Detection of Recombinant Epoetin and Darbepoetin Alpha after Subcutaneous Administration in the Horse

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Abstract

A direct detection method for anti-doping control of recombinant human erythropoietin (rHuEPO) abuse in racehorses is proposed. This method involves screening of plasma (or serum) by an enzyme-linked immunosorbent assay specific for human EPO and confirmation in urine samples by characterization of the urinary EPO isoelectric profile. This method was tested on horses that were administered epoetin alpha (rHuEPO) and the hyper-glycosylated form of this drug (darbepoetin alpha).

Introduction

By stimulating erythropoesis, recombinant human erythropoietin (rHuEPO) increases the number of circulating red blood cells. Therefore, some endurance athletes use it as a doping agent to enhance oxygen transport and aerobic power. Anti-doping control in humans is based on the characterization of the isoelectric profile of this hormone in urine (1).

Administering this human drug to racehorses is controversial. The supposed advantages of doing so have not been proven, and as suggested by the observation of two cases of autoimmune anemia in horses after experimental administration of this hormone (2), it may even be dangerous. However, antibodies to EPO were found in certain racehorses during controls performed in the United States in 2003, and this was interpreted as an indicator that EPO was indeed being used. However, direct detection of the administered substance is required to report an adverse finding in anti-doping control, and there is no validated method for this in equine sport.

We propose here a direct detection method based on the screening of plasma (or serum) by an enzyme-linked immunosorbent assay (ELISA) specific for human EPO and confirmation in urine samples by characterization of the urinary EPO isoelectric profile.

This method was tested on horses that were administered rHuEPO (epoetin alpha) and the hyper-glycosylated form of this drug (darbepoetin alpha).

Experimental

The study was conducted at the administration and sampling center of the Fédération Nationale des Courses Françaises (FNCF). The four horses used in this study were all thoroughbreds (1 male, 2 geldings, and 1 female) in good general health, and they were maintained at rest for three days before and during the experiment.

Three horses (B, H, and C) received 36 IU/kg/day of epoetin alpha (Eporex®, Janssen-Cilag, France) for six consecutive days, and one horse (RS) received a single dose of 0.37 µg/kg of darbepoetin alpha (Aranesp®, Amgen), corresponding to about 29 IU/kg when estimated with the ELISA used in this study. The drugs were administered by subcutaneous injection in the neck.

Heparin plasma (and serum) obtained by jugular venipuncture and urine samples were taken at 24-h intervals, over a 48-h period prior to the injections. Blood and urine samples were then collected daily for the 13 days following the first injection of epoetin alpha or the single dose of darbepoetin alpha. All the samples were stored frozen at -20°C until assayed.

Plasma (and serum) samples were assayed for their EPO concentration using the ELISA for human EPO Quantikine IVD from R&D Systems. The standard curve was established from recombinant human EPO calibrated against the Second International Reference Preparation (67/343), a urine-derived form of human erythropoietin.

The isoelectric profiles of EPO in urine were monitored through the isoelectric focusing and “double blotting” method described for human anti-doping control (3). Briefly, urine samples (20 mL) were submitted to ultrafiltration using membranes with a molecular weight cut-off of 30 kDa in order to concentrate the hormone in the retentate. This retentate was assayed for its EPO level by ELISA and submitted to isoelectric
focusing (IEF) (pH gradient of 2–6). The isoforms of EPO were specifically revealed by the double-blotting method using monoclonal anti-human EPO AE7A5 from R&D Systems. The final result was a chemiluminescent image of these isoforms.

Results and Discussion

In all four horses, the plasma (or serum) EPO concentrations were less than 2.5 IU/L before injections were given. These concentrations are probably underestimated because of the poor cross-reactivity of equine EPO and the anti-human EPO antibodies used in ELISA.

The day after the first injection of epoetin alpha, the EPO concentrations substantially increased in the different plasma (69–86 IU/L) and remained above 38 IU/L over the six consecutive days of treatment. After the last injection, the concentrations decreased rapidly and returned to initial values in 2–3 days (Figure 1).

In the horse treated with a single dose of 200 μg darbepoetin alpha, the EPO plasma concentration reached 156 IU/L in the 24 h following injection and decreased regularly over the subsequent days. It returned to basal value on the 10th day following administration (Figure 1).

After concentration of urine by ultrafiltration, the retentates obtained from the samples taken before treatment presented low levels of EPO, indicating that urinary EPO (estimated from its level in the retentate and the concentration factor of ultrafiltration) ranged from less than 0.1 to 0.9 IU/L. Though the AE7A5 monoclonal antibody used in the double-blotting method was different from both the capture and the conjugated antibodies used in ELISA, the results of the isoelectric analysis correlated well with the ELISA. In the case of calculated EPO levels under 0.5 IU/L, no image was detected by the IEF analysis. In the case of higher values, an image of endogenous equine hormone was observed in IEF. It thus appeared that the horse urinary EPO was highly heterogeneous, being composed of a great number of isoforms with pI from 4 to 5.6, and quite different from the administered human recombinant hormones epoetin and darbepoetin alpha (Figure 2).

Administration of these recombinant hormones was followed by the appearance in urine of the isoforms corresponding to the injected drugs. The urinary EPO profiles were characteristic of the administered hormone even in the cases where endogenous natural EPO was detected in urine before the treatment. This indicated that, as in humans, recombinant
EPO molecules are excreted in horse urine without noticeable change in their isoelectric profiles.

In the three horses that received epoetin alpha, the characteristic isoforms of this drug were unambiguously recovered in the urinary patterns from the day after the first injection to the end of treatment. The only slight modification in the isoelectric pattern of the injected epoetin was an intensification of its two most acidic isoforms when excreted in urine. The same modification has been observed in humans (unpublished results). After the last injection, the pattern of excreted EPO disappeared so quickly that the urine samples reverted to their initial type after 48 h with either no EPO or only natural endogenous hormone detected.

The characteristic acidic isoforms of darbepoetin alpha (due to hyper sialo-glycosylation of this drug) were evident and constituted the main bands of the urinary pattern over the 5 days following the single injection of Darbepoetin. No isoelectric pattern could be detected in the samples taken at the 6th and 7th days after injection. The initial endogenous pattern observed before the injection (endogenous EPO was detectable in the urine of the horse treated with darbepoetin) was recovered on the 8th day following injection and remained stable over the subsequent days.

These results show that the presence of recombinant EPO in horse urine can be clearly established by the method of isoelectric focusing and double-blotting developed for human anti-doping control. No ambiguity with natural endogenous equine hormone appeared in this study because the latter was either not detectable or, if detectable, differentiated from recombinant EPO by its isoelectric pattern.

Based on these observations, we propose the use of an estimation of plasma EPO concentration by the Quantikine IVD human erythropoietin from R&D Systems as a screening process and the isoelectric focusing analysis of urinary EPO as a confirmation method.

References


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