

An efficient sample preparation for CERA analysis in serum

Institute of Biochemistry, German Sport University, Cologne, Germany

Introduction

The analysis of erythropoietin (EPO) is mainly based on the detection of isoelectric profiles and their differentiation in endogenous or recombinant patterns[1]. While the first- and second- generation of recombinant EPO were detectable in urine, the third generation of erythropoiesis stimulating agents (ESAs) CERA (Continuous Erythropoietin Receptor Activator), marketed under the name MIRCERA® favors the analysis in blood[2,3]. This is caused by the low excretion in urine, which relies on the high molecular mass (approximately 60kDa), limiting the glomerular filtration. The plasma half life of 120-140h is 15 to 20 times longer compared to other ESAs[4]. Therefore CERA has a prolonged timeframe for detecting its misuse.

In WADA accredited laboratories an enzyme-linked immunosorbent assay (ELISA) is used as screening method to detect CERA-misuse in blood[5]. False negative results [6] and false positive results in our lab have shown less reliability of this procedure. Confirmation analysis is performed by isoelectric focussing (IEF). Due to the high protein concentration in serum a purification step is necessary which is performed by immunoaffinity chromatography[7,8], liquid chromatographic separation[9], by magnetic beads combined with a specific anti-hEpo antibody[10] or by protein precipitation with perchloric acid[6]. This presentation shows a further procedure to remove most of serum proteins, using a precipitation by acetonitrile[11], while CERA and other EPOs stay solubilised. The method was optimized for 0.1 mL of serum.

Experimental

For this study serum samples originating from a former excretion study [10] of a male person who received a single subcutaneous injection of 50µg Mircera® were analyzed. These samples were collected from day 0 to day 14 and have been stored for more than three years

at -20°C. Furthermore the analysis was carried out with human serum samples (0.1 mL) which were spiked with 0,1 ng CERA (1 ng/mL).

Sample preparation for CERA is described in Figure 1.

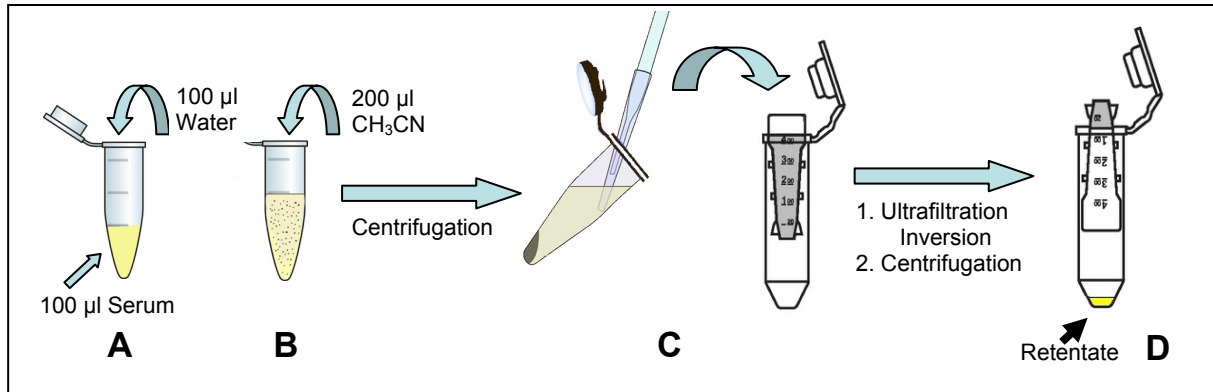


Figure 1: Sample preparation for CERA

A) 0.1 mL of serum (spiked or from excretion study) and 0.1 mL water were transferred to an Eppendorf Protein LoBind Tube.

B) 0.2 mL CH₃CN were added and the sample was vortexed. The protein precipitate was separated by centrifugation at 13.000 g for 10 min at 20°C.

C) The supernatant was transferred to an Amicon Ultra 0.5 mL (30K), water was added to a total volume of 0.5 mL. Ultrafiltration was performed at 10.000 g for 20min at 20°C.

D) The retentate was submitted to IEF-analysis.

Results and Discussion

A rapid procedure for depleting high abundance serum proteins to detect CERA has been developed. Due to its heavy glycosylation ESAs and human EPO stay solubilised. Darbepoetin alfa (NESP), Epoetin alfa, beta and delta in first experiments have shown comparable recovery rates to CERA. Based on the purpose of this research further tests were only performed for CERA. The new sample preparation shows no change in the isoelectric pattern of CERA. The high recovery for CERA analysed by IEF was estimated via densitometry (see Figure 2).

IEF Analysis

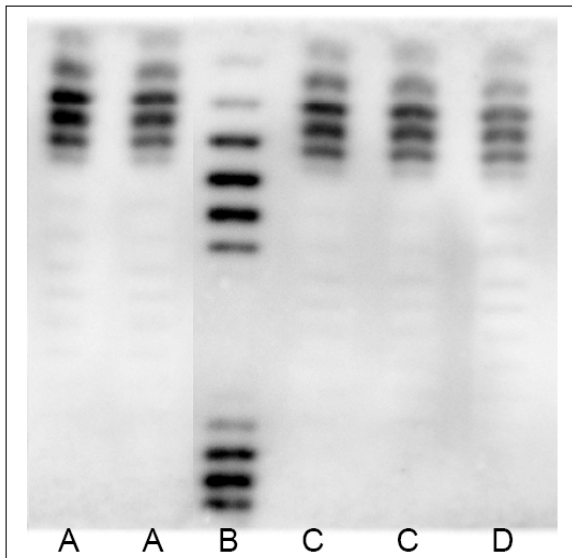


Figure 2: IEF-PAGE (immuno double blot) of CERA from spiked serum samples and a CERA-Quality-Control (QC)

- A) Serum 0.1 mL, spiked with 0.1 ng CERA (≈ 1 ng/mL), purified with CH₃CN precipitation
- B) BRP + NESP
- C) Serum 0.1 mL, purified with CH₃CN precipitation, 0.1 ng CERA added to the retentate
- D) CERA-QC: diluted serum sample (excretion study) estimated with 0.53 ng/mL CERA ("Mircera ELISA" from MicrCoat Biotechnology)

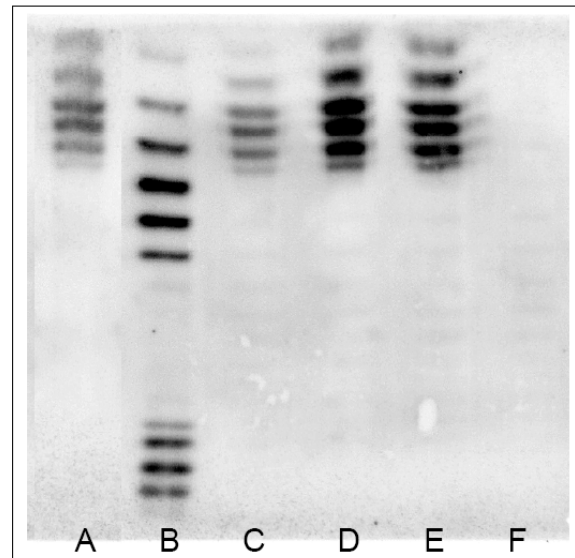


Figure 3: IEF-PAGE (immuno double blot) of CERA from serum of an excretion study and CERA-STD 0.1 ng, applied directly

- A) CERA STD 0.1 ng
- B) BRP + NESP
- C) Serum samples (0.1 mL)
- D) day 14
- E) day 6
- F) day 3

Serum sample of day 14 (Figure 3, lane C) contains 0.9 ng/mL CERA (CERA ELISA). A dilution series of this sample show a limit of detection (LOD) of 0.16 ng/mL using 0.1 mL of serum. This was confirmed during the validation of the new CERA-method.

The LOD was achieved at a concentration of 0.1 ng/mL serum, using 0.1 mL of spiked serum. This method is applied in our laboratory for CERA detection in blood (serum and also plasma) as an easy to use screening method and confirmation as well.

Sarcosyl-PAGE Analysis

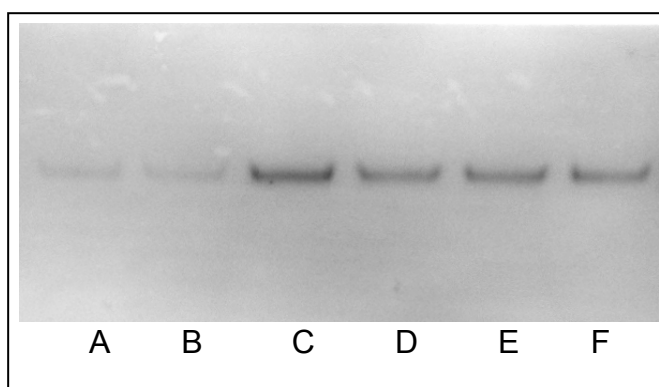


Figure 4: Sarcosyl-PAGE - (immuno double blot) of CERA from spiked serum samples precipitated with different volumes of CH₃CN
Serum 0.1 mL, spiked with 0.1 ng CERA
A-E) 300, 295, 290, 285, 280 µl CH₃CN
F) CERA-QC: diluted serum sample (excretion study) estimated with 0.53 ng/mL CERA ("Mircera ELISA" from MicrCoat Biotechnology)

The SARCOSYL-PAGE^[9] is compatible with the acetonitrile precipitation and can be used complementary to IEF for CERA detection. Due to lower protein loading capacity of the SARCOSYL-Gel, the acetonitrile volume should be between 260 µl and 290 µl to sufficiently deplete protein abundance. As shown in Figure 4 the acetonitrile volumes higher than 290 µl are critical as CERA signal intensity is being lost.

References

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