A rapid and simple assay to determine pegylated erythropoietin in human serum

Tom Van Maerken,[1] Annemieke Dhondt,[2] and Joris R. Delanghe[1]

[1]*Department of Clinical Chemistry; and* [2]*Department of Nephrology, Ghent University Hospital, Ghent, Belgium*

Correspondence: Joris R. Delanghe, Department of Clinical Chemistry, Ghent University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium. Phone: +32-9-332-2956; Fax: +32-9-332-4985; E-mail: joris.delanghe@ugent.be

Running title: Determination of pegylated erythropoietin in serum

Word count: 2272

Number of Figures: 3

Number of Tables: 0

Number of Supplemental Tables: 5

Abstract

Stimulation of erythropoiesis by the third-generation erythropoietin drug CERA, a pegylated derivative of epoetin β, has provided valuable therapeutic benefits to patients suffering from renal anemia, but has also rapidly found application as an illicit performance-enhancing strategy in endurance sports. We present here a novel method for selective determination of CERA in serum, based on polyethylene glycol precipitation followed by a commercial homogeneous immunoassay. The developed method was highly discriminating between serum samples from CERA-treated patients and control subjects, as the covalently linked polyethylene glycol chain in CERA strongly enhanced the solubility of the protein in a polyethylene glycol–containing medium. Intravenous administration of CERA could be detected for several weeks in the majority of subjects tested. This assay outperforms the currently available CERA detection methods in terms of simplicity, convenience, cost, and throughput, making it ideal as a screening tool for doping control.

Keywords

CERA, doping, endurance sports, detection

The recent introduction of long-acting pegylated erythropoietin (Epo) is an important improvement for the treatment of anemia in end-stage renal disease (9). Continuous Epo receptor activator (CERA) is synthesized by linking a methoxy-polyethylene glycol polymer to epoetin β, resulting in an extended plasma half-life and prolonged stimulation of erythropoiesis. In CERA, polyethylene glycol (PEG) accounts for ~50% of the molecular mass of the compound (60 kDa).

Illicit use of recombinant Epo and Epo analogues, designated hereafter as erythropoiesis-stimulating agents (ESAs), for blood doping in endurance sports is currently detected by a method that combines isoelectric focusing (IEF) separation with double-blotting (1). This assay is technically capable of detecting CERA in both blood and urine specimens, but the poor urinary excretion of pegylated Epo may hamper the identification of CERA abuse when only a urine sample is analyzed (6). Blood testing has therefore been recommended as the method of choice (6). At present, the vast majority of samples collected for doping control purposes are urine specimens, but there is a growing awareness that blood may be the best matrix for detecting CERA and other forms of ESA-doping (12). The standard IEF-based detection method has proven its value, but is complicated and labor-intensive, and there is also a clear need for a novel and robust CERA assay in blood given the requirement for anti-doping laboratories to report an adverse analytical finding only when demonstrated by two different assay principles (15).

PEG precipitation is widely used in analytical protein chemistry as a fractional precipitating agent and has proven valuable for the detection of serum macro-analytes, e.g. macroprolactine (14) and macro-enzymes (2, 7). We hypothesized that differences in physicochemical characteristics between CERA and endogenous Epo or non-pegylated

ESAs may lead to a different solubility in a PEG-containing medium, and set out to develop a test for specific determination of CERA in serum based on PEG precipitation followed by a homogeneous chemiluminescent immunoassay.

Methods

Subjects and serum samples

A total of 96 patients (41 men and 55 women, aged 16–89 years) at Ghent University Hospital, Belgium, were included in this study. These patients belonged to one of the three following groups: (1) hemodialysis patients treated intravenously with CERA (Mircera®, Roche, Welwyn Garden City, United Kingdom) once every four weeks (dose range 50–350 µg) (n = 40), (2) non-renal patients not treated with CERA or other ESAs (n = 49), and (3) hemodialysis patients not treated with CERA or other ESAs (n = 7). Sex, age, and medication details for all individual patients are presented in Supplemental Tables 1–5. For 25 CERA-treated hemodialysis patients, serum samples were collected at week 1 (day 6, 7 or 8) following CERA administration. The other 15 CERA-treated hemodialysis patients were analyzed either at week 1 (day 6, 7 or 8), week 2 (day 14 or 15), and week 4 (day 27 or 28) after CERA administration (n = 8), or at week 1 (day 7 or 8), week 3 (day 13, 14 or 15), and week 4 (day 27 or 28) following CERA injection (n = 7). A single serum sample was collected for all other patients. The study was approved by the local ethics committee, and written informed consent was obtained according to institutional protocols 2009/250 and 2009/253.

CERA and epoetin β standard solutions

CERA (Mircera®) and epoetin β (Neorecormon®) were kindly provided by Roche. Standard curves were prepared by spiking a serum pool, obtained from persons with a normal hematocrit and not receiving ESAs, with 1000 IU/L CERA or 1000 IU/L epoetin β followed by serially diluting, in twofold increments, the spiked serum with unspiked serum from the same pool. Three separate standard curves were prepared for each ESA.

Experimental protocol of the CERA assay

For each patient sample or standard point, 150 µL serum was supplemented with 150 µL of either a 50% (w/v) PEG-6000 solution or the solvent for PEG-6000 (saline 0.15 mol/L) in separate microcentrifuge tubes. Following vortexing, incubation (37 °C, 15 min), revortexing, centrifugation (9300 *g*, 10 min), and dilution of the supernatant (1:4 in saline 0.15 mol/L), Epo concentration was measured using the Access EPO assay (Beckman Coulter, Brea, CA) on an Access analyzer (Beckman Coulter). The Access EPO assay is a paramagnetic-particle chemoluminescent immunoassay developed for the quantitative determination of Epo levels in human serum and plasma (measurement range: 0.6–750 IU/L). Results are depicted as the PEG/control ratio, i.e. the ratio between the Epo concentration in the PEG-6000–pretreated aliquot and in the saline-pretreated aliquot.

Statistical analysis

All data were analyzed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). One-way ANOVA followed by Tukey's multiple comparison

test was performed to analyze the serum samples for differences in PEG/control ratios among groups (hemodialysis patients treated with CERA, non-renal patients not treated with ESAs, hemodialysis patients not treated with ESAs). Statistical differences in PEG/control ratios between CERA and epoetin β standard solutions were assessed by two-sided two-sample *t*-tests. The level of statistical significance was set at $P < 0.05$ for all analyses.

Results and Discussion

Pegylation of a protein increases its water solubility as a result of the binding of two to three water molecules per ethylene oxide unit of PEG (10), and we therefore reasoned that the presence of a PEG chain in CERA may provide an opportunity for selective detection based on fractional precipitation. PEG itself was chosen as a suitable precipitant, since PEG solutions cause virtually no denaturation of proteins (13) and thus can be expected to preserve the native conformation of relevant epitopes. We decided to combine PEG precipitation with a commercially available, homogeneous immunoassay, with the aim to develop a convenient and simple method for selective determination of CERA in serum samples.

In a first experiment, we analyzed serum samples that were collected from hemodialysis patients one week (at day 6, 7 or 8) following intravenous administration of CERA (dose range 50–350 μg), serum samples taken from non-renal patients not receiving CERA or other ESAs, and sera from hemodialysis patients not treated with CERA or other ESAs. Fig. 1 shows the overall results for each group of patients, while

the clinical characteristics and individual results of all patients are presented in Supplemental Tables 1–3. In CERA-treated hemodialysis patients (n = 25), PEG pretreatment at a final concentration of 25% (w/v) PEG-6000 resulted in a 2.15-fold (95% confidence interval of the mean [95% CI]: 2.09–2.22) change in serum Epo levels relative to control pretreatment with saline. This increase in Epo concentration after PEG pretreatment presumably corresponded to a co-volume effect (voluminous pellet after PEG precipitation; no visible pellet after saline pretreatment). In contrast, the same procedure of PEG precipitation on serum samples from non-renal patients not treated with ESAs (n = 49) yielded a PEG/control ratio, determined as the ratio between the Epo level in the PEG-6000–pretreated aliquot and in the saline-pretreated aliquot, that was, on average, 0.92 (95% CI: 0.87–0.97). Similarly, serum samples from hemodialysis patients not treated with ESAs (n = 7) were characterized by a mean PEG/control ratio of 0.82 (95% CI: 0.63–1.01). These results demonstrated that PEG precipitation of serum samples followed by immunoassay-based measurement of Epo concentration was highly effective in discriminating CERA-treated patients from control patients not treated with ESAs (CERA-treated hemodialysis patients *versus* non-renal patients not receiving ESAs: $P < 0.001$; CERA-treated hemodialysis patients *versus* hemodialysis patients not receiving ESAs: $P < 0.001$).

We next evaluated whether the different solubility of CERA in 25% (w/v) PEG-6000 was due to the presence of the covalently linked PEG chain, by directly comparing CERA with its non-pegylated counterpart, epoetin β. To this purpose, a serum pool, derived from individuals with a normal hematocrit and not treated with ESAs, was spiked with either 1000 IU/L CERA or 1000 IU/L epoetin β. The endogenous Epo level of the

serum pool was 9.12 IU/L. The spiked serum was subsequently serially diluted, in twofold increments, with unspiked serum from the same pool, down to a concentration of 1.95 IU/L of the ESA (corresponding to a total Epo concentration of 11.07 IU/L). As shown in Fig. 2, our assay was capable of discriminating between the standard curve of CERA and the dilution series of epoetin β over the whole concentration range tested ($P <$ 0.01 for each concentration point). A higher PEG/control ratio was consistently observed for the CERA standard solutions compared to the corresponding epoetin β solutions, indicating that the PEG chain in CERA effectively increases the solubility of the molecule.

Finally, we performed a time-course experiment aimed at exploration of the detection window of the assay after intravenous administration of CERA. Fifteen hemodialysis patients, different from those presented in Fig. 1, were selected for this experiment. It was decided, for the patient's comfort, to investigate only leftovers from serum samples that were taken for routine diagnostic purposes, and this resulted in one group of patients that could be analyzed at week 1 (day 6, 7 or 8), week 2 (day 14 or 15), and week 4 (day 27 or 28) following intravenous injection of CERA (n = 8, dose range 50–150 μg), and in another group of patients that could be evaluated at week 1 (day 7 or 8), week 3 (day 13, 14 or 15), and week 4 (day 27 or 28) after intravenous CERA administration (n = 7, dose range 50–300 μg). Fig. 3*A* shows the distribution of the PEG/control ratios that were obtained at each time point for the 8 patients analyzed at weeks 1, 2, and 4 after CERA injection. Clinical characteristics and individual results of each patient are presented in Supplemental Table 4. The minimum PEG/control ratio observed was 1.86 at week 1 (mean PEG/control ratio: 1.95; 95% CI: 1.89–2.01), 1.57 at week 2 (mean PEG/control

ratio: 1.76; 95% CI: 1.64–1.88), and 1.47 at week 4 (mean PEG/control ratio: 1.77; 95% CI: 1.54–2.00). All these values were higher than the maximum PEG/control ratio of the 56 control patients analyzed in Fig. 1, which was equal to 1.30. For the 7 patients evaluated at weeks 1, 3, and 4 after CERA injection, PEG/control ratios lower than 1.30 were not observed at week 1 (mean PEG/control ratio: 1.89; 95% CI: 1.75–2.03), but were recorded for 1 patient at week 3 (mean PEG/control ratio: 1.95; 95% CI: 1.50–2.39) and for 3 patients at week 4 (mean PEG/control ratio: 1.55; 95% CI: 1.04–2.05) (Fig. 3*B* and Supplemental Table 5). Taken together, these data demonstrated that a simple PEG precipitation followed by immunoassay-based Epo measurement was highly efficient in detecting the presence of CERA in the first two weeks after intravenous CERA administration and capable of detecting the majority, but not all, of the CERA-treated subjects at weeks 3 and 4 after injection (dose range 50–300 μg).

We present here a rapid and simple method for selective determination of CERA in serum samples. A possible limitation of the study is that only CERA-treated hemodialysis patients have been included, which is due to ethical and practical considerations that hamper the recruitment of healthy sportsmen for a study with a prohibited doping substance. Another caveat is that we have not analyzed the serum samples in parallel by the conventional IEF-based detection method (1, 6), partly because this test has a number of pitfalls and is not always easy to interpret (3, 4, 8), and partly because the controlled medical setting of this study did not leave any uncertainty on whether CERA had been administered or not. From a practical point of view, the developed assay seems to offer several advantages for CERA doping detection compared to the standard ESA detection procedure. While the latter method is based on a complex and laborious workflow

consisting of immunoaffinity chromatography, ultrafiltration, IEF, and double-blotting, the assay proposed here is extremely simple, straightforward, more economical, and allows a high throughput, making it ideal as a screening tool. It should be noted that other alternative tests for CERA detection have recently been developed. Lamon *et al.* examined an enzyme-linked immunosorbent assay (ELISA) that relies on the combination of an anti-Epo and an anti-PEG antibody to specifically detect CERA doping in blood (5). Reichel *et al.* developed a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) method that allows the detection of endogenous Epo and various ESAs, including CERA, in urine, serum, and plasma samples (12). A drawback of the latter assay is that the sensitivity for CERA detection is relatively low, because binding of SDS to the PEG chain impairs the recognition of CERA by an anti-Epo antibody. This problem has recently been solved by exchanging the SDS for sodium N-lauroyl sarosinate (SARCOSYL), which does not interact with PEG (11). The ELISA and SARCOSYL-PAGE methods for CERA detection have been reported to be sensitive, specific, and easier to perform than the sophisticated IEF-based assay, but they remain more cumbersome and time-consuming than the approach presented here. The availability of various methods with complementary detection principles offers opportunities for improving doping control. When serum samples have been collected from athletes, PEG precipitation combined with immunoassay-based Epo measurement may hold promise as a first-line assay to screen for the presence of CERA in view of its simplicity and speed, followed by one or more confirmatory methods. In addition, it can be anticipated that future drug development efforts will increasingly focus on Epo modifications that allow for enhanced stability, which may, in principle, be detectable by

this assay. In conclusion, the developed method presents a conceptually new approach for selective detection of pegylated Epo in serum and may prove a valuable adjunct in the fight against doping in sport.

Acknowledgments

We wish to thank Roche for their gift of CERA and epoetin β, and C. Wehlou for help in preparing the manuscript.

References

1. **Ayotte C, Gmeiner G, Lasne F, Pascual JA, Reichel C, Saugy M.** WADA Technical Document – TD2009EPO version 2.0: Harmonization of the method for the identification of recombinant erythropoietins (i.e. epoetins) and analogues (i.e. darbepoetin and pegserpoetin) [Online]. World Anti-Doping Agency. http://www.wada-ama.org/rtecontent/document/TD_EPO_2009.pdf [Jan. 17, 2010].
2. **Davidson DF, Watson DJ.** Macroenzyme detection by polyethylene glycol precipitation. *Ann Clin Biochem* 40: 514–520, 2003.
3. **Delanghe JR, Bollen M, Beullens M.** Testing for recombinant erythropoietin. *Am J Hematol* 83: 237–241, 2008.
4. **Franke WW, Heid H.** Pitfalls, errors and risks of false-positive results in urinary EPO drug tests. *Clin Chim Acta* 373: 189–190, 2006.
5. **Lamon S, Giraud S, Egli L, Smolander J, Jarsch M, Stubenrauch KG, Hellwig A, Saugy M, Robinson N.** A high-throughput test to detect C.E.R.A. doping in blood. *J Pharm Biomed Anal* 50: 954–958, 2009.
6. **Lasne F, Martin L, Martin JA, de Ceaurriz J.** Detection of continuous erythropoietin receptor activator in blood and urine in anti-doping control. *Haematologica* 94: 888–890, 2009.
7. **Levitt MD, Ellis C.** A rapid and simple assay to determine if macroamylase is the cause of hyperamylasemia. *Gastroenterology* 83: 378–382, 1982.

8. **Lundby C, Achman-Andersen NJ, Thomsen JJ, Norgaard AM, Robach P.** Testing for recombinant human erythropoietin in urine: problems associated with current anti-doping testing. *J Appl Physiol* 105: 417–419, 2008.
9. **Panchapakesan U, Sumual S, Pollock C.** Nanomedicines in the treatment of anemia in renal disease: focus on CERA (Continuous Erythropoietin Receptor Activator). *Int J Nanomedicine* 2: 33–38, 2007.
10. **Parveen S, Sahoo SK.** Nanomedicine: clinical applications of polyethylene glycol conjugated proteins and drugs. *Clin Pharmacokinet* 45: 965–988, 2006.
11. **Reichel C, Abzieher F, Geisendorfer T.** SARCOSYL-PAGE: a new method for the detection of MIRCERA- and EPO-doping in blood. *Drug Test Analysis*; doi: 10.1002/dta.97.
12. **Reichel C, Kulovics R, Jordan V, Watzinger M, Geisendorfer T.** SDS-PAGE of recombinant and endogenous erythropoietins: benefits and limitations of the method for application in doping control. *Drug Test Analysis* 1: 43–50, 2009.
13. **Simpson RJ.** Concentrating solutions of proteins. In: *Purifying proteins for proteomics: a laboratory manual*, edited by Simpson RJ. New York, NY: Cold Spring Harbor Laboratory Press, 2004.
14. **Vieira JG, Tachibana TT, Obara LH, Maciel RM.** Extensive experience and validation of polyethylene glycol precipitation as a screening method for macroprolactinemia. *Clin Chem* 44: 1758–1759, 1998.
15. **World Anti-Doping Agency.** International standard for laboratories – version 6.0 [Online]. World Anti-Doping Agency. http://www.wada-

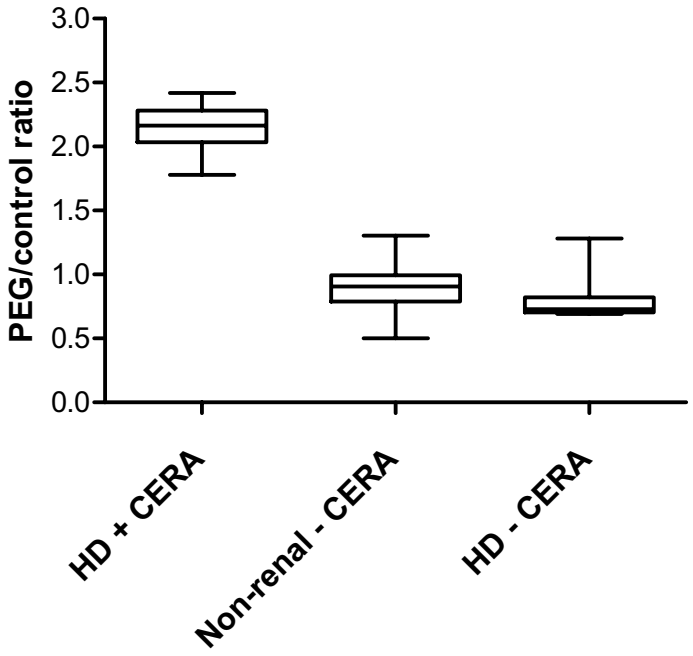
ama.org/rtecontent/document/International_Standard_for_Laboratories_v6_0_January_2009.pdf [Jan. 17, 2010].

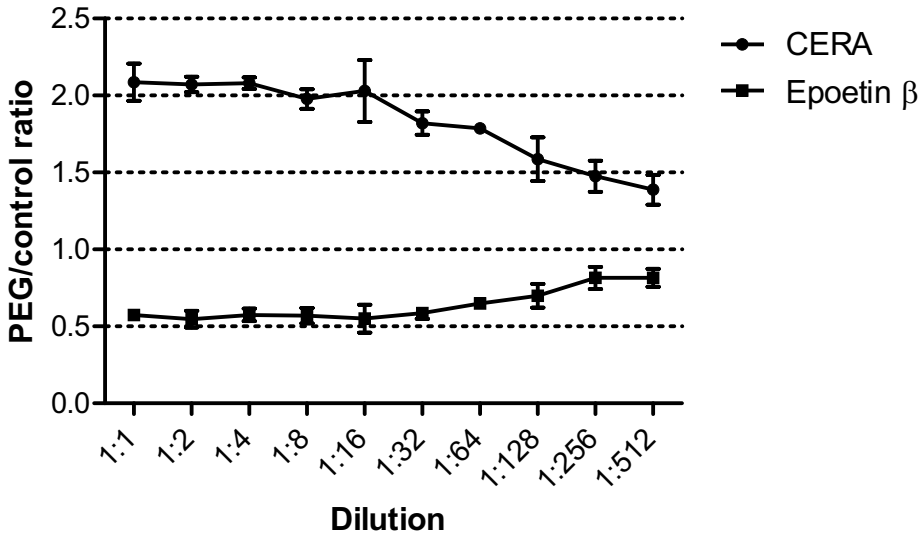
Figure legends

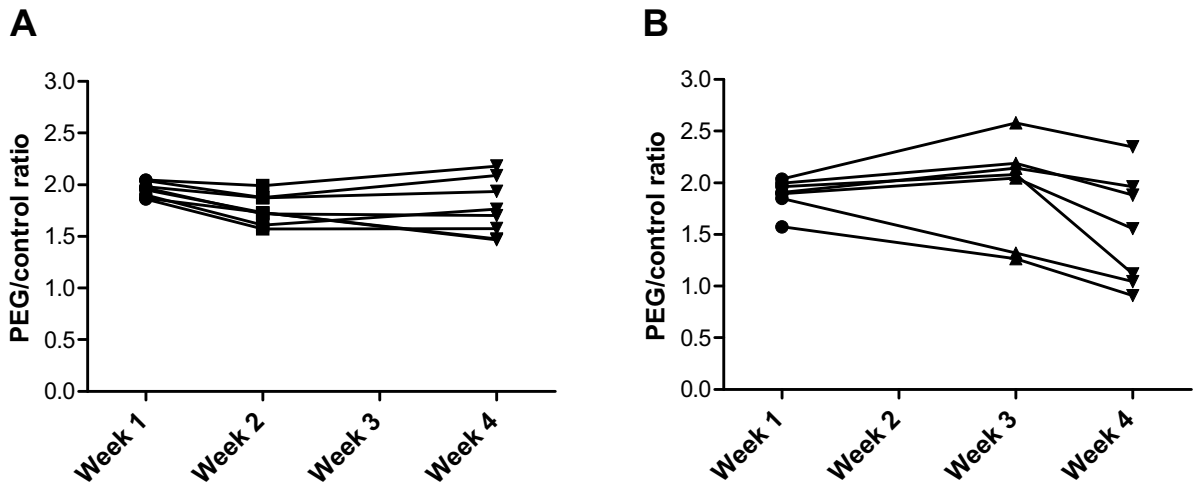
Fig. 1. PEG/control ratios for serum samples from CERA-treated patients and control patients. Serum samples were obtained from hemodialysis patients one week after intravenous administration of CERA (n = 25), from non-renal patients not treated with ESAs (n = 49), and from hemodialysis patients not treated with ESAs (n = 7). PEG/control ratios were determined as described in Methods. The horizontal line, box, and whiskers of each boxplot represent the median, the interquartile range, and the upper and lower range of the data, respectively. HD, hemodialysis.

Fig. 2. PEG/control ratios for CERA- and epoetin β–spiked serum. For each ESA, three separate dilution series were prepared and PEG/control ratios were determined, as described in Methods. Points represent the mean PEG/control ratios of the three experiments, and error bars indicate standard deviations.

Fig. 3. PEG/control ratios for serum samples at different time points following intravenous administration of CERA. Serum samples were collected from hemodialysis patients at 1, 2, and 4 weeks after intravenous CERA administration (*A*; n = 8, dose range 50–150 μg) and at 1, 3, and 4 weeks following intravenous CERA injection (*B*; n = 7, dose range 50–300 μg). PEG/control ratios were determined as described in Methods.


PEG/control ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
HD + CERA
Non-renal - CERA
HD - CERA


2.5
2.0
1.5
1.0
0.5
0.0
PEG/control ratio
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512
Dilution
CERA
Epoetin β


A
B
PEG/control ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Week 1
Week 2
Week 3
Week 4