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Detection of Mircera in blood plasma using isoelectric focusing combined with magnetic beads purification

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C.E.R.A., a continuous erythropoietin receptor activator, is a new third-generation erythropoiesis-stimulating agent (ESA). ESAs are prohibited doping substances. In doping control the current detection method uses concentrated urine samples, which are analysed by isoelectric focusing (IEF) (Lasne *et al*., 2002). Mircera contains a methoxy polyethylene glycol moiety, which is covalently conjugated to recombinant human EPO (rhEPO) beta. Pharmacokinetic studies revealed slower renal elimination of Mircera compared to rhEPO (Macdougall, 2005). The plasma half-life of Mircera is approximately 15 to 20 times longer. Thus it seems to be more reasonable to use plasma or serum samples for the detection of Mircera instead of urine. Due to the high protein concentration in plasma it is necessary to purify the EPO variants before IEF. Recently, different purification methods for plasma samples by immunoaffinity have been reported (Lasne *et al*., 2007, Reichel *et al*., 2009). The methods exploit both possibilities of separating EPO from plasma proteins: either EPO or high abundant proteins are retained on the affinity column.
Here we present an alternative purification method that uses magnetic beads and a specific anti-hEPO antibody.

Method

We established a purification method using magnetic beads (30-50 µL per 0.5 to 1 mL serum or plasma; Dynabeads® M-280 Sheep anti-Mouse IgG, Invitrogen, Oslo, Norway) and the anti-hEPO mouse monoclonal IgG_1 (Clone 9C21D11, R&D Systems, Minneapolis, MN, USA) (see Figure 1). For enhanced elution the samples were heated. Heating of urea can lead to carbamylation of proteins, which induces a change in the isoelectric point in the acid pH region (McCarthy *et al*., 2003). In our study we could not detect a change of the isoelectric profile of Mircera (Figure 2 and 3). Subsequently the purified samples were analysed by IEF. The method was applied to plasma samples from a Mircera excretion study.

In the study 50 µg Mircera were administered to a male person. Additionally the total EPO concentration in the samples was determined by ELISA (StemCell, Vancouver, Canada). We also performed a screening of serum samples with ELISA. Samples with strong EPO-signals were purified and analysed by IEF.

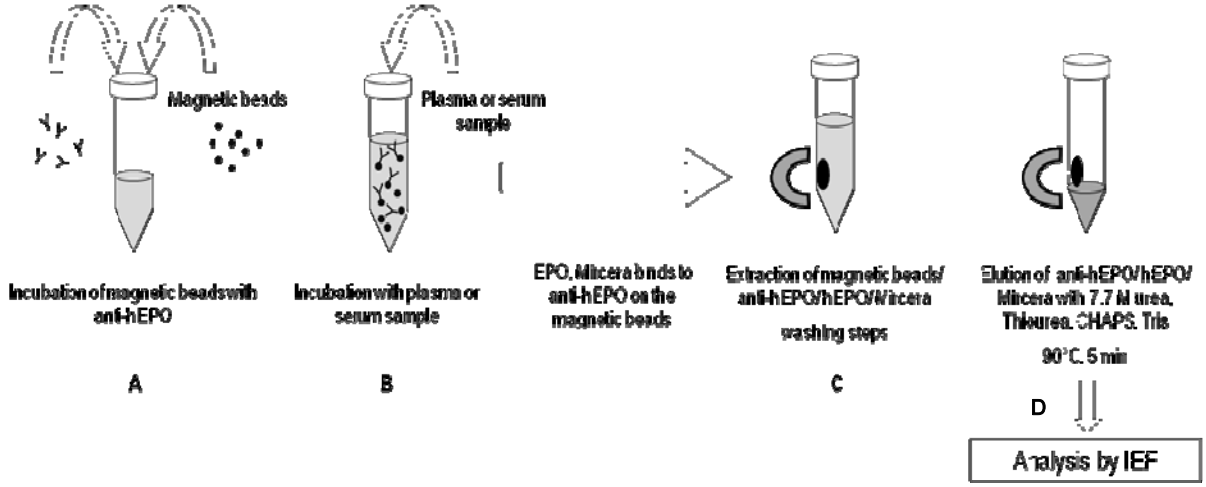

Figure 1: Procedure of Mircera and hEPO purification from plasma / serum by magnetic beads A) Mouse monoclonal anti-hEPO is linked to the anti-mouse antibodies on the surface of the magnetic beads; B) Addition of plasma samples; C) Extraction of the complex (magnetic beads - EPO-antibody - EPO forms) from the plasma by a magnet, discard of the supernatant, washing; D) Elution of the complex (anti-hEPO/ hEPO/ Mircera) with 7.7 M urea, 2.2 M thiourea, 4.4 % CHAPS, 44 mM Tris. Analysis by IEF.

Results

With the described purification method it was possible to extract the EPO variants, including Mircera, from human plasma samples. The detection limit for Mircera from plasma was below 1 ng/mL and the recovery was approximately 35% (see Figure 2). The Mircera isoform patterns on IEF were clearly visible.

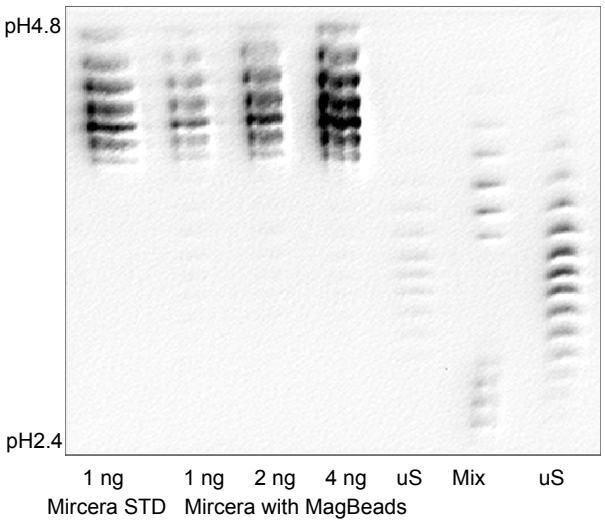

Figure 2: IEF-gel image of Mircera from spiked plasma samples
Mircera STD: 1 ng Mircera as a standard was applied on IEF-gel directly.
Mircera with MagBeads: the indicated amount of Mircera was spiked to plasma samples and purified with magnetic beads.
uS: endogenous EPO from urinary samples.
Mix: Standard composed of epoetin α and β and darbepoetin α.
The displayed gel section covers a pH range from approx. 2.4 to 4.8.

In the excretion study the expected decrease of the intensity during the washout phase could be observed (see Figure 3, day 1 to day 14).

The serum sample prior Mircera injection (day 0) contained only endogenous hEPO. Consistent results were found for the total EPO concentration that was determined by hEPO-ELISA (see Figure 4). The maximum total EPO concentrations (day 3) were about ten fold higher than the basic value (day 0).

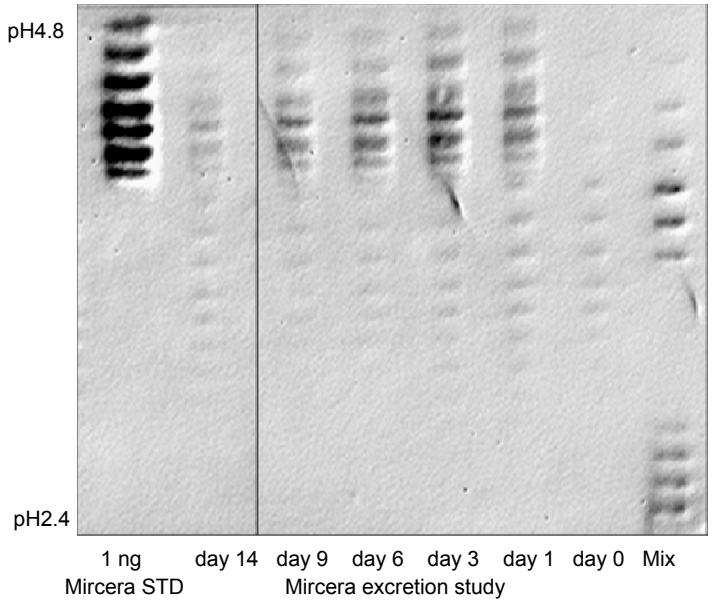

Figure 3: IEF-gel image of Mircera from plasma samples of the excretion study
Plasma samples were taken before 50 µg Mircera application (day 0) and then at day 1, 3, 6, 9 and 14 after the application. The decrease of the intensity during the washout phase is clearly visible. The standard (STD) is 1 ng Mircera which was applied directly onto the IEF-gel.

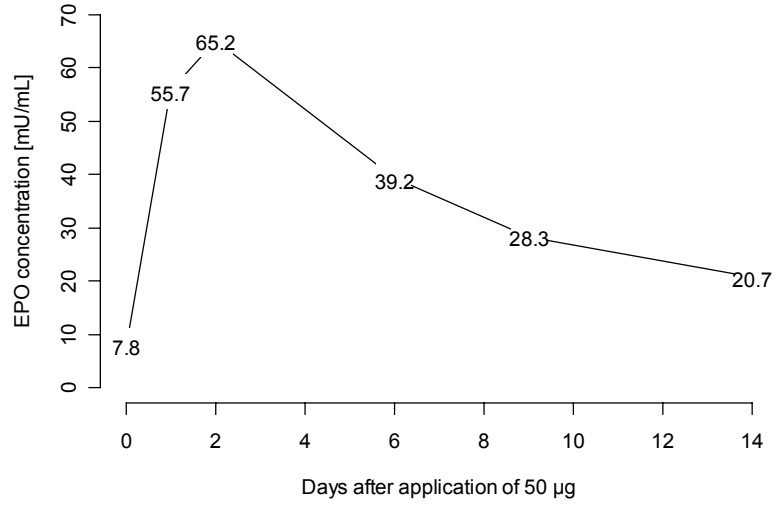

Figure 4: EPO concentration in plasma samples of the excretion study, determined by ELISA
Plasma samples were taken before 50 µg Mircera application (day 0) and then at day 1, 3, 6, 9 and 14 after the application. The decrease of the intensity during the washout phase is clearly visible.

It was possible to detect Mircera with the hEPO-ELISA. Thus we concluded that elevated EPO concentrations can be a consequence of Mircera application. With the hEPO-ELISA we

screened 316 serum samples for elevated EPO concentration (Figure 5). We considered the normal range of hEPO concentration in serum to be 3-17 mU/mL (Lasne *et al*., 2007) and analysed a set of 15 samples with EPO concentrations between 17 and 85 mU/mL by IEF. In this set the IEF confirmation revealed no Mircera abuse.

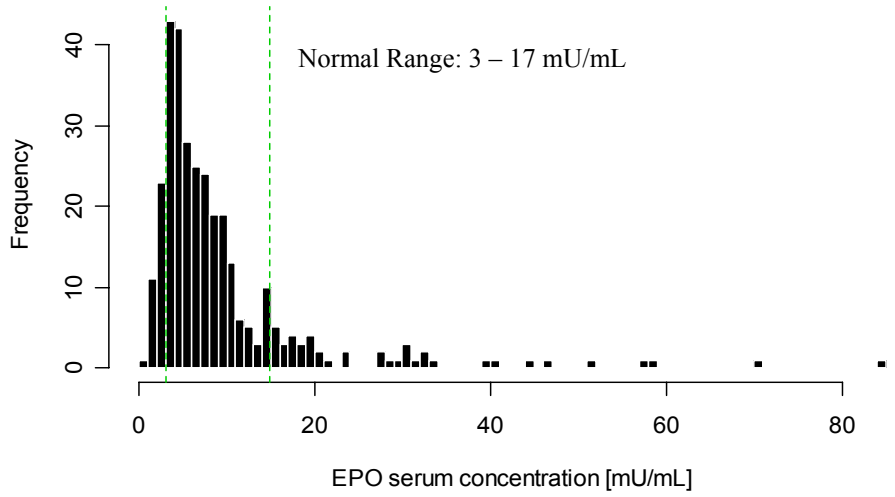

Figure 5: Histogram of EPO concentration determined by hEPO-ELISA
Elevated EPO concentrations might also be due to Mircera abuse.

References

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