

Detection of the Administration of Human Erythropoietin (HuEPO) to Canines

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Abstract

Recombinant human erythropoietin (rHuEPO) doping is prohibited in animal (canine and equine) sport. The effectiveness of a range of immunoassay screening methods for the detection of rHuEPO in canine urine was evaluated. The excretion profiles following rHuEPO administration to dogs were investigated. The presence of rHuEPO in postadministration samples was confirmed using the World Anti-Doping Agency (WADA)-approved isoelectric focusing immunoblotting confirmatory technique. Following the administration study, a screening program involving approximately 6000 greyhound sport (mostly racing) samples was undertaken for rHuEPO. This resulted in the detection of the first rHuEPO positives in the world of canine or equine sport. In an additional case, endogenous HuEPO was detected in a sample submitted as greyhound urine. It was determined that this arose from the submission to control stewards, as greyhound urine, of a substance that was, in fact, human urine. This was a particularly welcome development as definitive confirmatory evidence of such sample switching can be difficult to obtain in the case of greyhounds.

Introduction

In human sports events or strenuous exercise lasting more than 1 min, the predominant mode of energy production is aerobic. This means that performance is limited by the oxygen that is delivered and utilized by the active muscles. The level of performance is determined by the level of training, which affects both central and peripheral factors. When the muscles are well trained, as in the case of elite endurance athletes, the limiting factors in determining the maximal oxygen uptake are the cardiac output and the oxygen-carrying capacity of the blood. The oxygen-carrying capacity of the blood is determined by the hemoglobin content, which helps bind oxygen within the red blood cells. It is the total body hemoglobin, rather than its relative concentrations, which is correlated with the maximal oxygen uptake. When hemoglobin levels fall, exercise performance is impaired. Athletes and their coaches are cognizant of this, and many performers regularly

take iron supplements to prevent anemia. It is also well recognized by sports practitioners that "blood boosting" can enhance performance. Consequently, various ways have been devised of augmenting the oxygen carrying capacity of the blood of athletes. These methods include the so-called procedures of "blood doping" as well as altitude training (1).

The mechanisms by which blood doping and altitude might work are analogous. They operate by elevating the number of red blood cells, either by infusion or by increased production via the process of erythropoiesis. This latter process is controlled by the circulating level of erythropoietin (EPO). Human EPO (HuEPO) consists of a single 165 amino acid peptide chain that is heavily glycosylated by one O-linked (L-serine) and three N-linked (L-asparagine) carbohydrate side chains corresponding to 40% of the total molecular mass (30,400 Daltons). It is produced primarily by the kidney. An increase in the level of HuEPO in the human body will lead to an increase in red blood cell production and consequently increased aerobic endurance.

Since the cloning of the EPO gene in 1985, it has been possible to produce large quantities of recombinant forms of human EPO (rHuEPO), with high purity, for controlling anemia in patients with chronic renal failure. Epoetin alpha, epoetin beta, and epoetin omega are the non-proprietary names for the forms of rHuEPO currently available. These three glycoproteins have an identical amino acid composition to that of endogenous human EPO (2), but the carbohydrate content differs depending, in part, on the *in vitro* mammalian cell line used for production. Epoetin alpha and beta are synthesized from Chinese hamster ovary (CHO) cells, whereas epoetin omega is produced from baby hamster kidney (BHK) cells.

Following translation of the polypeptide, oligosaccharide chains are added to specific amino acid residues of the protein, contributing to the stability of its tertiary structure. Epoetin omega has fewer O-linked sugars than alpha and beta (3). All recombinant forms have a relatively high content of tetra-sialylated oligosaccharides compared to endogenous human EPO (4).

Generally, the recombinant and endogenous human EPO

forms have very similar biological properties despite the differences in post-translational modification (5).

Several companies manufacture and license recombinant erythropoietic products around the world. Epoetin alpha manufactured by Amgen is available as EPOGEN[®] from Amgen and as PROCRT[®] from Ortho Biotech. EPREX[®] is a formulation of epoetin alpha manufactured by Ortho Biologics and sold by Janssen-Cilag and partners. Epoetin beta is available as NEORECORMON[®] and is manufactured and sold by Roche Pharmaceuticals. Epoetin omega is manufactured by Elanex Pharma and is available outside the U.S. as EPOMAX[®]. Because the patents on most of the products have nearly expired, generic sources of recombinant human erythropoietin are now becoming available.

In September 2001, Amgen began selling, under the trade name of ARANESP[®] (darbepoetin alpha), a novel erythropoiesis-stimulating protein (NESP) that has two additional N-glycosylation sites resulting from amino acid substitutions in the erythropoietin peptide backbone. The additional carbohydrate chains increase the molecular mass to approximately 37,000 Daltons and result in a drug that has a higher potency and approximately a three times longer half-life (mean terminal half-life following intravenous injection of 26.3 h) than that of epoetin alpha (8.5 h) (6).

The World Anti-Doping Agency (WADA)-accredited laboratories' evidence administered rHuEPO by a direct approach that separates and visualizes the isoforms of the EPO in urine to distinguish administered EPO from endogenous. The urine sample is first treated with a protease inhibitor and is then ultrafiltered to concentrate the EPO. The ultrafiltrate is applied to a gel that has been pretreated to establish a pH gradient. The isoforms of each EPO type move through an electric field across the pH gradient until they reach their isoelectric point (pI), at which their charge is neutral. Once separated, the proteins are transferred onto a membrane in an immunoblotting procedure. The membrane is then saturated with milk protein to block free binding sites before probing with a monoclonal anti-human EPO antibody (clone AE7A5, R & D Systems). This antibody is transferred to a second membrane in a second blot, and its position is revealed by adding a sequence of reagents terminating in an enzyme, which generates light in the presence of an appropriate chemiluminescent substrate (7–9). The light emitted enables the capture of a digital image that accurately reflects the position of the first antibody, and therefore the original position of the EPO after isoelectric separation. Recombinant human EPO from Chinese hamster ovary cells is composed of at least five isoforms that focus in the pI range 4.4–5.1, whereas endogenous human EPO is composed of 10 or more isoforms with a pI range of 3.7–4.7 (10).

Recombinant human erythropoietin is the only erythropoietin that is commercially available, and it could be abused in greyhound sport to stimulate red blood cell production and increase hemoglobin levels, thereby improving the aerobic-energy capacity of a greyhound. Similar fears have been expressed with regard to equine sport (11). The potential benefits to the horse have been questioned (11) because the horse has a splenic red cell reserve that can be released into circulation on exercise to increase the hematocrit by up to 33%. The misuse

of rHuEPO has the potential to compromise both the integrity of racing and the health of equine athletes (12). There have been reports that horses can develop non-regenerative anemia after multiple injections of EPO over a period of several weeks (13–15). It is speculated that the reason for this condition is that the horse's immune system recognizes the human EPO as a foreign protein and produces antibodies against it. These antibodies also cross-react with the naturally occurring equine EPO, thus preventing natural red blood cell production. Similar factors may be of importance in the case of greyhounds.

The aim of the present study was to develop screening and confirmatory analytical techniques for rHuEPO in canine urine samples. Greyhounds urinate readily after being released from their transporters. Thus, urine is the preferred sample matrix. Blood is rarely collected from the greyhound because of this relative ease of urine sampling. However, there is the problem of security. Switching of urine samples is clearly a possibility that must be avoided, and particular care is required during the period of waiting before a sample is obtained from a dog. In the case of equine sport, horse handlers responsible for collecting a urine sample from their horses have been known to substitute a urine sample of their own (16). The odor of urine and of the residues produced after solvent extraction can provide some evidentiary distinction between the species. The presence of appreciable quantities of nicotine, cotinine, caffeine, and uric acid in urine provides some evidence of a human source. However, definitive confirmatory evidence of such sample switching can be difficult to obtain in the case of greyhounds.

The screening technique adopted in this work was ELISA. Its application to equine urine (11) and canine blood (17) samples had been investigated previously. The screening method does not need to be quantitative, though it can be useful if it gives an estimate of the HuEPO concentration. The confirmatory method can be purely qualitative because rHuEPO is not licensed for use in the dog; therefore, the identification of rHuEPO in a canine urine sample would be sufficient evidence to call a positive. Prior to the present work, no canine sample had been reported positive for the presence of rHuEPO, despite rumors concerning its abuse. One reason for this is that no recognized confirmatory analytical procedure had been developed for use with canine samples. As of 2004, no equine sample had been reported as positive for the presence of rHuEPO, despite a few suspicious results in the U.S. (18). The aim of the present study with regard to rHuEPO confirmation in canine samples was to apply the WADA/International Olympic Committee laboratories' method to these samples. This approach had been attempted with equine samples (18).

Experimental

Immunoassay—R&D Systems Quantikine IVD Human EPO ELISA

The kit was purchased from R&D Systems (Abingdon, U.K.). This product is intended for serum/plasma analysis. Urine

aliquots were used in place of the prepared blood product in the manufacturer's instructions. The process is based on the double-antibody "sandwich" method. Microplate wells (96 per kit), precoated with monoclonal (mouse) antibody specific for HuEPO, are incubated with specimen or standard. The HuEPO binds to the immobilized antibody on the plate. After removing excess specimen or standard, wells are incubated with an anti-HuEPO polyclonal (rabbit) antibody conjugated to horseradish peroxidase. During the second incubation, the antibody-enzyme conjugate binds to the immobilized HuEPO. Excess conjugate is removed by washing. A chromogen is added into the wells and is oxidized by the enzyme reaction to form a blue-colored complex. The reaction is stopped by the addition of acid, which turns the blue to yellow. The amount of color generated is directly proportional to the amount of conjugate bound to the HuEPO antibody complex which, in turn, is directly proportional to the amount of HuEPO in the specimen or standard. The absorbance of this complex is measured and a standard curve is generated by plotting absorbance versus the concentration of the HuEPO standards. The HuEPO concentration of the unknown specimen is determined by comparing the absorbance of the specimen to the standard curve. The standards used in this assay are rHuEPO calibrated against the Second International Reference Preparation (67/343), a urine-derived form of HuEPO.

One-hundred microliters of R&D Systems assay diluent (a buffered protein base) was added to each well. This was followed by 100 μ L of standard, control, or specimen for each well. The plate frame was gently tapped for approximately 1 min in order to mix the well contents. The plate was covered with the adhesive strip provided. Wells were then incubated for 2 h \pm 5 min at room temperature (20–25°C). The contents of each well were then thoroughly decanted and blotted dry on clean paper towelling. Two-hundred microliters of the kit HuEPO conjugate was then added to each well. The plate was covered with a new adhesive strip and the wells incubated for a further 2 h \pm 5 min at room temperature. Each well was washed with 4 \times 400 μ L of the supplied wash buffer. Complete removal of liquid at each of the four steps is essential for good performance. After the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. Two-hundred microliters of freshly prepared "Substrate Solution" (a mixture of equal volumes of 0.005M hydrogen peroxide and 0.35 g/L tetramethylbenzidine) was added to each well. One-hundred microliters of "Stop Solution" (1M sulfuric acid) was added to each well, and the plate was tapped gently to ensure thorough mixing. The absorbance of each well was measured immediately at 450 nm, using wavelength correction at 600 nm in order to correct for optical imperfections in the plate. During each analytical run, all specimens, standards (0.0, 2.5, 5.0, 20.0, 50.0, 100, and 200 mIU/mL) and manufacturer's controls were run either in duplicate or triplicate. Replicate readings were averaged for each standard, and the average 0 mIU/mL standard absorbance was subtracted. A standard curve was created as per the manufacturer's instructions. Measurements were made on an Asys Hitech Expert Plus and on a Multiskan MS Version 3.0 microplate reader. In 60 runs spread evenly over a year and a half

period, no linear correlation coefficient less than 0.95 was recorded, and it was typically around 0.97.

Sensitivity. The minimum detectable dose is typically less than 0.7 mIU/mL. This was determined by adding 2 standard deviations to the mean absorbance of 20 replicates of the zero standard and calculating the corresponding concentration from the standard curve.

Specificity. The manufacturers state, in their product insert, that "the complete sequence of the EPO protein was compared with sequences in the Protein Identification Resource and the Swiss-Protein databases. Recombinant and natural human EPO sequences are identical; no significant homology with other human proteins was found. When assayed in the Quantikine IVD EPO ELISA, the World Health Organization (WHO) standard 87/684 (rHuEPO) showed essentially identical reactivity relative to WHO standard 67/343 (natural HuEPO)."

The manufacturer also spiked, at a concentration of 1.11 mIU/mL, the kit assay diluent with 51 different human and mouse biomolecules (e.g., growth factors such as IGF I). No cross-reactivity was observed. Interference was tested by the manufacturers by spiking human serum, plasma, or kit assay diluent with selected amounts of nine potentially interfering substances (including albumin, unconjugated bilirubin and triglycerides) and analyzing for the presence of HuEPO. Endogenous and additional levels of these substances were used. To determine if the added substance interfered with assay performance, the recovery of HuEPO levels in each specimen was calculated. Recoveries averaged 102.5 \pm 4.7% and ranged from 88.6 to 117.7%.

Accuracy. Recovery was estimated by addition of rHuEPO standard, at a concentration of 20 mIU/mL, to 10 blank greyhound urine samples. The percent recovery of the added rHuEPO was calculated from the equation:

$$\% \text{Recovery} = \left(\frac{\text{Measured value after addition} - \text{Measured value before addition}}{\text{Value of the added material}} \right) \times 100 \quad \text{Eq. 1}$$

The mean recovery was 97 \pm 16%.

Because findings of HuEPO in greyhound urine samples are rare and because the volume of such samples is very limited and they are needed for confirmatory analysis, it was not possible to check any of them for linearity of response on dilution.

Assay precision. This was estimated by addition of 2 rHuEPO standards to 10 blank greyhound urine samples. Samples were assayed in duplicate over the course of 20 days. The mean concentrations of the two standards were 7.9 and 19.4 mIU/mL with within-assay CVs of 9.5% and 10.2% and between-assay CVs of 9.4% and 10.6%, respectively.

Immunoassay—Neogen EPO ELISA

This kit (product no. 108810) was purchased from Neogen (Lexington, KY). This product is also intended for serum/plasma analysis. Urine aliquots were again used in place of the prepared blood product in the manufacturer's instructions. The process is a two-site ELISA for the measurement of the biologically active 165 amino acid chain of HuEPO. It uti-

lizes two different mouse monoclonal antibodies to HuEPO which, the manufacturer's product insert states, are specific to well-defined regions on the EPO molecule. One mouse monoclonal antibody to HuEPO is biotinylated and the other is labelled with horseradish peroxidase for detection.

Standards, controls, and samples are simultaneously incubated with the enzyme-labelled antibody and a biotin-coupled antibody in streptavidin-coated microplate wells (96 per kit). At the end of the assay incubation, the wells are washed to remove unbound components and the enzyme bound to the solid phase is incubated with the substrate, tetramethylbenzidine (K-Blue MAX). An acidic solution is then added to stop the reaction and convert the color to yellow. The intensity of this yellow color is directly proportional to the concentration of EPO in the sample. A calibration curve of absorbance versus concentration is generated using results obtained from the standards. Concentrations of EPO present in the controls and samples are determined directly from this curve. The standards have been calibrated by the manufacturer against the WHO standard 87/684 (rHuEPO).

Streptavidin-coated strips were placed in the holders provided. Two-hundred microliters of standards, controls, and samples were pipetted into the designated or mapped well. Then 25 μ L of Reagent 1 (biotinylated antibody) and 25 μ L of Reagent 2 (enzyme-labelled antibody) were added to each of the wells. The microplate was tapped firmly a minimum of five times on each of its four sides in order to achieve thorough mixing of the sample with reagents. The microplate was covered with aluminium foil or a tray so as to avoid exposure to light. It was then placed on a shaker for 2 h \pm 15 min at room temperature (20–25°C). The fluid was completely aspirated and each well washed five times with the supplied Working Wash Solution. ELISA Reagent B (tetramethylbenzidine substrate) (150 μ L) was then added to each well and the plate tapped as described earlier. With appropriate cover in place so as to avoid light exposure, the plate was placed on a shaker for 30 \pm 5 min at room temperature (20–25°C). One-hundred microliters of Stopping Solution (0.05M sulfuric acid) was added to each of the wells. The plate was again tapped as described earlier. The absorbance of the solution in the wells was read within 10 min, using a microplate reader set to 450 nm, against 250 μ L of distilled water. During each analytical run, all specimens, standards (typically 0, 7.5, 18, 41, 120, and 494 mIU/mL, though these concentrations vary a little from batch to batch) and manufacturer's controls were run either in duplicate or triplicate. Replicate readings were averaged for each standard and the average 0 mIU/mL standard absorbance subtracted. A standard curve was created as per the manufacturer's instructions. In three runs recorded, the linear correlation coefficient ranged from 0.95 to 0.97. Because of the results obtained with this kit from measurements on the administration samples (see Results section), no further validation was performed. However, the manufacturer's product insert provides a range of validation data for clinical applications. No definite information is available on the relative reactivities of this kit to the different variants of HuEPO.

Measurements were again made using an Asys Hitech Expert Plus and Multiskan MS Version 3.0 microplate reader.

Immunoassay—IMMULITE LKEP1 kit

This is a solid-phase, chemiluminescent immunometric assay used with the IMMULITE 1000 Analyzer. This product is intended for serum/heparinized plasma analysis. Urine aliquots were used in place of the prepared blood product in the manufacturer's instructions. Sample (100 μ L of adjustor, control, or canine urine) was added to a test unit containing a bead. Reagent A, containing a ligand-labelled murine monoclonal anti-EPO antibody, was added to the test unit, which was then incubated for 0.5 h. Reagent B was added to the test unit; this contains alkaline phosphatase conjugated to goat polyclonal anti-EPO antibody in a buffer. The reaction was incubated for 0.5 h. The test unit was then washed and luminogenic substrate added. Five minutes later, the reaction tube was processed by the photomultiplier tube, where the light generated by the luminogenic reaction was measured. In this reaction, the substrate (an adamantyl dioxetane phosphate) is dephosphorylated into an unstable anion intermediate by the alkaline phosphatase conjugate on the bead. The unstable intermediate emits a photon upon decomposition. The amount of light emitted is directly proportional to the amount of bound alkaline phosphate. As this is a "sandwich" assay, the amount of light emitted is directly proportional to the concentration of analyte in the sample. The kits (from Euro/DPC Limited, Glyn Rhonwy, Llanberis, Wales) provide 100 tests each. The manufacturer provides a range of validation data for clinical applications (19). These include calibration range (up to 200 mIU/mL), analytical sensitivity (0.24 mIU/mL), high-dose hook effect, precision, linearity, recovery and specificity. These results were comparable to the R & D immunoassay kit. No definite information is available on the relative reactivities of this kit to the different variants of HuEPO.

This assay was also used to establish the concentration of the EPO in the retentate following concentration by ultrafiltration prior to the gel electrophoresis. Samples are diluted if necessary with 50mM Tris HCl buffer in order to obtain an EPO concentration of 500 mIU/mL and 20 μ L loaded onto the gel.

Isoelectric focusing immunoblotting

The procedure used is as described previously (10). Because of limited volumes, less than 5 mL of urine was taken, rather than the 20 mL recommended by Lasne et al. (10). Immunoassay of a portion of the ultrafiltrate was used to ensure that sufficient EPO could be added to the gel for the iso-electric focusing.

After focusing and double blotting, the image visualized by chemiluminescence was recorded using the FluorChem 8800 Imaging System and then analysed using AlphaEase FC software (version 3.1.2), both from Alpha Innotech (San Leandro, CA).

Administration study

Four 20-kg (approx.) beagles were administered 2500 units of EPREX[®] (supplied in pre-filled ready-to-use syringes). It was administered subcutaneously to dogs 1 and 2 and intravenously to dogs 3 and 4. The active ingredient is epoetin alpha. The preparation was obtained from Cahill May Roberts (Cork, Ireland). EPREX[®] was the preparation administered by

Roberts et al. (11,18) in their equine studies, and anecdotal information from greyhound veterinary surgeons indicated that it would be appropriate in this study. It was not possible to use greyhounds because of the long delays being experienced in obtaining licences under European Union legislation for administration studies on animals such as these. Beagles were chosen because of the close genetic similarity between them and greyhounds (20). A pre-administration urine sample was taken from each dog. Samples were then taken at regular intervals for seven days and all collection times recorded. Samples were collected by catheterization, and 20 mL was collected each time.

Animal welfare

Administration studies were carried out in the BioResources Unit, Trinity College, Dublin, Ireland. The BioResources Unit and its premises are registered under the European Communities (Amendment of Cruelty to Animals Act, 1876) Regulations, 2002 (SI No. 566 of 2002).

It is a requirement of such registration that the conditions of care and housing of animals conform to annex (ii) of directive 86/609, that the conditions are appropriate to the species housed, that the animal care staff have appropriate experience and training, and that adequate security arrangements exist.

The premises are under the full time supervision of a veterinary surgeon.

Negative control samples

In order to establish normal canine background EPO response levels, urine samples were obtained from 50 HuEPO-free greyhounds (i.e., no HuEPO administration).

Results

R&D Systems kit

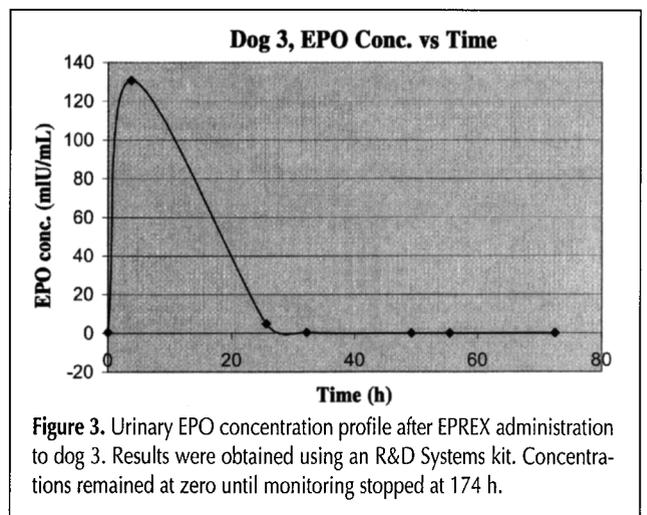
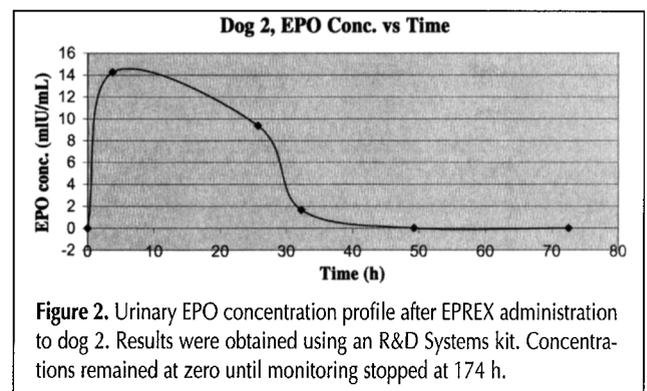
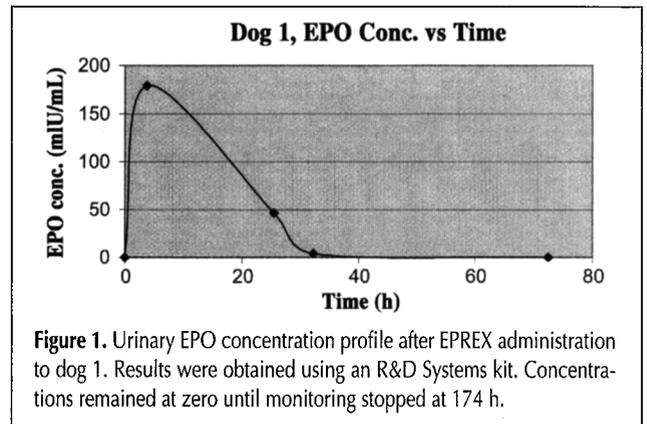
The pre-administration values measured in all four dogs were < 0.2 mIU/mL. The results obtained for dogs 1–3 are shown in Figures 1–3, respectively. The maximum levels of EPO observed in the urines from dogs 1, 2, and 3, at 3.75 h post-administration in each case, were 179, 14.3, and 131 mIU/mL, respectively. Thereafter, in all three cases, the observed EPO levels dropped until reaching a level less than 0.2 mIU/mL. This occurred between 32.3 and 49.3 h post-administration. The lack of urine samples obtained in this period makes it difficult to determine at exactly what time the EPO level dropped below 0.2 mIU/mL. No urine sample was obtained from dog 4 until 78 h after administration. Concentration peaked at 0.6 mIU/mL after 128 h. Values of < 0.2 mIU/mL were obtained for all 50 urine samples obtained from greyhounds that had not been administered EPO.

The measurements on the administration samples were repeated a year later. The samples had been stored frozen at a temperature of less than -18°C in the meantime. However, the positive administration samples had been repeatedly thawed and refrozen (for use as standards during routine lab screening) during that time. Although a considerable drop in

the EPO concentration of these latter samples was observed, it was still detectable in dog 1 up to 32.3 h and in dogs 2 and 3 up to 25.5 h.

Neogen kit

The EPO values observed using this kit were generally much lower than the corresponding values obtained using the R&D Systems kit (see Table I). In the case of dog 1, the 3.75 and 25.5 h post-administration samples, which gave concentrations of 179 and 46.5 mIU/mL, respectively, using the R&D Systems kit, gave values of 21.0 and 20.6 mIU/mL, respectively, using the Neogen Kit. The general shape of the excretion profile curve was similar for dog 1 using both kits. In the cases of dogs 2 and



3, no EPO was detected in any of the administration samples, and the same was true for dog 4.

Immulate

Using this kit, EPO was detectable for post-administration time periods similar to those obtained using the R&D Systems kit. However, the absolute concentration values obtained were lower in many cases (see Table I). The Immulate product insert warns, in regard to clinical testing, that "Because results obtained with any EPO assay may differ significantly from any other, it is recommended that any serial testing performed on

the same patient over time should be performed with the same EPO test." In the light of such known variation between immunoassay systems, the differences observed between the R&D and Immulate systems in the present study do not, for the most part, seem unexpectedly large.

In the case of dog 4, the concentration was again found to peak at 128 h for the small number of late samples obtained, giving a value of 2.2 mIU/mL (somewhat larger than the value obtained using the R&D Systems Kit).

Discussion

The excretion profiles following EPO administration to dogs indicated that EPO administration could be easily detected in urine using the R&D Systems kit up to 25.5 h after administration and was still significantly higher than background levels up to 32 h after administration. Between 32 and 49 h, EPO levels become indistinguishable from the background. Similar comments apply to the Immulate kit. However, the latter was found to be more expensive. Several of the post administration samples were submitted to the WADA-approved method for EPO. Less than 5 mL of urine was required for the samples tested since the concentration of EPO shown to be present by immunoassay of the ultrafiltrate indicated sufficient to be present for analysis of the isoforms. The aim of the immunoassay of the ultrafiltrate prior to electrophoresis is to establish the concentration of EPO. This assay measures total EPO and does

not distinguish between endogenous and the different recombinant products.

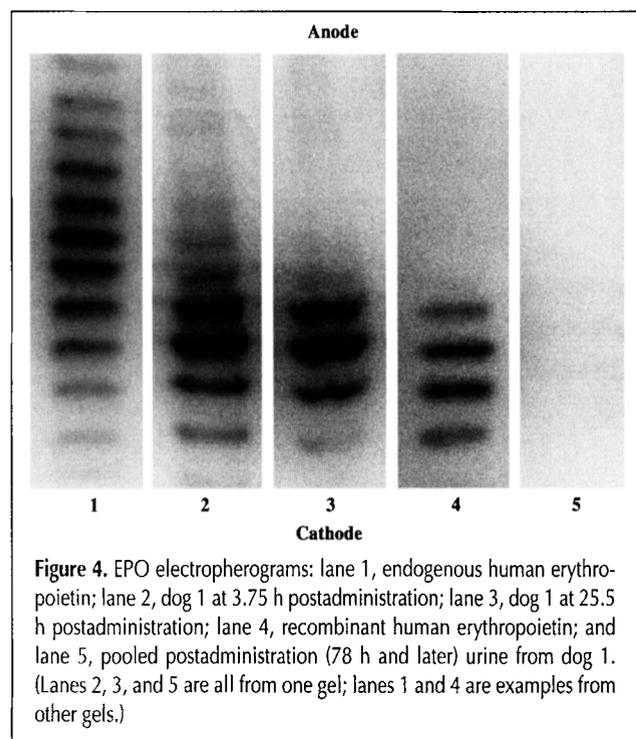
Previous work by us has shown that loading 10 mIU of EPO onto the gel (typically using a 20- μ L volume of retentate, diluted if necessary to contain 500 mIU/mL of EPO) readily produces a clear isoform pattern. In some samples, as little as 2 mIU is sufficient. The amount of EPO in the retentate is determined using the Immulate assay. The Immulate results do not exactly correlate with the intensity of the bands observed. In this case, the two samples (dog 1, 3.75 h and 25.5 h post-administration) presented EPO concentrations by immunoassay of 1460 mIU/mL and 792 mIU/mL in the retentate after ultrafiltration from initial volumes of urine of 1.5 and 1 mL, respectively. These equate to concentrations in the original urine of 22.4 mIU/mL and 19.8 mIU/mL. Normally for human work, 20 mL of urine is routinely used. We were fortunate that these canine samples had sufficient EPO present to enable the smaller volumes to be used successfully.

Representative electropherograms are shown in Figure 4. Lane 1 is from authentic endogenous human EPO and shows several intense bands midway between anode and cathode corresponding to the various isoforms of EPO. Lanes 2 and 3 show a profile with intense bands in the basic region of the gel

Table I. Urinary EPO Concentrations (where samples were obtained) after EPREX® Administration to Dogs 1, 2, and 3*

Time (h)	Concentration EPO Found (mIU/mL)								
	R&D			Immulate			Neogen		
	Dog 1	Dog 2	Dog 3	Dog 1	Dog 2	Dog 3	Dog 1	Dog 2	Dog 3
0	0	0	0.1	0	0	0	0.1	0	0
3.75	179	14.3	131	5.1	11.3	70.4	21.0	0	0
25.5	46.5	9.4	4.8	3.0	6.7	18.2	20.6	0	0
32.3	4.0	1.7	0.4	3.6	2.7	2.4	10.8	0	0
49.3	–	0	0	–	<1	<1	–	0	0
55.5	–	–	0	–	–	1.0	–	–	0
72.5	0	0	0	<1	<1	1.1	0	0	0
78	–	0	0.6	–	<1	<1	–	0	0
97	0	0	0	<1	<1	<1	0	0	0
128	0	0	0	<1	<1	<1	0	0	0
150	0	0	0	<1	<1	<1	0	0	0
174	0	0	0	<1	<1	<1	0	0	0

* Results obtained using the R&D Systems kit, the Immulate kit, and the Neogen kit.



(near to the cathode) consistent with the profile of rHuEPO isoforms comparable with those shown in lane 4 from authentic recombinant human EPO. Lane 5 shows the absence of bands consistent with the absence of human EPO observed from the immunoassay results. Following the administration study, a screening program for EPO, using the R&D Systems kit, was introduced at the National Greyhound Laboratory. At the time of writing, approximately 6000 greyhound sport (mostly racing) samples have been tested, of which four have tested positive. The presence of human EPO in all four samples was then confirmed using the WADA-approved method. Three of the four samples were found to contain rHuEPO. The fourth represented an unexpected, but decidedly welcome, outcome of the present work in that it involved the detection of endogenous HuEPO in a sample submitted as greyhound urine. In the subsequent Control Committee hearing, it was determined that this arose from the submission of human urine, as greyhound urine, to the control stewards. The sample in question gave an EPO concentration reading of 8.0 mIU/mL on the ELISA plate. The electropherogram obtained had an isoform profile typical of HuEPO and similar to that obtained for the endogenous HuEPO standard in lane 1 of Figure 4.

The specificity of the ELISA kit as a screening technique would appear to be borne out by the fact that no false positives occurred.

Acknowledgments

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