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Effect of single doses of methoxypolyethylene glycol-epoetin beta (CERA, Mircera™) and epoetin delta (Dynepo™) on isoelectric erythropoietin profiles and haematological parameters

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Erythropoietin (EPO) has been misused in sports for many years due to its performance-enhancing effect. In the last decade, detection of abuse has been possible with isoelectric focusing (IEF) based on the different isoform profiles of endogenous and recombinant EPO. The release of new EPOs on the market, such as the recombinant erythropoietin epoetin delta (Dynepo™) and the chemically modified EPO, CERA (Mircera™) potentially represents analytical challenges to the fight against doping. This study set out to investigate the possibility of and the time window for detecting the administration of a single dose of Dynepo™ and CERA. Our results are in agreement with earlier findings that detection of Dynepo™ is best achieved by combining IEF with SDS-PAGE. Haematological parameters were monitored for possible effects due to the long half-life (130 hours) of CERA in blood. Interestingly, although several haematological parameters were significantly changed after the injection of CERA, the endogenous EPO signal was still present in all collected samples. Due to the long half-life and the large size of the CERA molecule (about 60 kDa), it was uncertain whether CERA would be excreted into urine in detectable amounts unless urine collection was preceded by strenuous physical exercise. We find that CERA can be detected in urine without prior exercise in several, but not all, subjects. CERA is nevertheless best detected in serum with regard to both probability and length of detection, in addition to stability in matrix over time.

Keywords: erythropoietin; CERA; Dynepo™; doping control; haematological parameters; isoelectric focusing; SDS-PAGE; Mircera ELISA

Introduction

Erythropoietin (EPO) is a glycoprotein hormone produced mainly in the kidneys, which stimulates the proliferation and maturation of red blood cells in the bone marrow.[1] Due to the increased oxygen transport capacity in blood, elevated erythropoiesis will increase an athlete's performance, especially in endurance sports.[2–6] Recombinant human EPO (rhEPO) products (aka epoetins) developed for the treatment of anaemia are readily available and have been widely misused in sports for many years. The use of rhEPO or other erythropoiesis-stimulating agents in sports was prohibited by the International Olympic Committee (IOC) in 1988. Since 2001, misuse of first-and second-generation epoetins (e.g. epoetin alfa and beta, and darbepoetin alfa (Aranesp™)) has been detected by the isoelectric focusing (IEF) and double-blotting procedure.[7] Due to the short half-life of these epoetins in the blood, they are only detectable in urine 3–7 days after the last injection.[8–10] The physiological effect, on the other hand, lasts for several weeks.[2] The discrepancy between the detection window and the persistence of effect has made out-of-competition testing of athletes throughout the calendar year and in the weeks leading up to a competition especially important. The long physiological effect caused by doping practices enhancing oxygen transfer, such as the administration of rhEPO, is indirectly measurable as several haematological parameters will reflect the elevated erythropoiesis. The World Anti-doping Agency (WADA) and the International Cycling Union (UCI) have put their efforts together to establish individual haematological passports. [11,12] Such passports will create profiles of each athlete over time, and since individual variation will be much smaller than the variation of a population, this will markedly increase the sensitivity of the blood tests.[13]

Recently, new EPO-analogues, including biosimilars with varying IEF-profiles and chemically and genetically modified epoetins which in different ways challenged the anti-doping community in terms of method of detection, entered the market. One of these, Epoetin delta (Dynepo™), was the first rhEPO to be produced in a human cell line.[14] Epoetin delta was therefore expected to have a more similar isoelectric profile to endogenous EPO, thereby hampering the EPO-analysis which is based on the difference in isoelectric profiles between endogenous and recombinant EPO. Epoetin delta turned out to be more similar to traditional epoetins than anticipated, and differentiation from endogenous EPO after IEF is possible and is supported with the addition of

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SDS-PAGE analysis due to a difference in apparent molecular weight.[15,16] Epoetin delta was taken off the market in March 2009 for commercial reasons. The other product, methoxypolyethylene glycol-epoetin beta or CERA (Continues Erythropoietin Receptor Activator, Mircera™), is an epoetin beta with a 30 kDa polyethylene glycol (PEG) chain attached to it, making this a molecule of more than 60 kDa. CERA binds to and activates the EPO receptor, but due to a lower affinity it is not internalized with the receptor and degraded like endogenous EPO.[17] The excretion of such large molecules from blood to urine is minute under normal circumstances, so even if the PEGylated epoetin were recognized by the EPO-antibodies used in the IEF-method, the concentration of CERA in urine was anticipated to be too low for detection. Detection of CERA in the urine from two cyclists during the 2008 Tour de France [18] showed that the excretion of CERA into urine will exceed the detection limit after strenuous exercise, at least in some athletes.

This study set out to look at the possibility for detection of a single injection of CERA in both urine and serum and to determine the window of detection in the two matrices, as well as to look at the urine EPO profile after a single injection of epoetin delta. Several haematological parameters are sensitive to drugs that increase the oxygen transport capacity of blood, and we wanted to monitor these possible effects in the first two weeks following the injection of CERA, due to its long half-life in blood.

Experimental

Materials

Mircera™ and Creatinine Plus enzymatic creatinine assay was obtained from Roche (Welwyn, Garden City, UK) and Dynepo™ from Shire (Hampshire, UK). Blood was collected in Vacuette K2E tubes and Vacuette Z Serum Sep Clot Activator tubes, both from Greiner Bio-One GmbH (Kremsmünster, Austria). Devices for microfiltration (Steriflip (0.2 µm), HPF Millex-HV filters (0.45 µm), Millex GV filters (0.22 µm), ultrafiltration (Centricon Plus-20, Microcon YM-30), and polyvinylidene difluoride (PVDF) membranes (Durapore, Immobilon-P) were purchased from Millipore (Billerica, MA, USA). Complete Protease Inhibitor Cocktail Tablets were obtained from Roche (Mannheim, Germany) and PBS-tablets were from OXOID (Hamshire, UK). Tris(hydroxymethyl)aminometane and glycine were obtained from Merck (Darmstadt, Germany). Acrylamide/bisacrylamide solution for IEF (PlusOne ReadySol IEF, 40% T, 3% C) and urea was from GE Healthcare (Uppsala, Sweden). Carrier ampholytes (Servalytes 2–4, 4–6, and 6–8) were from Serva (Heidelberg, Germany). The primary antibody used, mouse anti-human EPO antibody (Clone AE7A5), was from R&D Systems (Oxford, UK). Dithiothreitol (DTT), ammonium peroxidisulfate (APS), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), goat anti-mouse IgG-Biotin antibody (B6649) and chemiluminiscent peroxidase substrate (CPS 160) were from Sigma-Aldrich (St Louis, MO, USA). Streptavidin-Horse Radish Peroxidase (HRP) complex was obtained from BioSpa (Milano, Italy). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using ready-made BisTris-gels (NuPAGE, 10%), lithium dodecyl sulfate (LDS) sample buffer, 4-morpholinepropanesulfonic acid (MOPS) electrophoresis running buffer and NuPAGE transfer buffer (Invitrogen, Carlsbad, CA, USA). NIBSC (human urinary erythropoietin, second international reference preparation) was from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK) and BRP (second Biological Reference Preparation of erythropoietin (rhEPO)) was from the European Directorate for the Quality of Medicines (Strasbourg, France). The EPO Purification Kit containing anti-EPO monolith columns, UPD (Urine Precipitate Dissolvation) buffer, washing buffer, desorption buffer, detergent aid, exposure aid and adjustment buffer were from MAIIA Diagnostics (Uppsala, Sweden). The Coomassie® Protein Assay Reagent Kit was obtained from Pierce Biotechnology (Rockford, IL, USA), and the Retinol-binding protein (RBP)/RBP4 ELISA kit from Immundiagnostik AG (Bensheim, Germany). The EPO ELISA-kit used was from StemCell (Vancouver, Canada) andthe enzyme-linked immunosorbent assay (ELISA) for detection of CERA (Mircera ELISA) was purchased from MicroCoat Biotechnologie GmbH (Bernried, Germany).

Methods

Thirteen healthy and recreationally active volunteers were recruited for the study, of which eight were well-trained, non-competing students from the Norwegian School of Sports Sciences. Eight subjects (four women (P#9, P#11, P#14 and P#15) and four men (P#4, P#7, P#8 and P#10) received CERA, and five subjects (one woman (P#2) and four men (P#1, P#3, P#5 and P#6) received epoetin delta. The inclusion criteria were normal haematological parameters, including haemoglobin between 12.5 and 16.5 g/dl for men, 11.5 and 15.5 g/dl for women, and blood pressure $\leq$ 90 (diastolic) and $\leq$ 140 (systolic). Each participant received a single subcutaneous injection of either CERA (75 µg to men, 50 µg to women) or epoetin delta (4000 IU), after which they stayed seated for 30 min to make sure there was no adverse reaction to the injected drugs. This study was approved by the local Data Protection Officer and the Regional Ethics Committee. Informed consent was obtained in writing after informing all participants orally and in written form about the drugs and the study.

Control urine, and EDTA blood and serum samples were collected prior to the injections. Urine was collected every morning for seven days and after exercise on three days in the period between two and seven days post injection of the subjects that received CERA, and every morning and evening for seven days after injection of epoetin delta. Blood was collected on days 3, 5, 7, 10, and 14 post-injection of CERA. Two of the CERA-receiving subjects (#14 and #15) were recruited later than the first six, and urine and serum were collected for direct detection purposes approximately on days 5, 7, 10, 14, 17, and 21 post-injection. Blood was sampled on day 4 from those that received epoetin delta, to monitor the haematological parameters. Collection of blood was standardized as non-fasting samples and the participants stayed seated for 10 min prior to sampling. Blood in the Vacuette Z tubes was allowed to coagulate for 30 min before centrifugation at 1500g for 10 min, RT. The separated serum was transferred to a separate tube and stored at $-20\,^{\circ}$C until measurements of EPO (Immulite 2500 EPO immunoassay), serum transferrin receptor (sTfR) (BN ProSpec nephelometry, Siemens Healthcare Diagnostics) and CERA (Mircera ELISA) were performed, or until EPO was affinity purified on EPO affinity columns (EPO Purification Kit). EDTA blood samples were homogenized on a roller for 15 min after collection and subsequently analyzed on a Sysmex 2000i XT haematology analyzer (Sysmex, Norway).

The urine samples were subjected to either concentration by ultrafiltration or affinity purification on EPO affinity columns (MAIIA AB), prior to the isoelectric focusing and the double blotting

procedure for EPO isoform analysis.[7] Briefly, 2 ml 3.75 M Tris-HCl pH 7.5 and 400 µl Complete™ was added to 20 ml urine to dissolve urine precipitates prior to filtration and to prevent proteolytic degradation. The urine samples were then passed through Steriflip filters (0.2 µm), followed by ultrafiltration on Centricon Plus-20 and Microcon YM-30 filters, both with MWCO of 30 kDa. The urine samples were concentrated 400 times to a final retentate of about 50 µl. The total protein concentration in the urine samples was measured with Coomassie Protein Assay Reagent prior to ultrafiltration.

Affinity purification on the recently validated EPO affinity columns[19] was performed according to the manufacturer's instructions (MAIIA AB). Briefly, 2 ml UPD-buffer was added to urine aliquots of 20 ml, the mixtures incubated for 10 min at RT to dissolve precipitates followed by heating on a simmering water bath for 9 min. After cooling, the samples were diluted with 20 ml mix (1 : 20 Protection Aid, 1 : 100 Detergent Aid in H_2O) and filtered through HPF Millex-HV filters. Urine samples were passed through the monolith columns at 1 ml/min using a vacuum manifold at a pressure of −5 kPa. After washing, the isolated EPO was eluted with 50 µl desorption buffer into 5 µl adjustment buffer and stored at −20 °C until use. Serum (0.5–2 ml) was diluted 1 : 10 in dilute UPD-buffer (1 : 20 in H_2O), filtered through Millex GV filters (0.22 µm) and then affinity purified using the same columns and desorption buffers as for urine.

IEF and double-blotting was performed as described.[7] Briefly, 20 µl retentates or eluates were saturated with urea and focused on an IEF-gel with a pH-range of 2–6. Focused proteins were electro blotted to a PVDF-membrane, incubated with 5 mM DTT in PBS (1 h, RT) and blocked with 5% skimmed milk powder in PBS (1 h, RT). After incubation with primary antibody (1 : 1000), the bound EPO-antibodies were blotted in semi-dry, acidic conditions (0.7% acetic acid) to a second PVDF-membrane. After incubation with biotinylated secondary antibody (1 : 4000) and strepdavidine-HRP complex (1 : 1500), EPO isoforms were visualized by chemiluminescence in a CCD (Charge Coupled Device) camera (FUJIFILM LAS 1000 Plus, Science Imaging Scandinavia, Sweden). EPO isoform profile analysis was performed using the GASepo software (v1.3, ARC Seibersdorf Research GmbH, Austria).[20]

Prior to SDS-PAGE, EPO was isolated using the human EPO ELISA, as described previously.[16] Briefly, 20 µl retentate was mixed with 80 µl Buffer A, loaded in a plate well and incubated overnight on a shaker at 4 °C. The wells were washed with 4 × 300 µl PBS, and EPO was eluted by incubation in 30 µl 1× LDS-sample buffer under reducing conditions at 95 °C by pipetting a few times up and down. Affinity purified samples were separated by SDS-PAGE at constant voltage (125 V) for 110 min on 10% TrisBis (1 mm) gels, and the separated proteins were blotted to PVDF-membranes using the XCell SureLock Mini-Cell and XCell II Blot Module from Invitrogen (Carlsbad, CA, USA). All subsequent steps were as described for the IEF gels.

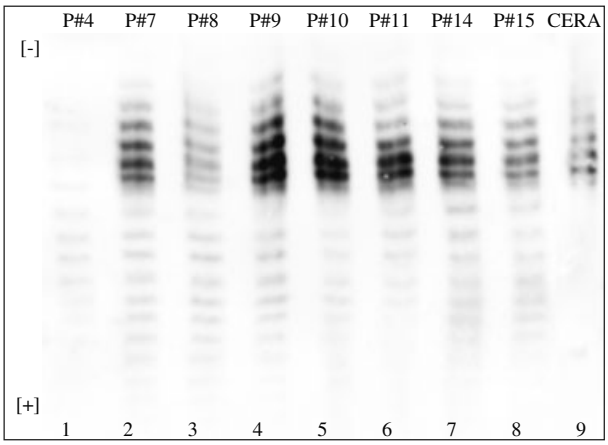
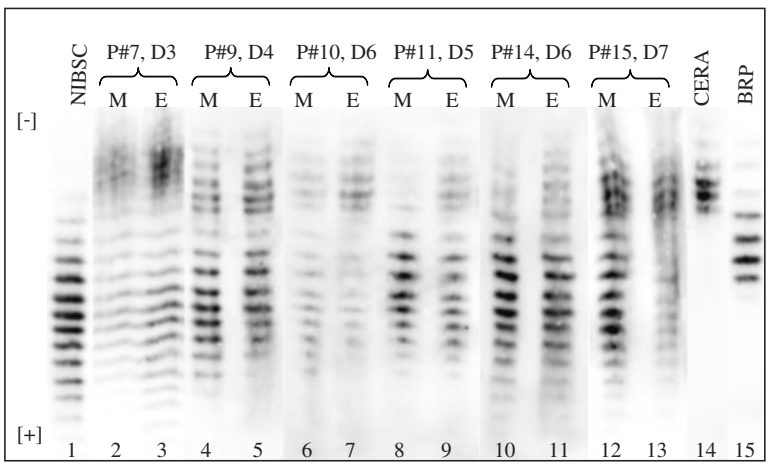


Figure 2. IEF of serum samples after CERA injection. Affinity purified serum samples collected 7 days post injection from all the CERA receiving subjects. Note that CERA is detectable a week after injection in serum from all subjects apart from P#4.

A



B

P#	Urine	SG	CR (mmol/L)	RBP (µg/L)	UP/CR (mg/mmol)	RBP/CR (µg/mmol)
7	M	1.030	20.4	233	5.0	11.4
	E	1.023	13.7	60	5.6	4.4
9	M	1.024	8.1	63	6.8	7.8
	E	1.012	6.6	33	11.7	5.0
10	M	1.019	8.5	27	8.0	3.2
	E	1.019	8.9	51	14.5	5.7
11	M	1.020	9.5	81	6.6	8.6
	E	1.020	11.3	129	11.2	11.4
14	M	1.015	7.2	55	13.9	7.6
	E	1.010	5.0	34	18.6	6.8
15	M	1.018	6.5	17	6.9	2.7
	E	1.020	7.4	12	7.3	1.6

Figure 1. IEF and protein measurements of urine samples after CERA injection. A. Urine samples were collected in the morning (M) and post-exercise (E). Lane 1: NIBSC (0.3 ng); lanes 2–13: Samples from the persons (P) receiving CERA, collected on different days (D) post injection; lane 14: CERA (0.4 ng); lane 15: BRP (0.25 ng). The urine samples were either concentrated by ultra filtration (lanes 2, 3, 12 and 13) or affinity purification (lanes 4–11), and loaded onto the IEF-gel. Affinity purification resulted in better separation of the CERA bands. Note the stronger CERA signal after exercise for most subjects. B. Specific gravity (SG), creatinine (CR) and retinol binding protein (RBP) concentrations, the total urine protein to creatinine ratio (UP/CR) and retinol binding protein to creatinine ratio (RBP/CR) of the morning and post-exercise urines displayed in A.

Table 1. Detection window for CERA in urine and serum samples after IEF and double blotting

	Urine		Serum	
Subject	Last positive	First negative	Last positive	First negative
P#4	none	all	none	All
P#8	none	all	D7	D10
P#10	D6 M+E	none	D14	none
P#7, P#9, P#11	D7 M+E	none	D14	none
P#14	D6 M+E	D11 M	D14	D17
P#15	D11 M+E	D14 M	D14	D17*

D=day of sample collection; M=morning urine; E= first urine after exercise. * =CERA bands detectable, but not fulfilling the criteria for an adverse analytical finding.

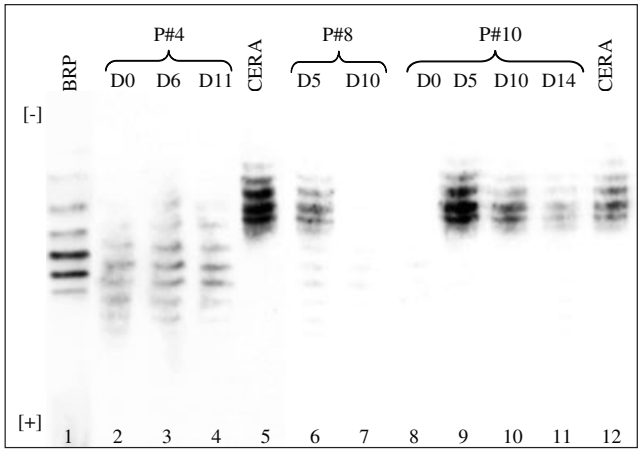


Figure 3. Highly variable detection window for CERA in serum. Lane 1: BRP (0.25 ng); lanes 2–4: P#4 on days 0, 6 and 11 post-injection; lane 5: CERA (0.6 ng); lanes 6 and 7: P#8 on days 5 and 10; lanes 8–11: P#10 on days 0, 5, 10 and 14, respectively; lane 12: CERA (0.4 ng). The window of opportunity for CERA in serum varies highly between individuals: while CERA was detectable for 14 days in 6 of the 8 subjects (illustrated with P#10, lanes 8–11), the concentration fell below the detection limit much faster for one subject (P#8, lanes 6 and 7) while we were never able to directly detect CERA in serum from P#4 (lanes 3 and 4).

The Mircera ELISA kit, recently validated for CERA measurements in serum,[21] was used according to the manufacturer's instructions.

Graphing of the hematological parameters was done in Prism5 software from GraphPad (La Jolla, CA, USA).

Results and Discussion

Electrophoretic data

Using the standard EPO test with IEF and double-blotting, we were able to detect CERA in urine collected in the morning without prior exercise, in six out of eight subjects on days 2–7 after injection (Figure 1A). This was an unexpected result, as we believed the excretion of CERA into urine would be too low for detection with the IEF-method. When using ultrafiltration as the method for concentrating protein, the CERA bands from the urine retentates where often smeared and fuzzy (Figure 1A, lanes 2–3) or streaked (lanes 12–13) compared to standard deposited directly on the gel (lane 14). As affinity purification of the serum samples on anti-EPO monolith columns gave rise to nicely separated CERA bands (Figure 2), the CERA excretion urines were affinity purified on the same columns with the addition of PEG-polymers (Exposure aid, part of the EPO purification kit) in order to expose the antigenic site for the antibodies. This greatly improved the resolution of the CERA bands, and the bleeding of bands between lanes disappeared as well (Figure 1A, lanes 4–11).

The amount of CERA present in urine varied greatly amongst the subjects (Table 1). While we were never able to detect CERA in urine from two of the eight CERA-receiving subjects (P#4 and P#8), CERA was still detectable in urine from P#15 collected 11 days after injection.

The IEF signal of CERA appeared stronger in the post-exercise urine samples than in the morning urines (Figure 1A), indicating

Table 2. Measured concentrations of EPO (Immulite 2500 EPO) and CERA (Mircera ELISA) in serum samples. Serum samples in which CERA was detectable after IEF are marked with grey

	D0		D3		D5		D7		D10		D14	
	ng/ml		ng/ml		ng/ml		ng/ml		ng/ml		ng/ml	
Subject	EPO	CERA	EPO	CERA	EPO	CERA	EPO	CERA	EPO	CERA	EPO	CERA
P#4	0.12	0.013	0.15	0.20	0.17	0.11	0.12	0.10	NM	0.04	0.11	0.02
P#7	0.09	0.012	0.62	3.70	0.48	2.59	0.34	1.27	0.05	0.03	0.07	0.03
P#8	0.09	0.044	0.38	1.51	0.42	1.18	0.09	0.20	0.08	0.07	0.07	0.06
P#9	0.14	<0.011	1.27	5.51	0.96	4.30	0.73	3.28	–	–	0.30	0.80
P#10	0.12	<0.011	1.33	6.17	0.81	3.40	0.66	3.05	0.48	1.10	0.27	0.76
P#11	0.07	0.014	1.27	5.47	0.85	4.29	0.61	2.50	0.27	0.82	0.17	0.49

	D0		D7		D10		D14		D17		D21	
	ng/ml		ng/ml		ng/ml		ng/ml		ng/ml		ng/ml	
Subject	EPO	CERA	EPO	CERA	EPO	CERA	EPO	CERA	EPO	CERA	EPO	CERA
P#14	0.15	<0.011	0.59	2.14	0.17	0.15	0.14	0.05	0.14	0.02	–	–
P#15	0.19	0.13	–	–	0.43	1.25	0.38	0.67	0.21	0.40*	0.21	0.33*

* = CERA bands detectable after IEF, but not fulfilling the criteria for an adverse analytical finding.

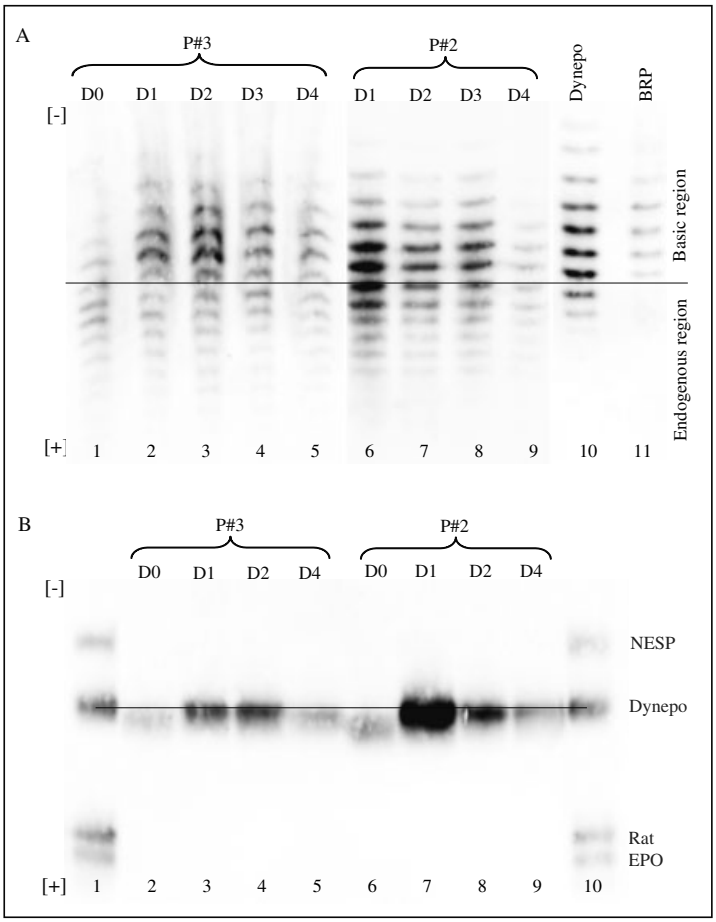


Figure 4. IEF and SDS-PAGE of affinity purified urine samples after epoetin delta injection. A. Lanes 1–5: morning urine samples from P#3 collected prior to injection (D0) and on days 1-4 post injection; lanes 6–9: morning urine samples from P#2 collected on days 1–4 post injection; lane 10: epoetin delta(0.3 ng); lane 11: BRP (0.25 ng). B. Urine was collected prior to (D0) and the days following injection (D1–D4). The shift in apparent Mw for the EPO band was visible D1 and remained until D4 post-injection for P#2, while it had moved towards its D0-position for P#3 on D4. Urine retentates (20 µl) were affinity purified with the StemCell EPO ELISA kit prior to SDS-PAGE. Standards (rat EPO (0.7 ng), epoetin delta (0.4 ng) and NESP (0.1 ng)) were loaded directly on the gel (lanes 1 and 10) in LDS-sample buffer after heating.

increased excretion of CERA after exercise in agreement with a previous report.[18] The phenomenon of transient proteinuria after strenuous exercise (athletic pseudonephritis) is thought to be caused by reduced tubular reabsorption and/or increased glomerular permeability.[22] To find out whether the stronger post-exercise CERA signal was due to transient proteinuria or just more concentrated urines, we measured total urine protein (UP), retinol binding protein (RBP), and creatinine (CR) concentrations along with specific gravity (SG), and calculated the ratios UP/CR and RBP/CR of the collected pairs of morning and post-exercise urines (Figure 1B). Creatinine excretion is a fairly constant process,[23,24] not significantly affected by exercise,[25] and the increase in UP/CR seen for P#9, #10 and #11 after exercise suggests that a transient proteinuria of either glomerular (decreased RBP/CR) or a mixed glomerular/tubular (increased RBP/CR) origin has taken place, allowing an increased excretion of larger molecules like CERA.

An inter-individual variation was also seen in the serum concentration of CERA (Tables 1 and 2). Direct detection of CERA was possible 14 days post-injection in five subjects and for 7 days in the remaining two responsive subjects (Table 1, Figure 2). We were never able to directly detect CERA in any of the collected serum samples from P#4. The CERA concentrations for D17 and D21 from P#15 were well over the cut-off limit when measured with the Mircera ELISA (Table 2), but after IEF the concentration of CERA was too low to fulfil the positivity criteria, as only the two least basic CERA bands were still visible (not shown).

None of the collected serum samples from P#4 contained CERA above the limit of detection (Figure 3, lanes 2–4), and there was no effect on the haematological parameters (Figure 6). To make sure there were no technical faults around the injection that caused the lack of response, this individual was later re-injected with 75 µg CERA. Since the possibility existed that this subject had measureable amounts in his serum the first few days following injection which were missed in the first round, we collected blood daily the first five days after his re-injection. Nor this time were we able to detect CERA with IEF in any of the early or the later (up to day 14) serum samples (not shown), and once again there was no measurable haematological effect. A very slow release of CERA into the systemic circulation of this individual might explain these results. This is supported by the fact that we measured CERA above the cut-off limit (100 pg/ml) with the Mircera ELISA in his serum samples collected between day 3 and 7 (Haematological data and Figure 7).

The continued presence of CERA did not seem to turn off the endogenous EPO production in our subjects during the collection period, as hEPO was present in all the CERA positive

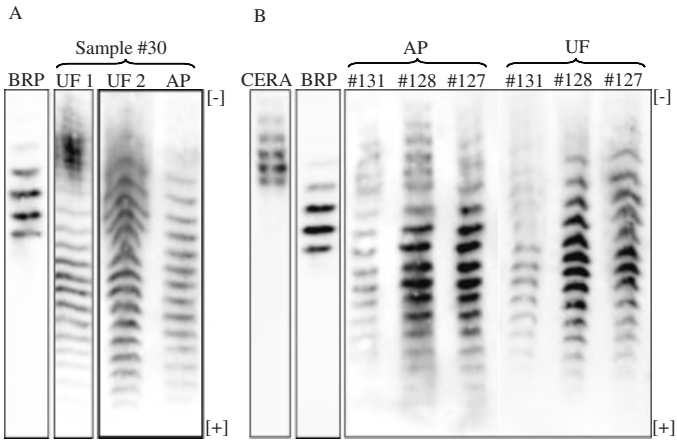


Figure 5. CERA in urine is sensitive to degradation during long-term storage. A. Urine sample prepared at different times (UF1 and UF2, 2 weeks and 10 months post collection, respectively) and with different methods (UF: ultrafiltration, AP: affinity purification) prior to IEF. Note the disappearance of the CERA after storage (−30 °C) and re-thawing, which took place in some, but not all, CERA positive urines. B. Urines (#127, #128 and #131) originally positive for CERA (not shown) were stored for several months and then concentrated by both affinity purification and ultra filtration. Note that in these urines the CERA-bands are only visible using affinity purification as preparation method for IEF.

samples (Figure 1A). This was somewhat surprising to us as the haematological data indicated increased erythropoiesis caused by the injected drug, which previously has been shown to coincide with a shift from an endogenous to a basic (recombinant) EPO profile. [10] Due to the relatively high concentration of CERA in the serum samples, the amount of serum had to be reduced from the initially used 2 ml to 0.5–1 ml, in order to make the endogenous EPO profiles visible.

We noticed that the CERA in some of the urine samples degraded after storage. This phenomenon is illustrated with sample #30 shown in Figure 5A, which had a clearly visible presence of CERA a few weeks after collection (lanes marked UF1). After several months storage (at −30 °C) and an extra round of thawing, CERA is no longer visible (lanes marked UF2), and in some samples the basic bands 1–4 seemed stronger (as seen here). The latter observation may indicate breakdown of CERA into its components epoetin beta and PEG. Affinity purification of these samples confirmed that the amount of CERA, if any, was now below the detection level (lanes marked AP). In other urine samples, CERA was still detectable with double blotting after months in the freezer when affinity purified on EPO columns, but not when concentrated by ultrafiltration (Figure 5B, AP and UF, respectively). CERA was readily detected in all tested serum samples after nine months in the freezer (Figure 2), and thus seem more stable in serum compared to urine, at least in our hands. This may simply be due to the far higher CERA concentration in serum compared to urine, making the CERA more sensitive to degradation in urines with a higher enzyme and/or bacteria concentration.

SDS-PAGE analysis was also conducted on both urine and serum samples, revealing a narrow CERA band with a higher apparent molecular weight than seen for other EPO analogues. The sensitivity for CERA on SDS-PAGE was poor though; even in serum samples where the measured CERA concentration was 5–10 times higher than the EPO concentration, the endogenous EPO band was labelled much stronger than the CERA band (not shown). Reichel *et al*. has since then shown SARCOSYL-PAGE to be a more sensitive method for the detection of CERA in serum.[26,27]

Because we did not expect to detect CERA in the urine samples; especially when collection was not preceded by exercise, the first six volunteers were only asked to collect urine the first seven days after the injection. The surprising presence of CERA even in the morning urines, as well as the high concentration of CERA in three of six sera on day 14, prompted us to recruit new volunteers in order to collect samples for an extended period of time. Therefore, looking at the respective windows of opportunity for CERA in serum and urine, comparison is only possible in three subjects (P#8, P#14, and P#15), since for the remaining four subjects the urine samples were all positive up to the last day of collection (Table 1). For P#8, CERA was never detected in urine, while the sera collected up to day 7 were all positive. The fact that the CERA concentrations in serum for P#9, P#10, and P#11 on day 14 were in the same range as that of P#15 (Table 2), makes it tempting to speculate that CERA would have been detectable in urine from these three individuals for at least 10–12 days, just as it was for P#15. Nevertheless, detection of CERA has a higher probability and a longer window of opportunity in serum than in urine.

A single injection of 4000 IU epoetin delta was detectable in the urine samples collected from the five subjects from day 1 to 4, but due to the presence of endogenous EPO in the urines, IEF alone was not sufficient to fulfil the criteria for an adverse analytical finding (AAF) set by WADA (Figure 4).[28] The importance of additional evidence like SDS-PAGE gels for epoetins like epoetin delta[16] is clear from Figure 4B, where the increase in apparent molecular weight, as well as a gradual narrowing of the protein band compared to the bands of endogenous EPO and negative control NIBSC, is visible.

Haematological data

The haematological profiles displayed large inter-individual variation in the response to a single injection of CERA (Figures 6 and 7), ranging from no response in one subject to significant effect on several haematological parameters in others. The effect seen in the individual parameters differed in size but had similar time-pattern: early markers of increased erythropoiesis, like immature reticulocyte fraction (IRF) and percent reticulocytes (%Ret), were first to peak between day 5 and day 7. Haemoglobin (Hgb), hematocrit (Hct) and serum soluble transferrin receptor (sTfR) all peaked at day 10, though the effect in Hgb and Hct were both slower and of a lesser magnitude than in sTfR.

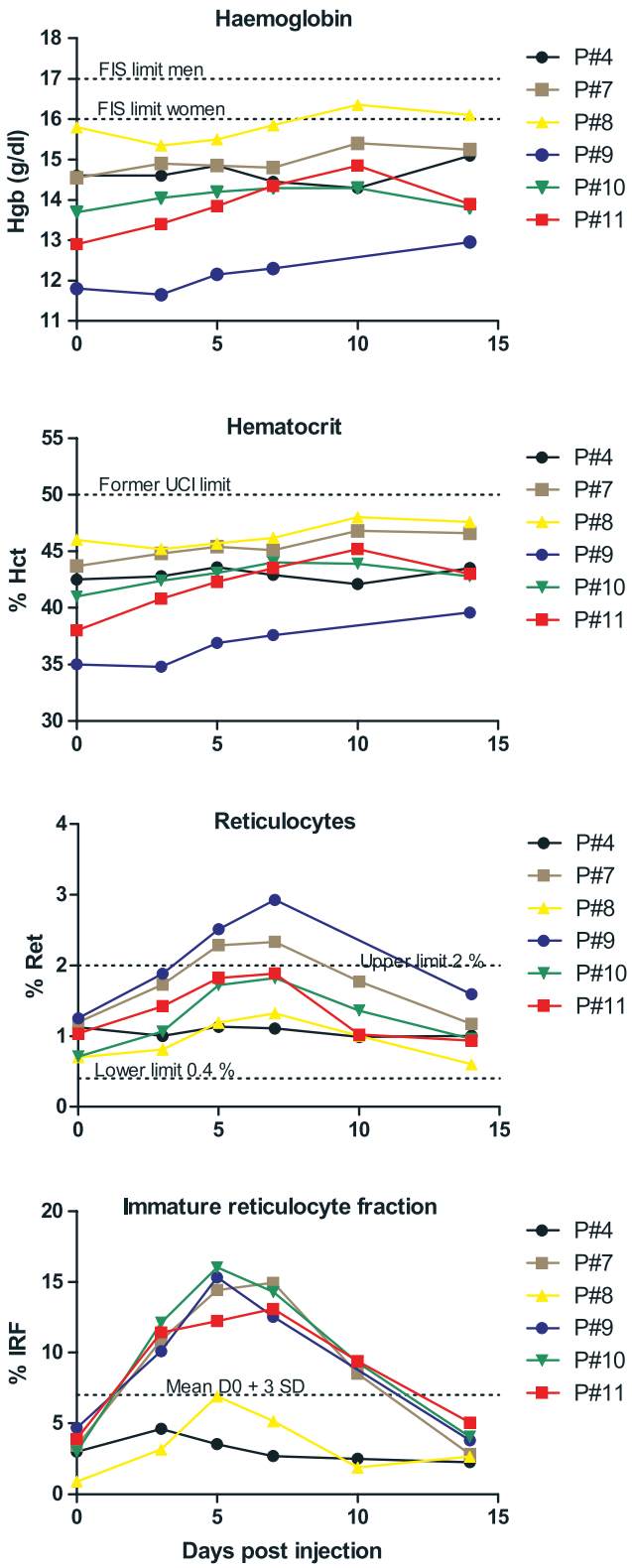


Figure 6. Effect of CERA on haematological parameters. Haemoglobin (Hgb), hematocrit (% Hct), reticulocytes (% Ret) and immature reticulocyte fraction (IRF, Sysmex) were measured in six subjects prior to CERA injection (D0) and on the depicted days post-injection. A single injection had significant effect in several subjects, e.g. Hgb increased from 12.9 g/dl to 14.9 g/dl and % Hct increased from 38% to 45% for P#11 from D0 to D10, and by D7 % Ret had increased from 1.3% to 2.9% for P#9. No response was detectable for P#4. Data for day 10 is missing for P#9.

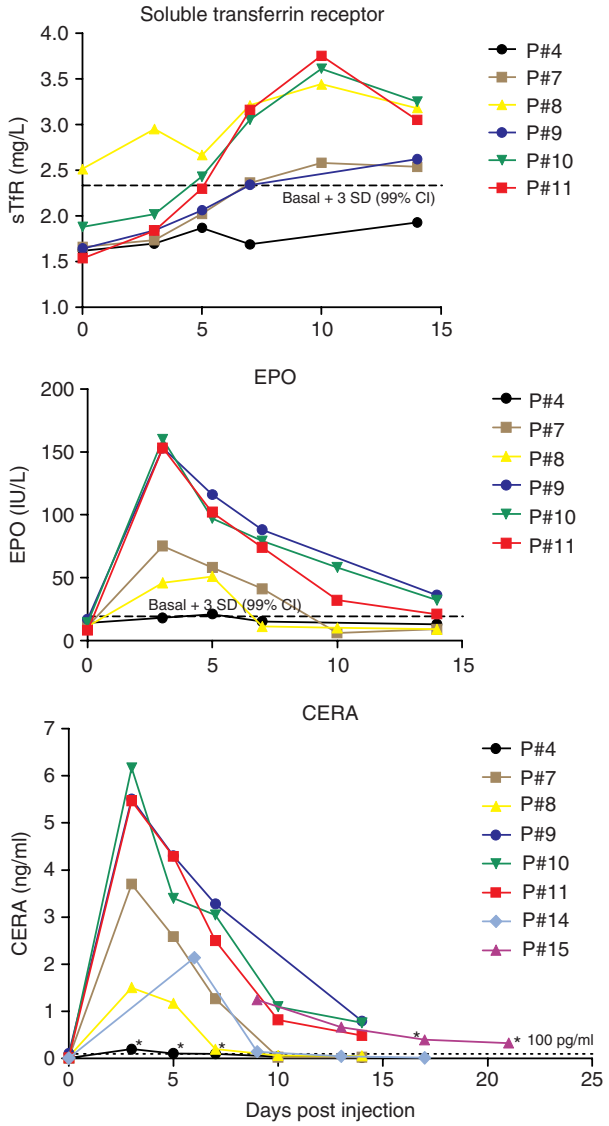


Figure 7. Serum levels of soluble transferrin receptor (sTfR) (top panel), EPO (middle panel) and CERA (lower panel) after a single CERA injection. EPO levels were measured with Immulite® EPO 2500 Immunoassay and CERA levels were measured with Mircera ELISA. Data for D7 is missing for P#15.

The increase in Hgb was most noticeable in the two females subjects: P#11 showed a significant increase in Hgb from 12.9 prior to injection to 14.9 on day 10; an increase of 15%, while P#9 had an increase of 10% on days 7 and 14 (Figure 6, no sample was collected on day 10 for this subject due to illness). The increase in Hgb amongst the males was 3–6%, which is within what is considered normal variation. The effect on % Hct and % Ret was also larger for the female than for the male subjects (Figure 6). While none of the subjects reached the (former) limits for Hgb and % Hct set by UCI or the International Skiing Federation (FIS), % Ret reached above 2% on day 5 in subjects P#7 and P#9. A decline in % Ret was evident by day 10 and this parameter was almost back to base value for all subjects by day 14. IRF was the parameter with the most rapid response; there was a marked increase on days 3 to 10 in four of five responsive subjects (Figure 6). By day 14, IRF was back to pre-injection levels for all subjects. IRF has been shown to be a sensitive marker of increased erythropoiesis

in athletes undergoing altitude training and the subsequent fall in EPO-levels on return to sea-level.[27] In addition, IRF values tend to be higher in elite athletes than in the general population.[29] Still, as information regarding training or stay at altitude for the individual athletes is known to the anti-doping authorities, and, as IRF values are stable throughout the season regardless of training programme and competitions,[30] IRF could be an interesting additional marker of erythropoietic status in the individual blood passports.

The concentration of sTfR increased steadily in all responsive subjects the first ten days, while a decline in the serum concentration was evident on day 14 (Figure 7, upper panel). Elevated sTfR levels have previously been positively correlated with basic (recombinant) profiles of urinary EPO and have therefore been considered a good indicator for when to conduct an EPO-analysis with the IEF-method.[10] The ON-hes model which includes serum concentrations of sTfR in addition to EPO, was found to be more sensitive in the detection of increased erythropoiesis than the model excluding this parameter (ON-he).[31] When applied to our data, the increase in ON-hes score was between 15 to 30% on days 5–10 for subjects P#9, P#10, P#11, and P#14.

In this study, the serum concentration of total EPO (measured with Immulite 2500) peaked already on day 3 in four of five subjects, but was still at or above the 99% CI at day 14 for three of five subjects due to the long half-life of CERA in blood (Figure 7, middle panel). It should be noted that the affinity for CERA measured by the Immulite 2500 EPO immunoassay used was found to be only about 20% of that for rEPO (BRP). When this is taken into account, the Immulite 2500 EPO results agrees quite well with the CERA concentrations measured with the Mircera ELISA (Table 2). The concentration of CERA peaked on D3 in all subjects, not on D5 as indicated by the manufacturer. CERA was even significantly elevated in P#4 (after the first injection) from D3 to D7, and on days 16 and 21 for P#15, indicating that the Mircera ELISA may detect lower concentrations of CERA than the standard EPO test. All serum samples with detectable CERA profiles after IEF (Table 2, shaded numbers), had CERA levels above 150 pg/ml. A few samples had CERA concentrations slightly above the suggested screening cut-off of 100 pg/ml,[21] but were just around or below the detection limit for IEF (Figure 7, lower panel, samples marked with *). One of the negative control samples (D0, P#15) had a CERA concentration above the 100 pg/ml cut-off (Table 2), indicating that at the present time the Mircera ELISA seems to be a more sensitive, but less specific screening test for CERA in serum than IEF and double blotting.

Conclusion

CERA was detectable in morning urine in six out of eight subjects with no prior exercise. The CERA signal was stronger in most post-exercise urines, probably due to transient proteinuria. A single injection of CERA did not turn off the endogenous EPO production despite the effect on haematological parameters, as the endogenous EPO profile was present alongside the CERA profile in all collected urine and serum samples. CERA seems to be more stable over time in serum samples compared to urine, even when urine is stored at −30 °C. Our results further indicate that the detection probability for CERA is much higher in serum than in urine. We find that the detection of epoetin delta in urine is greatly facilitated by the information gained from SDS-PAGE in addition to the IEF-profile, as previously reported by Reichel[16] and included in WADA's technical document for EPO analysis (TD2009EPO).[28]

We have shown here that a single injection of CERA can have a significant effect on haematological parameters in some individuals due to its long half-life in serum. Such changes will immediately be recorded by the recently implemented blood passports,[12] which will become an invaluable tool and addition to the direct methods in the fight against doping.

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