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How to detect blood doping with subcutaneous or intravenous injection of C.E.R.A.

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ABSTRACT

Purpose: 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. **Methods:** To evaluate the detection window of C.E.R.A. in healthy volunteers, six subjects received a 200 µg single injection either intravenously or subcutaneously. An ELISA was designed and used to detect C.E.R.A. in the serum of the subjects at intervals of up to 27 days. Physiological response to C.E.R.A. was determined by reticulocyte count. **Results:** While all subjects uniformly responded to C.E.R.A., its detection window varied greatly between individuals. Irrespective of route of administration, the molecule remained detectable for between 8 and more than 27 days in serum. **Conclusion:** We propose the use of this specific ELISA test in combination with an immunoassay that detects EPO and molecules sharing immuno-cross-reactive epitopes to improve the chances of detecting C.E.R.A. in serum samples. This protocol should make C.E.R.A. abuse relatively easy to detect and should prevent the future use of this drug as a doping agent.

Key words:

Erythropoietin; drug abuse; ELISA; ESA

INTRODUCTION

Erythropoietin (EPO) is a 30.4 kDa human glycoprotein hormone produced mainly in the kidney (5). Its chief physiologic effect is the stimulation of erythropoiesis (6), which finally results in the formation of red blood cells, and the consequent improvement of blood oxygen-carrying capacity.

In endurance sports, an increase in the number of erythrocytes is known to enhance athletic performance (4). Therefore ESAs (Erythropoiesis Stimulating Agents) are potent and widely used doping agents (12). As a result, the International Olympic Committee (IOC) Medical Commission banned these drugs in 1990. The current anti-doping urinary test (10–12) allows the differentiation between the various ESAs, such as endogenous EPO, rhEPO, and darbepoetin alpha. C.E.R.A., a continuous erythropoietin receptor activator, is the active ingredient of a new drug for anemia treatment (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 ($\leq 60\text{kDa}$). It has been reported that integration of PEG molecules may maintain in vivo biologic activity of some pharmaceutically active molecules, for C.E.R.A. integration of the PEG moiety has resulted in an increased half-life and increased biologic activity in vivo when compared with epoetin. To avoid illegal abuse of this new agent in sport, it is important to investigate and validate new detection techniques. To this end, we performed a study involving six healthy subjects, the main goals of which were to validate an enzyme-linked immunosorbent assay (ELISA) for detection of C.E.R.A. and to establish the detection window of the molecule, in order to discourage C.E.R.A. doping in sport.

METHODS

ELISA test principle

A specific enzyme-linked immunosorbent assay (ELISA) was used for the measurement of MIRCERA®, using microtitre plates (MTPs) pre-coated with Streptavidin. All material, reagents, and antibodies were provided by Roche Diagnostics (Roche Diagnostics GmbH, Penzberg, Germany). The entire ELISA principle was published elsewhere (7).

Assay validation

All validation experiments were performed at the Swiss Laboratory for Doping Analyses (LAD, Lausanne, Switzerland) during 2 consecutive weeks by two different technicians. For each ELISA assay, the calibration curve and sample concentrations were calculated according to a mathematical model (four-parameter Rodbardfunction). The lower limit of quantification (LLOQ) and the false-positive rate were evaluated. Intra-assay precision, accuracy, linearity, matrix effect and cross-reactivity data were also determined. The entire assay validation was published elsewhere (7).

Subject characteristics

Six healthy Caucasian men aged between 20 and 28 years participated in this study that was approved by the local ethical committee. All anthropometric data were published elsewhere (7).

Protocol

All subjects received one single injection of 200 µg of 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 (IV) injection. The entire protocol was published elsewhere (7).

Protein dosages

Total EPO, ferritin, and c-reactive protein (CRP) concentrations were measured using an IMMULITE 2000 immunoassay system (Siemens Healthcare Diagnostics, Deerfield, IL). Every measure was performed in duplicate. Performance characteristics of the IMMULITE EPO (9), ferritin (11) and CRP (10) assays were previously established.

RESULTS

Assay validation

The LLOQ was evaluated at 30 pg/mL. Therefore, the lowest quality control (LLQC) was defined as 50 pg/mL. Our measuring range was 30–1,000 pg/mL. Intra-assay and inter-assay precision were all lower than 10%. The intra- and inter-assay accuracies were all in the acceptance range defined by the manufacturer. The accuracy between all individual serum

samples was between 80 and 120% when spiked with C.E.R.A. concentrations corresponding to those of the high and the low QC. By extrapolating the obtained calibration curve, the mean measured C.E.R.A. concentration of the 140 tested blank serum samples was estimated at 3.6 pg/mL (median = 0, min = 0, max = 37) (see Figure 1). A receiver operating characteristic (ROC) curve illustrating the relationship between the sensitivity and specificity of the ELISA depending on the chosen cut-off limit was obtained. The cut-off limit of the assay was fixed at 100 pg/mL. According to this value, the assay had a 100% specificity and a 80% sensitivity over a 4-wk period following a single 200 µg C.E.R.A. injection.

A strong cross-reactivity with both epoetin beta and darbepoetin alpha molecules leads to a loss of signal in presence of high concentrations of EPO (1,000-fold the physiologic concentration) in a spiked serum sample. No interference was observed when both types of EPOs were present in physiologic concentration. In contrast, high concentration of PEG molecules in the samples resulted in a slight but not relevant increase in signal strength (data not shown).

C.E.R.A administration

An expected 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. (Figure 2). No significant hematocrit or hemoglobin concentration change was observed throughout the study. In parallel, a highly significant decrease in ferritin concentration ($P < 0.01$) was observed in all subjects following C.E.R.A. injection. These two effects were independent of the route of administration. CRP concentrations did not vary in a significant manner.

C.E.R.A. serum concentrations were determined using ELISA. At day 0, 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 C.E.R.A. IV, the day 1 samples contained the highest concentration of C.E.R.A., after which the concentration decreased rapidly. In contrast, after subcutaneous C.E.R.A. administration, C.E.R.A. concentration was highest between day 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 between 16 and 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 after 27 days (see Figure 3).

Total EPO serum concentration was quantified. Using linear dilutions of epoetin beta, darbepoetin alpha, and C.E.R.A., it was observed that, while the analyzer was very accurate for the measurement of epoetin beta and darbepoetin alpha, C.E.R.A. measurements were systematically underestimated (data not shown). Nevertheless, considering all the samples, a good correlation ($r^2 > 0.98$) existed between the measured C.E.R.A. concentration (ELISA) and the total EPO concentration (Figure 4). Figure 5 depicts the individual curves for total ESAs in serum, including C.E.R.A.

DISCUSSION

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 antidoping assay must be fixed in order to reach a specificity as close as possible to 100% (0% false positive). In our study, a ROC-curve was used to determine the cut-off limit of the C.E.R.A. assay at 100 pg/mL. We decided to 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 shows that when the cut-off limit is fixed at 100 pg/mL, the sensitivity of the assay over a 4-wk 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%).

In all subjects, reticulocyte count increased following C.E.R.A. administration. At the same time, ferritin level decreased rapidly. This response is characteristic of ESA treatment, as the stimulation of erythropoiesis involves extensive production of reticulocytes and iron store depletion. After 6–8 days, this trend reversed for both parameters. CRP concentrations did not indicate the occurrence of any inflammation that could have caused ferritin changes.

At the end of the study, reticulocyte count was lower than the original basal level. This may be due to the ability of ESAs to reduce reticulocytes' maturation period, resulting in an expected decrease in reticulocyte count at the end of the treatment. Note that, as expected in such a short period, this phenomenon is not linked to an increase of the total erythrocyte mass (1).

In accordance with the results of Macdougall et al. (8), the pharmacokinetic profile of the drug in serum differed considerably with route of administration, with IV administration allowing faster distribution of the molecule in blood. This fact has also been demonstrated previously with rhEPO (2). Surprisingly, no direct relation existed between C.E.R.A. concentration and reticulocyte count increases, the latter being independent of the route of

administration. It may be that the reticulocyte production process requires a minimal period to be activated, whatever the availability of the molecule in the organism is. Moreover, C.E.R.A.'s pharmacokinetic profile was not related to ferritin decrease or to any of the other physiologic parameters we measured in the subjects, including body mass index (BMI). Consequently, the huge 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.

It is possible that physical activity during the study period may have had an influence on the molecule behavior and its metabolic clearance. In our study, the subjects, all healthy young men, were not asked to monitor their physical activity, so there are no data available for multivariate analysis. We recommend that future studies should be specifically conducted to estimate the impact of physical activity on C.E.R.A. metabolic clearance, or that is should be included as an analysis in broader-based pharmacokinetic studies of MIRCERA®.

C.E.R.A. measurements from the automatic analyzer were systematically underestimated. The immunoassay for total EPO quantification is based on two different specific anti-EPO antibodies targeting different epitopes (3). C.E.R.A. has a different molecular structure and appears to interact differently with both EPO antibodies. It is possible that the differences in receptor interactions are carried over into differences in interactions with the assay reagents, thus explaining the lower signal obtained from the assay. Nevertheless, the total EPO assay provides a reliable quantitation of samples containing mixtures of C.E.R.A. and endogenous EPO because these molecule share immuno-crossreactive epitopes. Therefore, we recommend beginning C.E.R.A. analysis by screening the total EPO immuno-cross-reactivity of all samples. The reasons for this are twofold: firstly, taking into account the restricted dynamic range of our ELISA, a preliminary assay of total EPO-immuno-cross-reactivity would allow the calculation of the appropriate dilution factor to apply for a reliable quantitative result; secondly, if the ELISA test is positive and the analyzer returns a total EPO concentration above 71.3 mIU/mL (corresponding to a probability of less than 1/1,000 to be comprised of endogenous EPO only), this enhances the chances of the assay providing an adverse analytical finding.

In conclusion, our results clearly demonstrate that C.E.R.A. does not have the required properties of a doping agent for sports performance. This is despite its major clinical advantage of increased dosing intervals. This unsuitability for doping is compounded by much longer detection window for C.E.R.A., which has a detection window that varies considerably, ranging between 8 days and more than 4 wk for a single 200 µg injection in

healthy volunteers, than for rhEPO and darbepoetin alpha (5,9). The combination of a C.E.R.A.-specific ELISA test and an immunoassay determining total EPO immune-crossreactivity would be a considerable hurdle for athletes planning to use C.E.R.A. to increase their performance.

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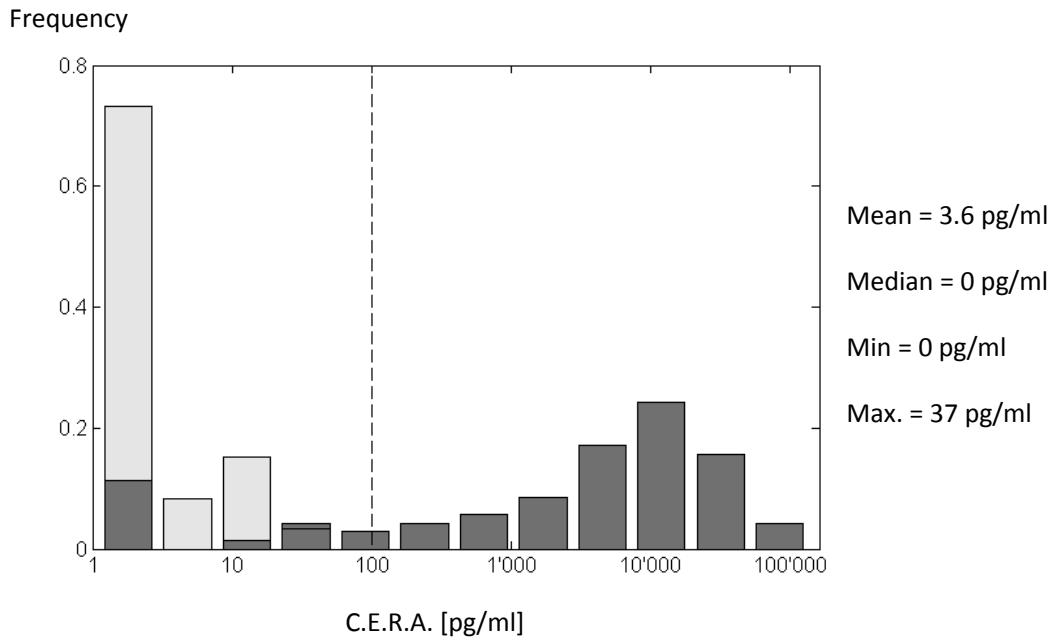


Figure 1: C.E.R.A. concentrations measured on 140 tested blank serum samples (dark grey) plotted together with all serum samples collected over a period of 4 weeks after one single injection of 200 µg of MIRCERA®. Vertical line corresponds to the cut-off limit of the assay (According to this value, the assay had a 100% specificity and a 80% sensitivity).

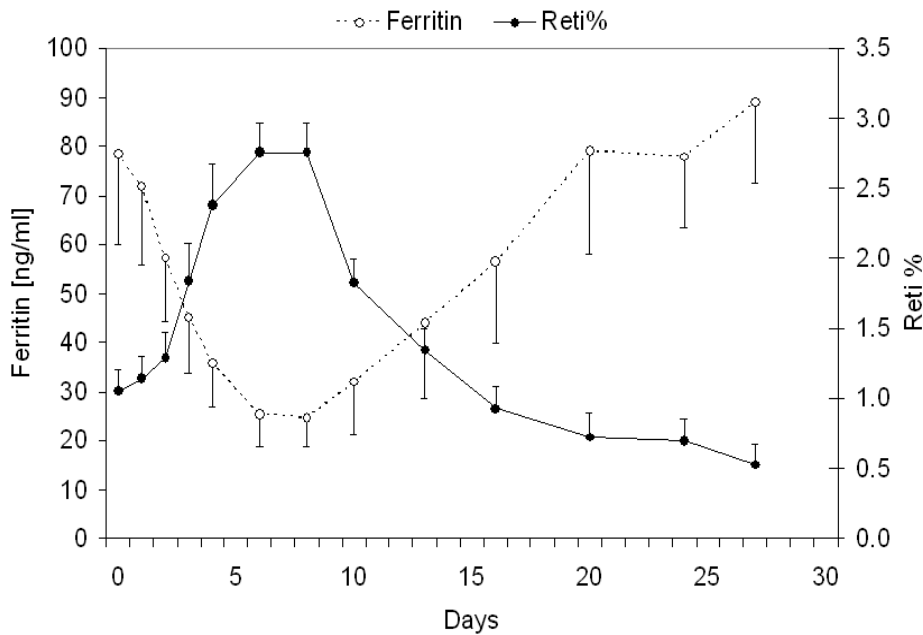


Figure 2: Mean ferritin concentration (ng/mL) and mean reticulocyte count (%) variations observed over time.

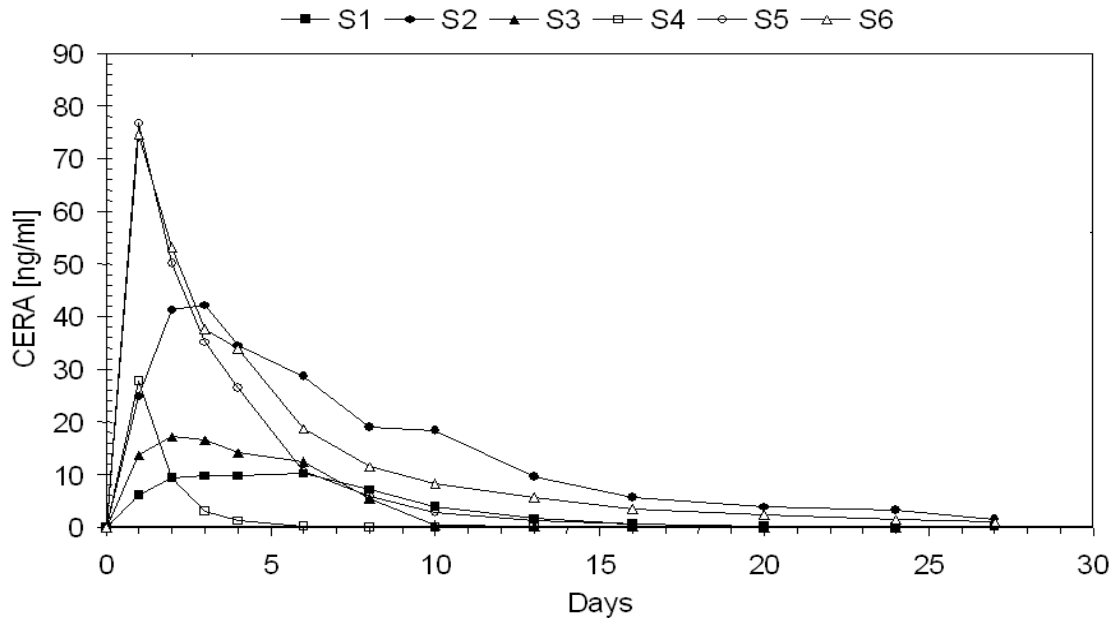


Figure 3: C.E.R.A. concentration (ng/mL) measured in serum over time following a single injection of 200 µg of MIRCERA®. S1, S2, S3 = subcutaneous, S4, S5, S6 = intravenous.

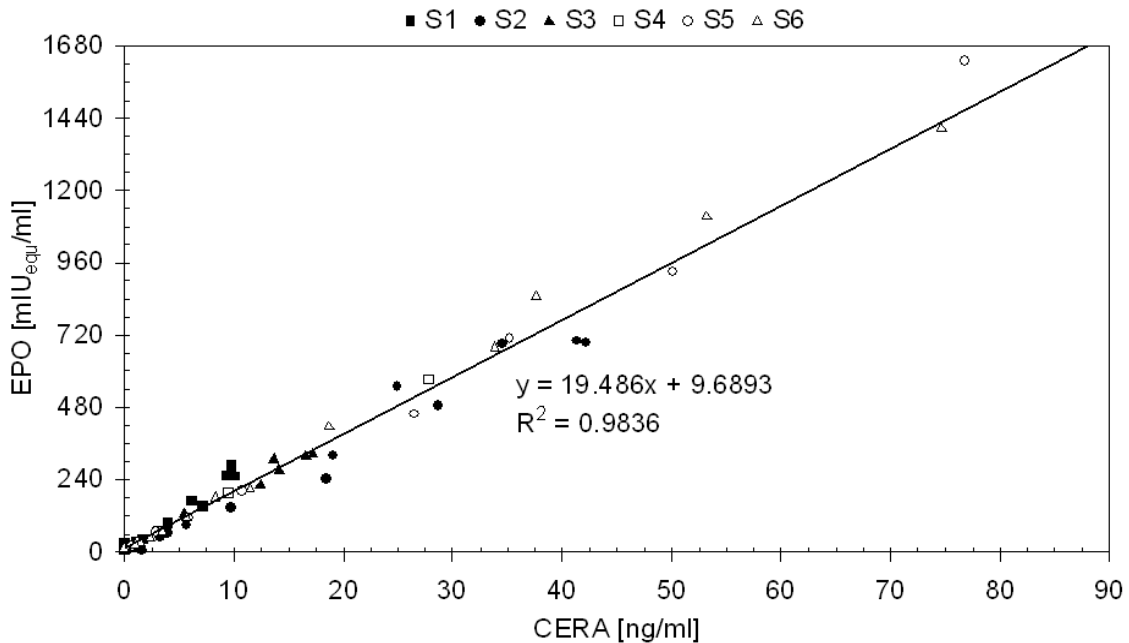


Figure 4: Two-by-two representation of C.E.R.A. concentration [ng/ml] versus total EPO concentration (mIU_{equ}/mL). For C.E.R.A. doping, total EPO measured values can be used to determine C.E.R.A. concentration if they are corrected by use of linear regression.

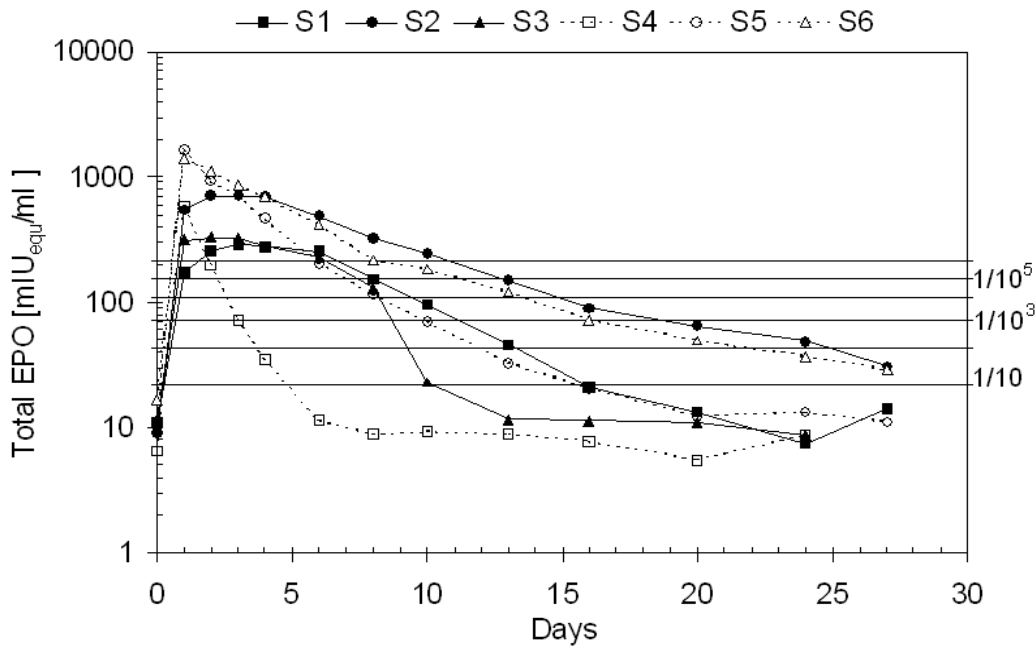


Figure 5: Total serum EPO concentration (mIUeq₁/mL) of the six subjects measured over time. Horizontal lines represent the probability of observing the corresponding endogenous EPO concentration within a normal reference population (9 to 99.999 percentiles). S1, S2, S3 = subcutaneous, S4, S5, S6 = intravenous.