

Reihlen P¹, Kempkes R¹, Blobel M¹, Völker-Schänzer E¹, Majer B¹, Schänzer W¹, Thevis M^{1,2}

Recovery of supposedly degraded CERA from urine samples

Institute of Biochemistry, German Sport University Cologne, Cologne, Germany¹; Centre of Preventive Doping Research, German Sport University Cologne, Cologne, Germany²

Abstract

The Continuous Erythropoiesis Receptor Activator (CERA) is a pegylated form of epoetin beta. Through pegylation CERA gains a prolonged serum half-life. As other epoetins CERA is able to stimulate erythropoiesis and thereby increase oxygen transport capacity. The misuse of CERA in sport as a performance enhancing drug has been well documented in the last years. Under frozen long term storage conditions CERA has been published to be unstable. A similar issue has been published for the also pegylated erythropoietin-mimetic peginesatide. It was shown to precipitate if stored frozen. Now it could be shown that the same is true for CERA. Prevalent preparation methods were not able to resolubilize CERA after precipitation. Using multiple chaotropic sample solution (MCSS) CERA was resolubilized from the precipitate and again detectable using immunoblots after separation by IEF-PAGE. Due to the harmful and costly nature of CHAPS which is the detergent component of the MCSS, CERA was resolubilized using only urea. Addition of 1M urea to urine samples can easily be incorporated to the prevalent preparation procedures. Any analysis of a frozen A or B sample bears the risk of false negative CERA results unless the samples have been prepared with a solubilizing agent.

Introduction

CERA is an epoetin beta linked to a methoxy polyethylene glycol polymer [1]. It is able to enhance oxygen transport capacity by increasing the amount of red blood cells. Numerous cases of CERA misuse in sports have been documented [2]. A recent publication has reported CERA in urine as being unstable when stored at -30°C [3]. The loss of a pegylated protein after freezing has also been reported for the EPO-mimetic peginesatide. After few days of storage at -18°C peginesatide has been shown to have a considerable decrease in concentration. Yet, peginesatide could be extracted from the protein precipitate using multiple chaotropic sample solution (MCSS) [4]. It was investigated if CERA could be recovered from frozen long term stored urines with a similar procedure.

Experimental

An excretion study was used to investigate the recovery of CERA from long-term stored urine samples. Samples were taken from a healthy volunteer (60 years, 80 kg) after a single subcutaneous application of 0.625 µg/kg bodyweight Mircera®. The samples were prepared and analyzed once before freezing and after 3 months of storage at -25°C.

Before storage 10 mL aliquots of the samples were prepared via ultrafiltration [5] followed by an immunoaffinity purification using ELISA-wells [6] (Stemcell; Vancouver, Canada). The samples were separated with IEF-PAGE, double immunoblotted and detected [5].

The samples were then stored 3 months at -25°C. After 3 months they were processed using 4 different preparation methods, namely Tris-, UPD-, MCSS- and Tris/Urea-preparation. The preparation names are derived from the buffers used to dissolve the precipitate in the samples. Preparations varied only the first step of preparation. Downstream ultrafiltration steps were identical for all preparations and will briefly be explained later on. 10mL of sample were used for each preparation.

Tris-Preparation:

Briefly, 3.75 M Tris/HCl and complete protease inhibitor were added and mixed thoroughly. Samples were then subjected to ultrafiltration procedure. The procedure is identical to the one published by Lasne *et al.* [5].

UPD-Preparation:

Briefly, samples were treated with urine precipitate desolvation buffer (UPD, MAIA Diagnostics, Uppsala) as described in the manual. Samples were then subjected to ultrafiltration [5].

MCSS-Preparation:

3.75 M Tris/HCl and complete protease inhibitor were added to the samples before centrifugation[5]. The supernatant was separated from the pellet. The pellet was then resuspended in 1mL MCSS [7.7M urea, 2.2M thiourea, 4.4% CHAPS (w/v), and 44 mM Trizma® base]. Afterwards the supernatant and the dissolved pellet were reunited and subjected to ultrafiltration.

Tris/Urea-Preparation:

3.75M Tris/HCl and complete protease inhibitor were added to the samples .Additionally urea was added to a concentration of 1M/L per sample. Samples were mixed thoroughly and then subjected to ultrafiltration [5].

Retentates of all preparations were applied to ELISA-wells and immunopurified [6].The samples were separated with IEF-PAGE, double immunoblotted and detected [5].

Results and Discussion

Long term storage of urine samples containing CERA, leads to loss of signal in these samples when prepared with Tris/HCl (Fig. 1, lanes 4 and 5) or UPD (Fig. 2, lanes 2 and 4). Through resuspension with MCSS CERA could be recovered from the pellet (Fig. 2, lanes 3 and 5). CERA seems to precipitate under long term storage conditions in a similar way as peginesatide. The precipitation of CERA seems to be matrix related. A loss of signal has not been observed in serum or plasma. The cause for this peculiarity is still under investigation. The MCSS preparation is not convenient for routine CERA analysis in urine due to the laborious resuspension of the pellet and the use of large amounts of costly and harmful CHAPS. A preparation using urea has been shown to successfully resolubilize CERA (Fig. 3, lane 2). This is an easy and efficient way to ensure proper CERA analysis for urine samples.

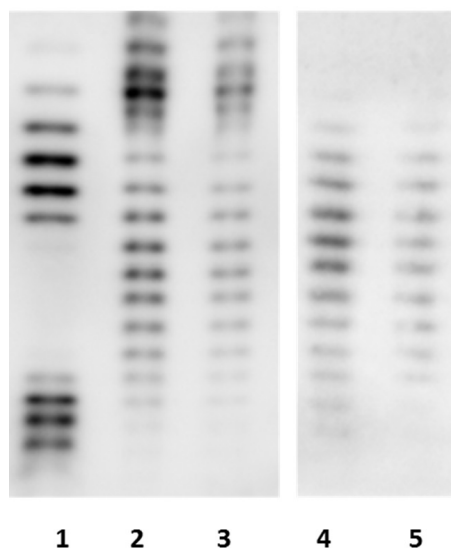


Fig 1: Double immunoblot of urine samples after IEF-PAGE showing BRP/NESP Standard (1) and Mircera excretion study urines 44h (3, 5) and 50h (2, 4) after application. Shown are the urines before (2, 3) and after 3 months (4, 5) of storage at -25°C. Urine samples were prepared with Tris/HCl.

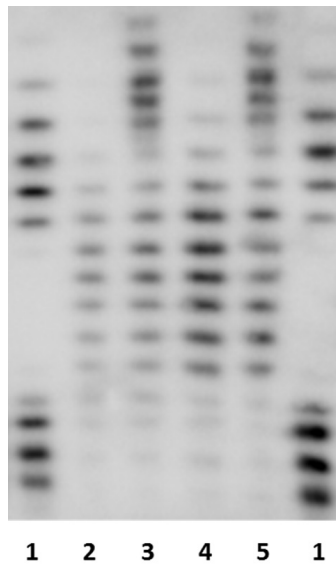


Fig 2: Double immunoblot of urine samples after IEF-PAGE showing BRP/NESP Standard (1) and long term stored (-25°C) Mircera excretion study urines 44h (4, 5) and 50h (2, 3) after application. Samples were prepared using either UPD (2, 4) or MCSS (3, 5).

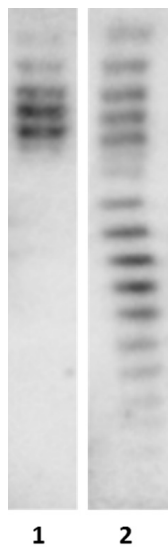


Fig 3: Double immunoblot of urine sample after IEF-PAGE showing CERA Standard (1) and long term stored (-25°C) Mircera excretion study urine 44h (2) after application. Sample was prepared with 1M urea in the sample.

Conclusions

CERA precipitation in frozen A or B-samples will lead to false negative results if samples are prepared without a solubilizing agent as shown here for urea. The method needs further investigation and validation to ensure proper use.

References

- [1] Jelkmann W. (2008) Developments in the therapeutic use of erythropoiesis stimulating agents. *British Journal of Haematology* **141**, 287-297
- [2] WADA Laboratory Statistics 2008-2010,
2008:http://www.wada-ama.org/en/Anti-Doping-Community/Anti-Doping-Laboratories/Laboratory-Statistics/WADA_LaboStatistics_2008.pdf (access date 29.10.2012)
2009:http://www.wada-ama.org/en/Anti-Doping-Community/Anti-Doping-Laboratories/Laboratory-Statistics/WADA_2009_LaboratoryStatisticsReport_Final.pdf (access date 29.10.2012)
2010:http://www.wada-ama.org/en/Anti-Doping-Community/Anti-Doping-Laboratories/Laboratory-Statistics/WADA_2010_LaboratoryStatisticsReport.pdf (access date 29.10.2012)
- [3] Dehnes Y, Hemmersbach P. (2011) Effect of single doses of methoxypolyethylene glycol-epoetin beta (CERA, Mircera) and epoetin delta (Dynepo) on isoelectric erythropoietin profiles and haematological parameters. *Drug Testing and Analysis* **3**, 291-299
- [4] Möller I, Thomas A, Delahaut P, Geyer H, W. Schänzer, Thevis M. (2012) Mass spectrometric detection of peginesatide in human urine in doping control analysis. *Journal of Pharmaceutical and Biomedical Analysis* **70**, 512-517
- [5] Lasne F, Martin L, Crepin N, Ceaurriz J. (2002) Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones *Analytical Biochemistry* **311**, 119-126.
- [6] Reihlen P, Völker-Schänzer E, Majer B, Schänzer W. (2012) Easy-to-use IEF compatible immunoaffinity purification of Erythropoietin from urine retentates. *Drug Testing and Analysis*. **4**, 813-817