent assay (ELISA) for the detection of C.E.R.A. in serum, using a negative and a positive reference population, with a pilot excretion study involving six healthy subjects. The described assay constitutes a rapid and reliable approach for the screening of C.E.R.A. in blood that will discourage the use of third-generation ESA doping in sport.

2. Methods

2.1. ELISA test principle

A specific ELISA was used for the measurement of C.E.R.A. using microtiter plates pre-coated with streptavidin. All material, reagents, and antibodies were provided by Roche Diagnostics (Roche Diagnostics GmbH, Penzberg, Germany). A biotinylated antibody against EPO was bound onto microtiter plates and samples were added. Serum samples were briefly centrifuged before incubation. The detection antibody used was a monoclonal anti-PEG antibody labeled with digoxigenin (DIG). After the secondary antibody incubation, an anti-DIG–Fab–HRP(poly) conjugate was added. Finally, bound conjugate was reacted with an ABTS substrate, leading to a colorimetric reaction that was detected at 405 nm (reference wavelength 492 nm). All incubation steps were performed at room temperature and lasted 1 h, except the biotinylated anti-EPO antibody incubation, which was performed overnight, and the ABTS substrate incubation, which was 40 ± 10 min. Between each incubation step, the plate was washed three times with a buffer containing 40 mM potassium phosphate buffer pH 7.4 and 0.5 g/L benzylglucoside. Every sample measurement was performed in duplicate and all the samples were diluted 5- to 250-fold to ensure that test samples had optical densities in the dynamic detection range of the test (30–1000 pg/mL).

2.2. Assay validation

All validation experiments were performed at the Swiss Laboratory for Doping Analyses (Lausanne, Switzerland) during 2 consecutive weeks by two different technicians. For each ELISA assay, the calibration curve was fitted and sample concentrations were calculated based on a polynomial mathematical model (four-parameter Rodbard-function) taking into account the non-linear behavior of the curve, especially in the high concentration range. Each absorbance value of standard, control, or serum sample was corrected by subtracting the value of the substrate blank. Each measurement was performed at least in duplicate.

Four independently prepared replicates of twofold serial dilutions of positive control (11 concentration points), as well as a zero standard, were measured on one plate in duplicate in order to define a suitable dynamic range for accurate measurements ($\leq$15% coefficient of variation [CV]). The lower limit of quantification (LLOQ) was defined by the first calibration point that was not blank where the signal at LLOQ $- 1.64 \times$ SD is larger than the signal of blank $+ 1.64 \times$ SD (95% confidence interval [95% CI], where SD is standard deviation). The lowest quality control (QC) sample (LLQC) was determined based on the LLOQ concentration. Three additional QCs (defined as low, medium and high QCs) were established at concentrations reflecting different regions of the calibration curve.

Intra-assay precision and accuracy were determined using five replicates of the four QC samples. Inter-assay precision and accuracy were determined using all values obtained from nine independent experimental assays of the same four QC samples. Precision was expressed as the % CV of the measurements performed, while accuracy was expressed as the percent recovery from the assigned value for the four QC samples. The acceptance range was defined as 80–120% for the four QC levels.

Dilutional linearity was evaluated by preparing spiked serum samples with a concentration that was 1000 times higher than the highest calibrator. This artificial sample was subsequently diluted 1:10, followed by an additional series of fourfold dilutions in matrix. The dilutions were also used to exclude a possible high-dose hook effect.

Matrix effect was assessed by testing 10 individual blank serum samples spiked with C.E.R.A. in concentrations corresponding to those of the high and the low QC or unspiked (blank).

The ability of the ELISA to differentiate and quantify the target analyte in the presence of other compounds in the sample, similar to the analyte, was tested. Cross-reactivity with epoetin beta (Recormon®, Roche Pharma AG, Reinach, Switzerland), darbepoetin alfa (Aranesp®, Amgen AG, Zug, Switzerland) and PEG (Roche Diagnostics GmbH, Penzberg, Germany) at two different concentrations (corresponding to the physiologic concentration of EPO, which was estimated at 125 ng/L [15 IU/L], and at 1000 times the physiologic concentration) was tested at the LLQC and blank (unspiked).

Antibody (anti-EPO antibody and DIG-anti-PEG antibody) stability was determined after six freeze-thaw cycles. The stressed reagents were compared with freshly thawed reagents using calibrators and QCs.

Two additional internal QCs were prepared by spiking a given concentration of C.E.R.A. into a serum matrix. The expected concentration for the negative QC was 0 pg/mL, while the expected concentration for the positive QC was 150 pg/mL. Both QCs, as well as a 100 pg/mL standard, were systematically deposited on each ELISA plate used for the validation process and the following assays. Concentrations for these three controls and standard measurements were recorded for evaluation.

2.3. Positive population: subject characteristics and study protocol

Six healthy Caucasian men, aged 20–28 years, participated in the pilot study. The study was conducted according to the Declaration of Helsinki as amended in the 41st World Medical Assembly and was approved by the local ethical committee (protocol #05/08). None of the subjects were involved in semi-professional or professional sport and all underwent a complete clinical examination before being included in the study. All participants gave their informed consent and agreed to blood collection. The subject population was relatively homogenous, the participants having a mean age of 23.0 years (SD 2.97) and a mean body mass index (BMI) of 23.3 kg/m^2 (SD 1.48). Each subject received a single injection of 200 μg C.E.R.A. (MIRCERA®, Roche Pharma AG, Reinach, Switzerland). Three randomly selected subjects received a subcutaneous injection, while the other three received an intravenous injection. Over 4 days post-injection, two blood samples per subject were systematically collected every morning. The first sample was collected in a serum gel 7.5 mL monovette, while the second was collected in a classical EDTA-coated 2.6 mL monovette (Saarstedt, Numbrecht, Germany). Thereafter, identical blood samples were collected on days 6, 8, 10, 13, 16, 20, 24, and for four of the subjects, day 27 post-injection (positive population).

A complete red blood cell count was performed, and the reticulocyte cell population was quantified using an automatic analyzer (XT-2000i analyzer, Sysmex, Norderstedt, Germany) to confirm that all subjects responded to the C.E.R.A. injection. In addition, these blood tests, which were carried out immediately after blood collection, allowed the control of hemoglobin concentration to avoid an excessive increase in blood viscosity. Serum samples were immediately aliquoted and frozen at $-80\,^{\circ}$C. Stability of C.E.R.A. in human serum was demonstrated previously for up to 6 h at ambient temperature, for up to three freeze-thaw cycles, and for up to 12 months at both $-20\,^{\circ}$C and $-70\,^{\circ}$C [13].

Table 1
Validation parameters of the ELISA assay.

QC	Conc. (pg/mL)	Assay	Intra-assay				
			n	Mean	SD	Precision [% CV]	Accuracy [% recovery]
HQC	800	1	5	819.2	39.2	4.8	102.4
		2		800.4	55.4	6.9	100.1
MQC	400	1	5	405.6	17.3	4.3	101.4
		2		424.6	6.3	1.5	106.2
LQC	200	1	5	190.8	7.1	3.7	95.4
		2		203.6	9.8	4.8	101.8
LLQC	50	1	5	50.4	4.3	8.6	100.8
		2		52.0	4.6	8.9	104.0
QC	Conc. (pg/mL)		Inter-assay				
			n	Mean	SD	Precision [% CV]	Accuracy [% recovery]
HQC	800		9	797.2	38.1	4.8	99.6
MQC	400			399.6	20.1	5.0	99.9
LQC	200			195.4	10.2	5.2	97.7
LLQC	50			46.0	4.1	8.9	92.0

ELISA: enzyme-linked immunosorbent assay; QC: quality control; HQC: high quality control; MQC: medium quality control; LQC: low quality control; LLQC: lower limit of quality control; SD: standard deviation; CV: coefficient of variation.

2.4. Negative population

In an anti-doping context, where false-positive results must be excluded, the establishment of a decisional limit (cut-off limit) taking into account the eventual matrix effect is mandatory. For this purpose, 140 blank serum samples were used to determine the cut-off limit of the assay. These samples were collected during a major cycling event in 2004, before C.E.R.A. was available on the market (negative population). These samples were aliquoted and stored at −20 °C.

2.5. Calculations and statistical analysis

A receiver operating characteristic (ROC) curve was established from the data obtained from both negative and positive (pilot study) populations using Matlab 7.7.0. (R2008b). The ROC was represented by plotting the sensitivity versus (1 − specificity) of the ELISA as its discrimination threshold varied. *P* values were assessed using Student's *t*-tests.

3. Results

3.1. Assay validation

Validation parameters for the ELISA assay are shown in Table 1. The LLOQ was determined as 30 pg/mL. Based on this, the LLQC was defined at 50 pg/mL. Our measuring range was 30–1000 pg/mL. Intra-assay and inter-assay precision were all less than 10%. The accuracy between all individual serum samples was 80–120% when spiked with C.E.R.A. concentrations corresponding to those of the high and the low QC. Dilutional linearity was assessed in the measurement range and no high-dose hook effect could be observed (data not shown). Fig. 1 shows that high concentrations of either epoetin beta or darbepoetin alfa (1000-fold the physiologic concentration) led to a loss of signal in spiked serum samples. No interference was observed when both ESAs were present in physiologic concentration. In contrast, a high concentration of PEG molecules in the samples resulted in a slight, but not relevant, increase in signal strength.

No significant degradation was observed after six freeze-thaw cycles for both anti-EPO and anti-PEG antibodies (data not shown).

Fig. 2 shows the concentrations of the 100 pg/mL standard and the positive internal QC from the validation process and the following assays. All the values were between their individual mean + 2SD and mean − 2SD values. For the 100 pg/mL standard, the 2SD values were between 80% and 120% of the target concentration. Follow-up of the positive control led to a value of ∼180 pg/mL, compared with the expected concentration of 150 pg/mL. However, values were always between mean + 2SD and mean − 2SD. Similar follow-up of the negative internal control resulted in absorbance levels well below the value corresponding to the LLOQ.

3.2. Incurred samples: C.E.R.A. administration

As expected, a significant ($P < 0.01$) increase in reticulocyte count was observed in all subjects following C.E.R.A. administration, suggesting that all subjects responded to C.E.R.A. (data not shown). No significant hematocrit or hemoglobin concentration change was observed throughout the study.

C.E.R.A. serum concentrations were determined using ELISA. Fig. 3 represents the serum concentration of the molecule during the 27 days of the study. At day 0 (pre-injection), C.E.R.A. was not detectable in any sample. A sharp increase in C.E.R.A. concentration was observed immediately after injection. In the three subjects who received intravenous C.E.R.A., the day 1 samples contained the highest concentration of C.E.R.A., after which the concentration

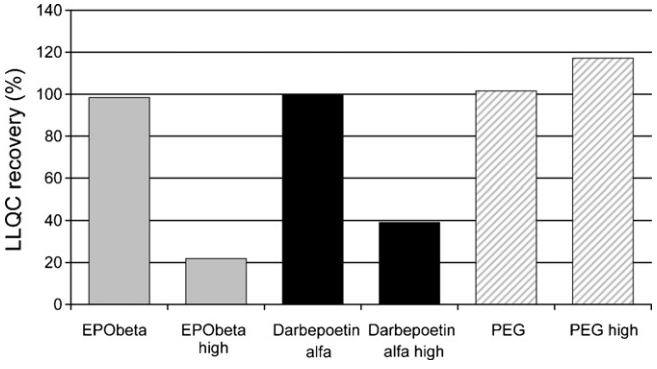

Fig. 1. Cross-reactivity test. LLQC recovery (%) in a blank serum spiked with 125 ng/L epoetin beta, darbepoetin alfa, and PEG or 125 mg/L ("high") epoetin beta, darbepoetin alfa, and PEG. EPO: epoetin; LLQC: lower limit of quality control; PEG: polyethylene glycol.

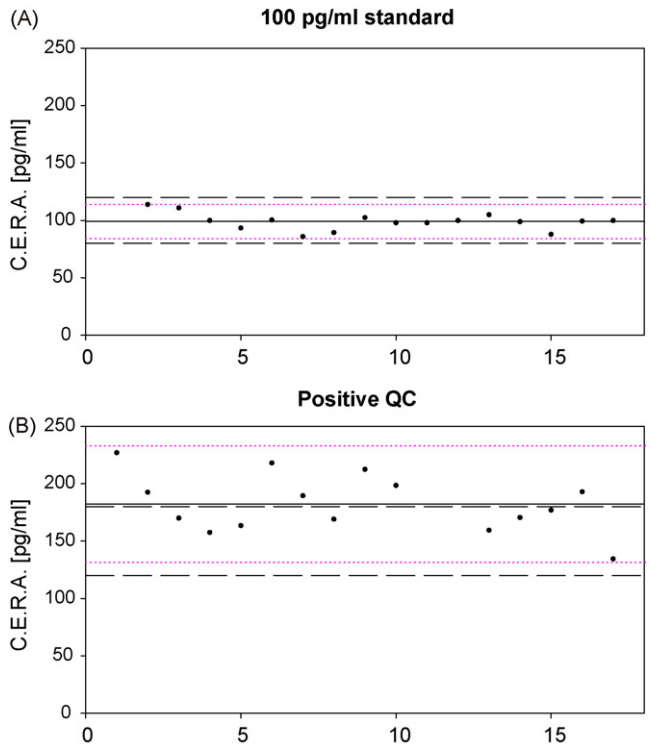

Fig. 2. Follow-up of 100 pg/mL standard and positive and negative QCs. (A) C.E.R.A. concentrations obtained for the 100 pg/mL standard over 2 months. Mean = 99.0, CV = 7.5%, min = 86, max = 114. (B) C.E.R.A. concentrations obtained for the positive control over 2 months. Target = 150, mean = 182, CV = 12.9%, min = 134, max = 227. The *x*-axis represents the number of plates. The continued line represents the mean value. The dashed red line represents the mean value ±2SD. The dashed line represents the target value ±20%. CV: coefficient of variation; SD: standard deviation; QC: quality control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

decreased rapidly. In contrast, after subcutaneous C.E.R.A. administration, C.E.R.A. concentration was highest between days 2 and 6. Thereafter, C.E.R.A. concentration decreased slowly. Moreover, the detection window of C.E.R.A. varied greatly among individuals, ranging from 16 to more than 27 days following a 200 μg subcutaneous C.E.R.A. injection. Among subjects who received a 200 μg intravenous injection, C.E.R.A. concentrations returned to basal levels after 8 days in one subject, while levels in another subject remained detectable at 27 days post-injection.

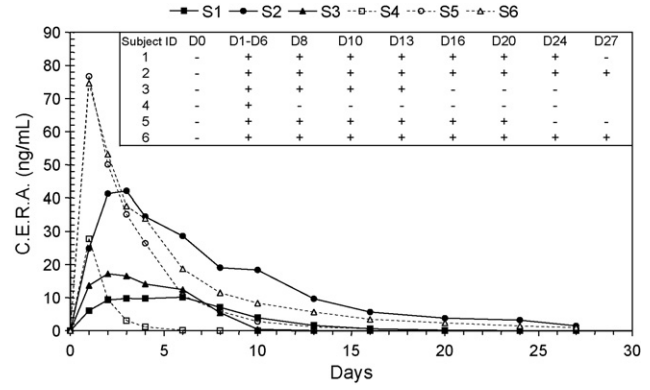

Fig. 3. C.E.R.A. concentration (ng/mL) measured in serum over time following a single injection of 200 μg of C.E.R.A. S1, S2, S3 = subcutaneous, S4, S5, S6 = intravenous. Table: detection window of each individual: +, higher than cut-off limit (100 pg/mL); −, lower than cut-off limit (100 pg/mL).

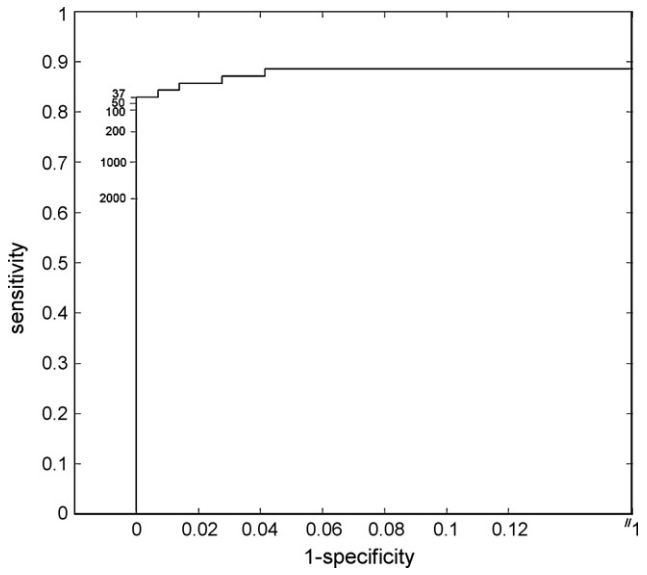

Fig. 4. ROC curve. Specificity and sensitivity of the ELISA according to the cut-off limit for a single 200 μg injection on week 4. ELISA, enzyme-linked immunosorbent assay; ROC, receiver operating characteristic.

3.3. Sensitivity of the method

Based on the absorbance levels, we extrapolated the mean measured C.E.R.A. concentration of the 140 tested blank serum samples (negative population). Of the samples tested, 138 were below the LLOQ (median = 0, min = 0, max = 37). The positive population constituted all samples from the pilot study. An ROC curve illustrating the relationship between the sensitivity and specificity of the ELISA depending on the chosen cut-off limit is shown in Fig. 4. The cut-off limit of the assay was fixed at 100 pg/mL. According to this value, the assay had 100% specificity and 80% sensitivity over a 4-week period following a single 200 μg C.E.R.A. injection.

4. Discussion

The described ELISA is a simple, rapid, and sensitive immunoassay specifically targeting C.E.R.A. molecules in serum based on the combination of an anti-EPO and an anti-PEG antibody. It allows an operator to analyze about 70 samples per day. This high throughput, along with the low cost of the test when compared with classic IEF separation, constitutes an undeniable advantage for a screening assay.

Cut-off limit determination of an immunoassay constitutes a major part of the validation process. In contrast to a diagnostic test, cut-off limit of an anti-doping assay must be fixed in order to reach a specificity as close as possible to 100% (no false-positives). In our study, an ROC curve was used to determine the cut-off limit of the C.E.R.A. assay at 100 pg/mL. Other standard methods of calculating cut-offs, such as SD multipliers, yielded values of approximately 50 pg/mL. Empirically, and taking into account the matrix effects, we set this limit at 100 pg/mL to ensure, with the greatest possible degree of certainty, that no false-positive results are reported. The ROC curve (Fig. 4) shows that when the cut-off limit is fixed at 100 pg/mL, the sensitivity of the assay over a 4-week period following 200 μg C.E.R.A. injection is 80%. Fixing the cut-off limit at 50 pg/mL results in slightly higher sensitivity (82%). If necessary, this value could be refined in the future with the availability of more data. However, it should noted that the dose injected in our pilot study is probably well above the doses used for performance enhancement. Therefore, further investigations may be needed to

assess both the sensitivity and the specificity of the assay with doses of C.E.R.A. likely to be used for doping in sport.

Follow-up of the 100 pg/mL standard and the positive internal control demonstrated the precision of the method, as all points were within individual mean + 2SD and mean − 2SD values. Values for the negative internal control were located well below the LLOQ. However, the mean concentration obtained for the positive control appeared to be overestimated by ∼20% when compared with the expected value. This apparent overestimation may be related to the serum matrix the QC is prepared in, as well as the multiple dilution steps necessary to obtain such low concentrations from a commercial C.E.R.A. product. Ring testing involving other laboratories should be performed to determine the most appropriate positive control value.

In accordance with the results of Macdougall et al. [14], the pharmacokinetic profile of the drug in serum differed considerably with route of administration, with intravenous administration allowing faster distribution of the molecule in blood. This phenomenon has also been demonstrated previously with rhEPO [15]. C.E.R.A.'s pharmacokinetic profile was not related to any of the other physiologic parameters we measured in the subjects, including BMI. Consequently, the large differences in the detection window observed between individuals cannot be explained by the route of administration of the product, or by any apparent physiologic characteristics of the subjects.

In summary, the results of the pilot study demonstrated that this assay could prove a strong disincentive to use C.E.R.A. as a doping agent for sports performance, despite its major clinical advantage of increased dosing intervals. This is compounded by the much longer detection window for C.E.R.A. (between 8 days and more than 4 weeks for a single 200 μg injection in healthy volunteers) than rhEPO and darbepoetin alfa [16,17]. This C.E.R.A.-specific ELISA constitutes a fast and reliable screening method that allows one technician to test for C.E.R.A. in about 70 samples in less than a day. This sensitive assay enables suspicious serum samples to be identified quickly for further analysis and confirmation using a complementary method such as IEF. The use of two different assay principles is a mandatory requirement for anti-doping laboratories to return an adverse analytical finding [18]. Consequently, a confirmation immunoaffinity purification test prior to the ELISA could also be considered. This fast ELISA screening method can also be used for the retrospective analysis of large numbers of samples from major sports competitions. Therefore, the combination of a C.E.R.A.-specific ELISA screening test and a confirmatory assay would make C.E.R.A. abuse relatively easy to detect, providing a strong disincentive for the use of C.E.R.A. as a performance enhancer in sport.

Conflict of interest

The authors declare no potential conflict of interests.

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References

[1] S. Donnelly, Am. J. Kidney Dis. 38 (2001) 415–425.
[2] J.W. Fisher, Exp. Biol. Med. (Maywood) 228 (2003) 1–14.
[3] W. Jelkmann, Physiol. Rev. 72 (1992) 449–489.
[4] I.C. Macdougall, Curr. Hematol. Rep. 4 (2005) 436–440.
[5] U. Wattendorf, H.P. Merkle, J. Pharm. Sci. (2008).
[6] I.C. Macdougall, Semin. Nephrol. 26 (2006) 313–318.
[7] B. Berglund, B. Ekblom, J. Intern. Med. 229 (1991) 125–130.
[8] D.J. Verbrugge, L.T. Goodnough, Scand. J. Clin. Lab. Invest. 54 (1994) 55–59.
[9] F. Lasne, L. Martin, N. Crepin, J. de Ceaurriz, Anal. Biochem. 311 (2002) 119–126.
[10] F. Lasne, J. deCeaurriz, Nature 405 (2000) 635.
[11] F. Lasne, J. Immunol. Methods 276 (2003) 223–226.
[12] F. Lasne, L. Martin, J.A. Martin, C.J. de, Haematologica 94 (2009) 888–890.
[13] S. Fishbane, A. Pannier, X. Liogier, P. Jordan, F.C. Dougherty, B. Reigner, J. Clin. Pharmacol. 47 (2007) 1390–1397.
[14] I.C. Macdougall, R. Robson, S. Opatrna, X. Liogier, A. Pannier, P. Jordan, F.C. Dougherty, B. Reigner, Clin. J. Am. Soc. Nephrol. 1 (2006) 1211–1215.
[15] P.G. Barclay, E.R. Fischer, D.C. Harris, Clin. Nephrol. 40 (1993) 277–280.
[16] A. Breidbach, D.H. Catlin, G.A. Green, I. Tregub, H. Truong, J. Gorzek, Clin. Chem. 49 (2003) 901–907.
[17] S. Lamon, N. Robinson, P. Mangin, M. Saugy, Clin. Chim. Acta 379 (2007) 145–149.
[18] World Anti-Doping Agency (WADA). International Standard for Laboratories v 5.0, Montreal, 2008.