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## **Detection of peginesatide by SDS-PAGE**

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### **Abstract**

Thanks to the valuable collaboration of Affymax that was initiated by WADA, two different methods, ELISA and SDS electrophoresis, have been developed and validated in the anti-doping laboratories of Switzerland and France respectively for detection of peginesatide in blood. The detailed method of SDS electrophoresis is described here. Using mAb anti-peginesatide clone 1G9 for immunoprecipitation and clone 11F9 for double immunoblotting after SDS-PAGE, the method proved to be sensitive (LOD=0.1 ng/mL) and specific for peginesatide in blood.

The method was applied to samples obtained from healthy volunteers treated by peginesatide. This drug was still detectable in plasma 21 days after a single IV injection of 0.05 mg/kg

### **Introduction**

Peginesatide (formerly known as Hematide®, commercialized as Omontys® by Takeda Pharmaceutical Company and Affymax, Inc.) a once monthly, pegylated, peptide-based erythropoiesis-stimulating agent (ESA), was approved by the US Food and Drug Administration (FDA) on March, 27th 2012 and is now available in the USA market. It is indicated for the treatment of anemia due to chronic kidney disease (CKD) in adult patients on dialysis. It is not indicated for use in patients not on dialysis and whose anemia is not due to CKD. Thanks to the valuable collaboration of Affymax that was initiated by WADA, two different methods (ELISA and SDS electrophoresis) have been developed and validated in the anti-doping laboratories of Switzerland and France for detection of peginesatide in blood. The detailed method of SDS electrophoresis is described here and is illustrated by the results obtained from samples obtained from healthy volunteers following an IV administration of 0.05 mg/kg of peginesatide.

### **Experimental**

#### **Reagents and samples**

The key reagents peginesatide and monoclonal antibodies (clones 1G9 and 11F9) to the peptide part of peginesatide were provided by Affymax (now available at ICI).

Plasma samples were collected from healthy volunteers by Takeda and Affymax during an IV administration study of peginesatide (0.05 mg/kg). However, the SDS-PAGE method was equally validated for serum.

#### **Method**

The method involved two steps:

##### **- Preparation of plasma samples**

The samples were submitted to immunoprecipitation (IP) of peginesatide, using agarose beads coated with 1G9 monoclonal antibodies. The beads were prepared using Direct IP kit according to the instructions of the manufacturer (Pierce).

For the preparation of 5 plasma samples:

250 µL of beads were incubated with 300 µL of 1G9 solution (200 µg/mL in coupling buffer) under slow rotating. After coupling, the beads were blocked by incubation with 1% bovine serum albumin in 0.005% (v/v) Tween20 phosphate buffer saline pH 7.4 (PBST) for 1 hour under slow rotating and submitted to a final washing (3x) with PBST.

For immunoprecipitation of peginesatide, 500 µL of plasma or serum were incubated with 100 µL of 1G9-beads for 1h30

under slow rotating, at room temperature. After washing (3x1 mL) with PBST, supernatant was removed and the pellet resuspended in 30  $\mu$ L of denaturing buffer (LDS sample buffer diluted 1:4 in water, containing 100mM DTT final concentration). After heating (7 min, 95°C), the tubes were cooled down and their content transferred into a spin device PTFE filter 0.45  $\mu$ m in order to remove the beads. After centrifugation (4 min at 7000 g), filtrate was ready to be analyzed by SDS-PAGE.

#### - SDS-PAGE and double immunoblotting

Electrophoresis was performed using Nu-PAGE®4-12% Bis-Tris Gel (Invitrogen) and MES-SDS as running buffer for 1h at 200V constant voltage.

Double-immunoblotting was performed as previously described [1] using 50 mM glycine/NaOH pH 11 buffer containing 50 mM NaCl, 0.04% SDS and 5% methanol, at 0.12 A constant current for 45 min for the first blot and 50 mM glycine/HCl, 0.1M NaCl, pH 2.6 buffer, at 0.04 A constant current for 10 min for the second blot. Incubation with the primary antibody of the first blotting membrane was performed using 11F9 antibodies (1  $\mu$ g/mL in 1% non-fat milk PBS). Chemiluminescent revelation of the second membrane was revealed as described for EPO [1].

## Results and Discussion

As proposed by Affymax, immunoprecipitation of peginesatide by 1G9 antibody was very efficient. However, this antibody proved to be unusable for double immunoblotting (low sensitivity).

11F9 antibody (that is used as detection antibody in ELISA method) was tested in single (SB) and double (DB) immunoblotting. SB gave rise to strong signals corresponding to light and heavy chains released from 1G9 antibodies during the elution step and faint, but unacceptable signals at the position of (or very near) the band corresponding to peginesatide in samples devoid of this drug (Figure 1). Using DB, as proved by the analysis of 61 serum and 19 plasma samples devoid of peginesatide, the only signal corresponded to light chains from 1G9 antibodies and was too far from the band of peginesatide to interfere with the result.

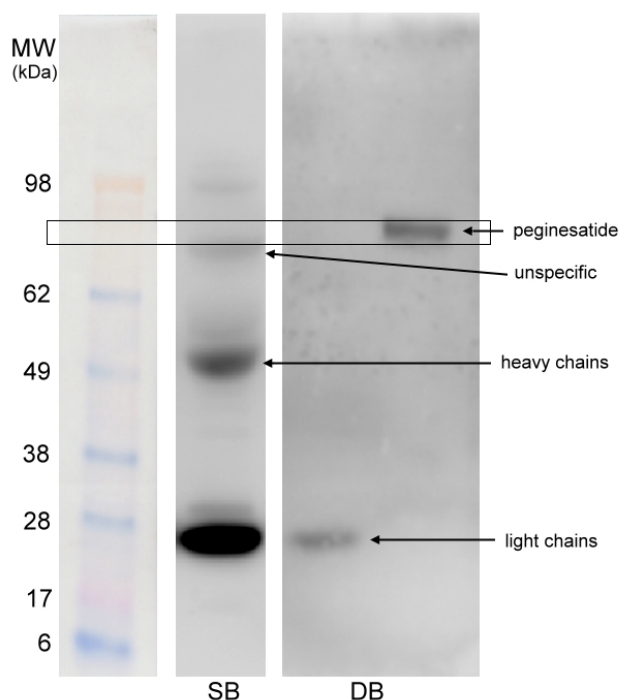


Figure 1: SDS-PAGE patterns obtained from a control serum sample (devoid of peginesatide) after single (SB) and double (DB) immunoblotting of peginesatide.

Sodium heparin plasma samples from a clinical study were provided by Takeda and Affymax (IV injection of 0.05 mg/kg of peginesatide). The SDS patterns were obtained by immunoprecipitation (1G9 antibodies) and DB (11F9 antibodies) as described here. The samples had been previously submitted to ELISA [2] for estimation of their peginesatide concentration and were diluted with blank plasma before immunoprecipitation in case of concentration > 20 ng/mL.

Peginesatide was still detectable in plasma 21 days after the IV injection (Figure 2). This last result showed that 0.5 mL of plasma containing 0.19 ng/mL were sufficient to detect the presence of this drug.

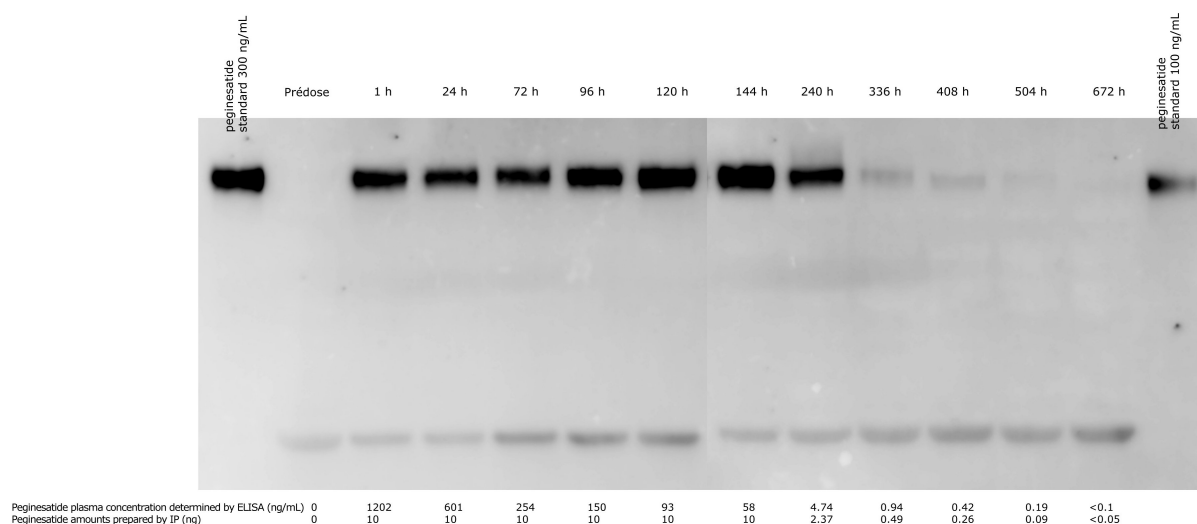


Figure 2: SDS-PAGE patterns obtained during 28 days following IV injection of peginesatide (0.05 mg/kg).

## Conclusions

SDS electrophoresis appears as a specific and sensitive method (LOD=0.1 ng/mL) to detect peginesatide in blood (serum or plasma) when immunoprecipitation using 1G9 antibodies is combined with double immunoblotting using 11F9 antibodies. These results clearly demonstrate the usefulness of collaboration with pharmaceutical industry for anti-doping control, demonstrating that detection methods can be ready even before a drug is commercialized.

## References

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2. Leuenberger N, Saugy J, Mortensen R, Schatz P, Giraud S, Saugy M. (2002) Methods for detection and confirmation of Hematide™/peginesatide in anti-doping samples. *Forensic Sci. Int.* **213**, 15-19.

## Acknowledgements

The authors want to thank the World Anti Doping Agency for support.