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Detection of Recombinant Human Erythropoietin in Urine by Isoelectric Focusing

Andreas Breidbach,[1] Don H. Catlin,[1,3*] Gary A. Green,[1,2] Inna Tregub,[1] Henry Truong,[1] and Jeffrey Gorzek[1]

Background: **Doping with erythropoietic proteins such as recombinant human erythropoietin (rHuEPO) and darbepoetin alfa is a serious issue in sport. There is little information on the time course of detection of rHuEPO in urine and on methods to evaluate electrophoresis-based data.**

Methods: **We used a recently described isoelectric focusing method for detecting rHuEPO and endogenous EPO in urine obtained from individuals treated with placebo or epoetin alfa. The latter was administered subcutaneously at 50 IU/kg on days 0, 2, 4, 7, 9, 11, 14, 16, and 18. Blood and urine samples were collected during the morning of study days −3, 0, 2, 4, 7, 9, 11, 14, 16, and 18 and on days 2, 3, 4, and 7 postadministration. We developed visual and numerical (two-band ratio) techniques to evaluate the electropherograms for the presence of rHuEPO.**

Results: **Compared with the placebo group, the epoetin alfa-treated group responded with increases in hematocrit, reticulocytes, macrocytes, serum EPO, and serum soluble transferrin receptor. The electropherograms showed that the pattern of bands arising from urinary rHuEPO is different from that of endogenous urinary EPO. Both the two-band ratio and the visual technique detected rHuEPO in all 14 epoetin alfa-treated individuals 3 days after the last dose. On the 7th day after the last dose, both techniques detected rHuEPO in approximately one-half of the participants. rHuEPO was not detected in the placebo-treated individuals.**

Conclusions: **The isoelectric focusing method detects rHuEPO in most urine samples collected 3 days after nine doses of epoetin alfa. The numerical two-band ratio was equivalent to a visual method for detecting rHuEPO in urine.**

Recombinant human erythropoietin (rHuEPO)[4] is a glycoprotein with 165 amino acids and three N-linked and one O-linked glycans that is expressed in Chinese hamster ovary cells transfected with DNA encoding HuEPO. HuEPO is the main factor responsible for the proliferation of erythrocytes in the human body. Approximately 90% of HuEPO production takes place in the kidneys whenever a tissue oxygen sensor detects oxygen depletion *(1, 2)*. For patients with kidney failure, the loss of the main HuEPO production site leads to severe anemia, which was treated by blood transfusion until 1988 when rHuEPO became available. Subsequently, rHuEPO has been approved for the treatment of anemia attributable to renal failure, cancer, and other types of anemia. Doping with erythropoietic proteins such as rHuEPO and darbepoetin alfa is perhaps the most complex and serious issue facing sport authorities today. A prodigious amount of anecdotal data indicates that such drugs are widely used in some sports *(3)*.

In endurance sports, the main performance-limiting factor is the oxygen-carrying capacity of the blood. rHuEPO enhances athletic performance by increasing the number of erythrocytes *(4)*. The International Olympic Committee added rHuEPO to its "List of Prohibited Substances" in 1990, although at that time no method existed to detect it in body fluids. A method for detecting rHuEPO in urine by electrophoresis was first described in 1995 *(5)*, and in 2000, Lasne and de Ceaurriz *(6)* introduced an isoelectric focusing (IEF) method coupled with a technique that reduced the nonspecific binding that accompanies immunoblotting. The technique, called "dou-

[1] UCLA Olympic Analytical Laboratory, [2] Department of Family Medicine, Division of Sports Medicine, and [3] Department of Molecular and Medical Pharmacology, University of California at Los Angeles, Los Angeles, CA 90025.

*Address correspondence to this author at: UCLA Olympic Analytical Laboratory, 2122 Granville Ave., Los Angeles, CA 90025. Fax 310-205-9177; e-mail Dcatlin@ucla.edu.

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[4] Nonstandard abbreviations: rHuEPO, recombinant human erythropoietin; IEF, isoelectric focusing; sTfr, soluble serum transferrin receptor; TBR, two-band ratio; QCN, negative quality control; and QCP, positive quality control.

ble-blotting", led to a practical method that is used to detect rHuEPO and darbepoetin alfa (a long-acting analog of rHuEPO) in the urine of athletes *(7, 8)*.

Very little is known about the time course of urinary rHuEPO after its administration. An increase in immunoreactive EPO has been reported during rHuEPO administration but not in samples collected 4 days after the last dose *(9)*, and Wide et al. *(5)* reported detecting rHuEPO in urine by electrophoresis up to 48 h after the last dose. In this study, our objective was to determine the time course of detectable rHuEPO in urine, using the improved IEF method *(6)*, after nine doses of rHuEPO. We also describe numerical and visual criteria for determining whether rHuEPO is present.

Materials and Methods

DRUGS, REAGENTS, AND CHEMICALS

rHuEPO (Epogen™) was obtained from Amgen Inc. The sources of all other drugs, reagents, and chemicals have been described elsewhere *(7)*.

STUDY PARTICIPANTS

We studied 13 men (9 Caucasians, 3 African Americans, and 1 Asian) and 12 women (11 Caucasians, 1 African American) between 23 and 40 years of age. The study participants were screened by medical history, physical examination, and blood and urine tests. Hematology screening tests included hematocrit, hemoglobin, macrocytes, and percentage reticulocytes. Serum screening tests were creatinine, ferritin, EPO, and soluble transferrin receptor (sTfr). The inclusion criteria included hematocrit of 36–45%, serum ferritin >30 μg/L, and regular physical exercise. Individuals were excluded if the history revealed major organ system disease, substance abuse, participation in any sport drug-testing program, or medications that affect the hematologic, hepatic, or renal systems. Females were screened for pregnancy and African Americans for sickle cell trait. The study was approved by the Institutional Review Board of the University of California at Los Angeles, and all study participants gave written informed consent.

DRUG-FREE CONTROL INDIVIDUALS

We collected one urine from each of 59 men and 37 women between 18 and 44 years of age. The ethnicity of the individuals was 10 African Americans, 7 Asian, 48 Caucasians, 18 Hispanics, and 13 undesignated. Forty-nine individuals lived in Los Angeles, CA, and 47 lived in Salt Lake City, UT. Medical history examination determined that the individuals had no active disease and were not taking any medications affecting the hematologic, hepatic, or renal system. The minimum hematocrits for males and females were 40% and 35%, respectively. The study was approved by the Institutional Review Board of the University of California at Los Angeles, and all study participants gave written informed consent.

PROTOCOL

After 25 individuals qualified for the study, they were paired according to ethnicity, gender, and similar hematologic weighted erythropoietic index *(10)*. One individual in each pair was randomly assigned to the epoetin alfa treatment group and the other to the placebo group. Five individuals who could not be paired were assigned to the epoetin alfa treatment group. The participants were blinded with regard to group. The 15 individuals in the epoetin alfa group (50 IU/kg) and the 10 in the placebo group (1 mL of 9 g/L NaCl) received epoetin alfa or saline subcutaneously between 0800 and 1100 on days 0, 2, 4, 7, 9, 11, 14, 16, and 18. All participants received 325 mg/day of ferrous sulfate (65 mg of elemental iron) by mouth from day −7 to day 25.

Blood and untimed urine samples were collected during the morning of study days −3, 0, 2, 4, 7, 9, 11, 14, 16, 18, 20, 21, 22, and 25. The last 4 study days corresponded to days 2, 3, 4, and 7 postadministration (washout). Blood and urines were collected immediately before administration of saline or epoetin alfa. Blood pressure, pulse, and queries regarding potential side effects were monitored on each visit. If the hematocrit exceeded 48%, the dose for that day was withheld; if the hematocrit exceeded 48% on two or more consecutive visits, the individual was excluded from the study. For all individuals, the red cell indices were measured within 8 h of collection. Serum and urine were stored at −70 °C until analysis. The methods for determining the erythropoietic index have been described *(10)*. In brief, hematocrit, percentage macrocytes, and percentage reticulocytes were determined with an ADVIA120 Hematology Analyzer (Bayer Diagnostics). The serum EPO concentrations were determined with an automated immunometric chemiluminescent assay (Immulite EPO; Diagnostic Products Corporation). The sTfr concentrations were determined by an automated immunonephelometric assay (Dade Behring GmbH). The performance characteristics of the serum EPO and sTfr assays have been described *(11, 12)*.

IEF

IEF was performed as described previously *(7)*. In brief, 20 mL of urine was subjected to ultrafiltration, and the retentate containing EPO was focused in an IEF gel (pH 2–6). The isoforms were double-blotted *(13)* and visualized by chemiluminescence. The emitted light was captured with a FluorChem 8000 CCD camera (Alpha Innotech Corp.).

BAND IDENTIFICATION

A representative example of an IEF gel with two lanes containing markers (lanes S) and four lanes of unknowns (lanes 1–4) is shown in Fig. 1. For the lanes representing samples whose rHuEPO content was unknown, band zero (0) is identified as the band with the same isoelectric point (pI) as the most acidic band in the rHuEPO marker. Starting from band 0, the bands toward the cathode are

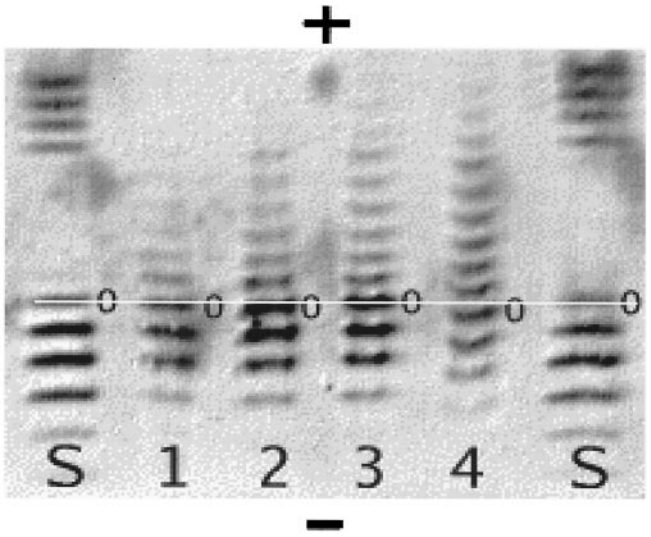

Fig. 1. Example of an IEF image illustrating the selection of the zero band (*0*) for six data lanes.

The two lanes containing markers (*lanes S*) were spotted with 2 fmoles each of rHuEPO and darbepoetin. *Lanes 1–4* are samples obtained from an epoetin alfa-treated individual. The *0* is placed immediately to the *right* of the band corresponding to the most acidic band of rHuEPO in *lanes S*, which in this image is the fifth band from the cathode. The most basic band is faint.

considered "basic" and the bands toward the anode are considered "acidic". Ideally, all bands with the same pI would line up perfectly horizontally across all lanes. In reality, the pH gradient created by ampholytes is subject to disturbances by sample constituents that lead to deflections of the lines of equal pH (iso-pH) and produce bands that have been characterized as "frowns" or "smiles". Smiles are bands with the left and right ends (tips) curved upward, such as band 0 in lane 4 of Fig. 1. Frowns are bands with the tips curved downward (not shown in Fig. 1). As seen in Fig. 1, the horizontal line connecting the 0 bands in the two lanes containing markers (lanes S) also passes through the outer tips of the band labeled 0 in lane 4, leading to the assignment of this band as band 0.

VISUAL DATA ANALYSIS

Three main criteria for identifying rHuEPO were developed. The first criterion was that bands that focus in the basic area of the lane, as determined by the location of the rHuEPO marker, must be darker than other bands in the same lane. The second criterion was that these bands must have the same pI values as the bands in the nearest lane containing a rHuEPO marker. The third criterion was that band 0 and the adjacent two bands in the direction of the cathode must be present. If all three of these visual criteria were met, the sample was considered to contain rHuEPO. If band 0 and the two adjacent basic bands were incompletely resolved or associated with any light or dark spots or imperfections, the sample was reanalyzed. If any of these criteria failed, the sample was categorized "rHuEPO not detectable". Four experienced readers followed the above three criteria to evaluate 48 images of lanes obtained from 24 individuals on days 3 and 7 postadministration. The images were randomized, and the readers were blinded. The instructions were to assign each image to the epoetin alfa or the placebo group.

NUMERICAL DATA ANALYSIS

Lane densitometry was carried out with FluorChem AlphaEase (Ver. 2.01) software (Alpha Innotech Corp), using the 1D-MultiAnalysis, Auto Grid option. The lane template was positioned such that it included two lanes containing markers with a maximum of four unknown lanes in between. For baseline adjustment, the default "Auto Base" method was used, in which the left boundary of the respective lane was broken into 16 regions. The mean background of each of those 16 regions was then used to construct the baseline. The resulting peaks were auto-integrated, using the default settings. Peaks that were not integrated by the software were manually integrated. We defined a peak as one in which the baseline-subtracted height was >30% of its total peak height, i.e., the corresponding band was at least 30% darker than its immediate surroundings. This process is illustrated in Fig. 2, which shows the densitogram of lane 3 of Fig. 1. For each lane in the gel, we calculated a ratio of peak areas. We referred to this ratio as the two-band ratio (TBR) where:

$$\mathrm{TBR} = \frac{\Sigma \mathrm{PA}_{2\text{-BASIC}}}{\Sigma \mathrm{PA}_{2\text{-ACIDIC}}}$$

$\Sigma \mathrm{PA}_{2\text{-BASIC}}$ is the combined peak area of the two bands immediately adjacent to band 0 on the basic side, and $\Sigma \mathrm{PA}_{2\text{-ACIDIC}}$ is the combined peak area of the two bands adjacent to band 0 on the acidic side. To avoid division by

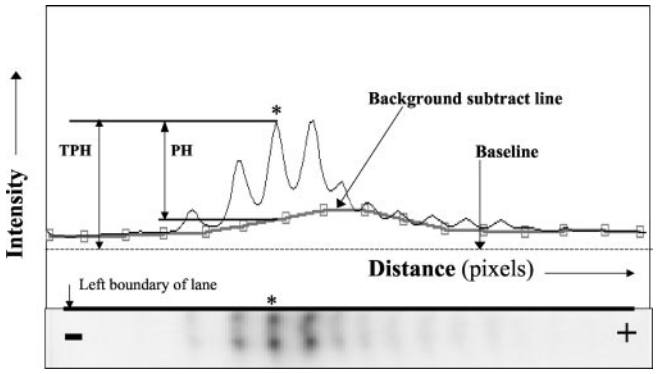

Fig. 2. Densitogram of lane 3 of Fig. 1 showing peaks produced when the CCD camera software integrates the pixel data shown in the electropherogram in Fig. 1.

The *x axis* represents the distance in pixels from the cathode edge of the integration grid. The *y axis* represents intensity. The *dashed horizontal line* is the baseline. □——□ is the background subtract line determined by the software. *Lane 3*, which has been rotated 90 degrees clockwise from its orientation in Fig. 1, is shown horizontally at the *bottom* of the figure. The left boundary of the lane, which is used to determine the background, is shown schematically as a *solid line* at the *upper edge* of the rotated lane. The + and − represent the anode and cathode. The *starred* (*) peak corresponds to the *starred* band. Total peak height (*TPH*) and background-subtracted peak height (*PH*) are indicated by *arrows*. A peak is accepted if PH is >30% of TPH.

0, if the latter two bands failed the 30% test, the denominator in the TBR was assigned the value of 1.

STATISTICAL ANALYSIS

All computations were performed on a PC with a Pentium™ chip utilizing the statistical software suite "R" *(14)*. We used the "survival" package with procedure "survreg" to calculate the geometric mean, *x*-axis scale, and various percentiles. Zero values were considered to be left-censored and the distribution to be log-normal. The Wilcoxon rank-sum test was used to determine differences between the epoetin alfa- and placebo-treated groups. *P* values were two-sided, and values <0.05 were considered statistically significant.

Results

CLINICAL EFFECT OF rHuEPO

The baseline characteristics of the placebo and epoetin alfa individuals are shown in Table 1. All individuals were between the ages of 23 and 40 and were well matched for age, weight, height, exercise, and erythropoietic index. The participants completed 347 of the possible 350 visits. Of 135 possible rHuEPO injections, 3 doses were withheld from one individual who was then excluded from the IEF analyses. Two other individuals missed one dose of epoetin alfa but remained in the study.

The efficacy of EPO was established by calculating an erythropoietic index (on-score), which is a weighted index of rHuEPO activity *(10)*. The five variables in the index are hematocrit, reticulocyte hematocrit, percentage macrocytes, serum concentration of EPO, and sTfr. The on-score was calculated using blood samples obtained 3 and 0 days before first drug administration (baseline); samples obtained on days 2, 4, 7, 9, 11, 14, 16, and 18 of treatment; and on days 2, 3, 4, and 7 after the last dose of epoetin alfa or placebo. The mean (SD) on-scores for all females and males in the placebo group on all days were 2.02 (0.045) and 2.27 (0.063), respectively, and there was no change over the 28 days of the study. For the females treated with epoetin alfa, the mean on-score steadily increased to a maximum of 2.77 on day 20 (day 2 postadministration) and then steadily decreased. For the male epoetin alfa group, the mean on-score steadily increased to a maximum of 2.91 on day 18 (last day of epoetin alfa administration) and then steadily decreased. For the male epoetin alfa group, the mean on-score was significantly higher than that of the placebo group for all days after day 2. For the female epoetin alfa group, the mean on-score was significantly higher than that of the placebo group on days 2 and 7–25.

Table 1. Demographic characteristics and baseline data of the individuals who received placebo and epoetin alfa.

	Placebo group		Epoetin alfa group	
	Mean	Range	Mean	Range
Age, years	31.6	23–40	30	23–40
Weight, kg	71.1	55.9–95.9	68.7	53.6–86.4
Height, cm	174	163–190	174	160–190
Exercise, h/week	11.7	5.0–22.5	10.2	5.0–25.0
Creatinine, mg/L	09	08–12	10	08–12
Hematocrit, %	41.8	35.8–44.9	41.2	35.8–44.8
On-score	2.1	1.9–2.3	2.1	1.81–2.38
Male, n	5		8	
Female, n	5		7	
Caucasian, n	9		11	
African-American, n	1		3	
Asian, n	0		1	

REPRESENTATIVE IMAGE OF MARKER AND URINARY ISOFORMS

Shown in Fig. 3 are representative examples of an IEF image obtained from the analysis of markers (lanes S), a negative quality-control urine (lane QCN), a clinical positive quality-control urine (lane QCP), and urines obtained from two epoetin alfa- and one placebo-treated individuals. The Lanes S (markers) contain 2 fmoles each of rHuEPO (the five bands closest to the cathode) and darbepoetin (the four bands closest to the anode).

Also shown in Fig. 3 are the isoform patterns of samples obtained on days 2, 3, and 4 postadministration from individual A in the placebo group (Fig. 3, section A). The placebo-group patterns were very similar to the patterns of the QCN. They were also similar to the patterns observed in the other 9 placebo-treated individuals and the day −3 and 0 samples from the 14 remaining individuals in the epoetin alfa group (not shown). Four samples obtained on days 2, 3, 4, and 7 postadministration from individual B in the epoetin alfa-treated group had patterns very similar to that of the QCP for all days (Fig. 3, section B). Four samples from individual C in the epoetin alfa-treated group, obtained on the same days, had a pattern that changed over time (Fig. 3, section C): close to QCP on day 2, close to QCN on day 7, and intermediate on days 3 and 4. Results for individual C were included in Fig. 3 to show such changes in patterns over time.

VISUAL DATA ANALYSIS

The four experienced readers properly identified the day 3 postadministration samples as being from the epoetin alfa group (n = 14) or the placebo group (n = 10). For the day 7 postadministration samples, the readers properly assigned the 10 placebo samples to the placebo group; however, for the 14 epoetin alfa samples, some were classified as from the placebo group (Fig. 4).

NUMERICAL DATA ANALYSIS

For 19 of the 96 controls, the TBR could not be calculated because of absent peaks (n = 15) and interfering spots (n = 4). Of the remaining 77 samples, 12 (16%) showed a TBR of 0. The 99th percentile was calculated to be 1.19. For the epoetin alfa group, the TBR values ranged from 0.42 to

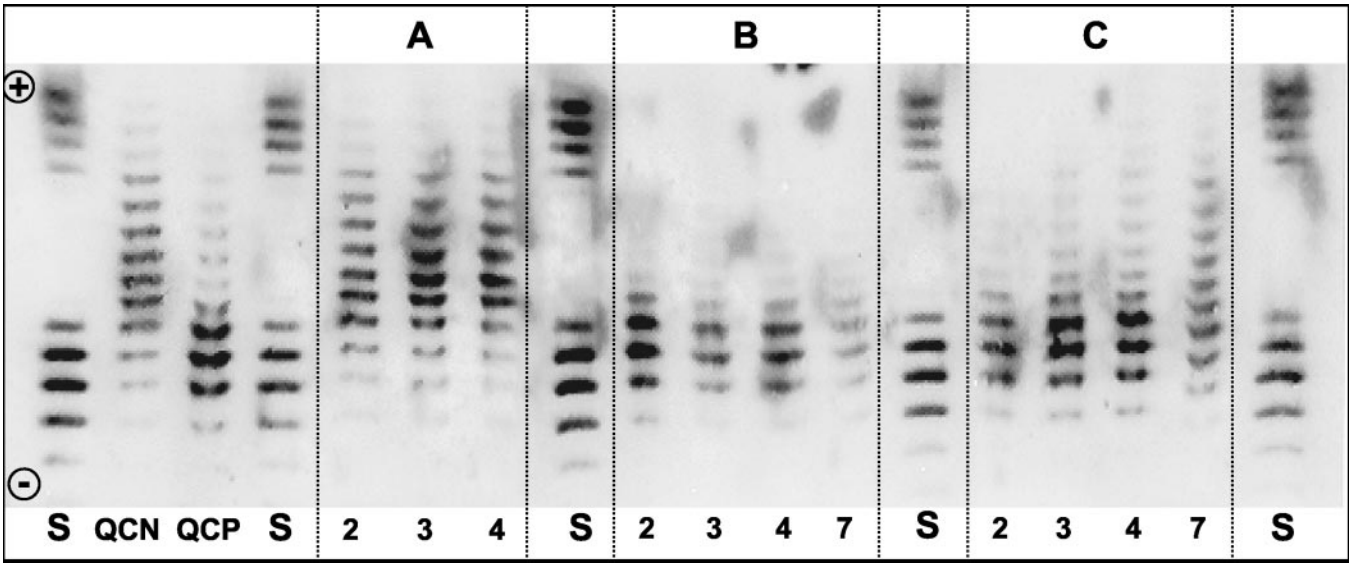

Fig. 3. Representative image of urinary EPO isoforms obtained by IEF analysis of urines from individuals treated with epoetin alfa and placebo.

The five lanes containing markers (*lanes S*) were spotted with 2 fmoles each of rHuEPO and darbepoetin. *Lanes QCP* and *QCN* represent urines from individuals known to be receiving rHuEPO and not to be receiving rHuEPO, respectively. The *lanes* in *section A* were obtained from a placebo-treated individual on postadministration days 2, 3, and 4. The *lanes* in *sections B* and *C* were obtained from epoetin alfa-treated individuals on days 2, 3, 4, and 7.

91 740 on the four postadministration days. None of the samples from the placebo group exceeded a TBR of 1.19 (range, 0–0.77). Of 95 samples analyzed from the epoetin alfa- and placebo-treated individuals on the 4 post-epoetin alfa days, only 3 samples (3%) contained too little EPO for evaluation. Those three samples came from three epoetin alfa-treated individuals on days 4 (n = 1) and 7 (n = 2) postadministration.

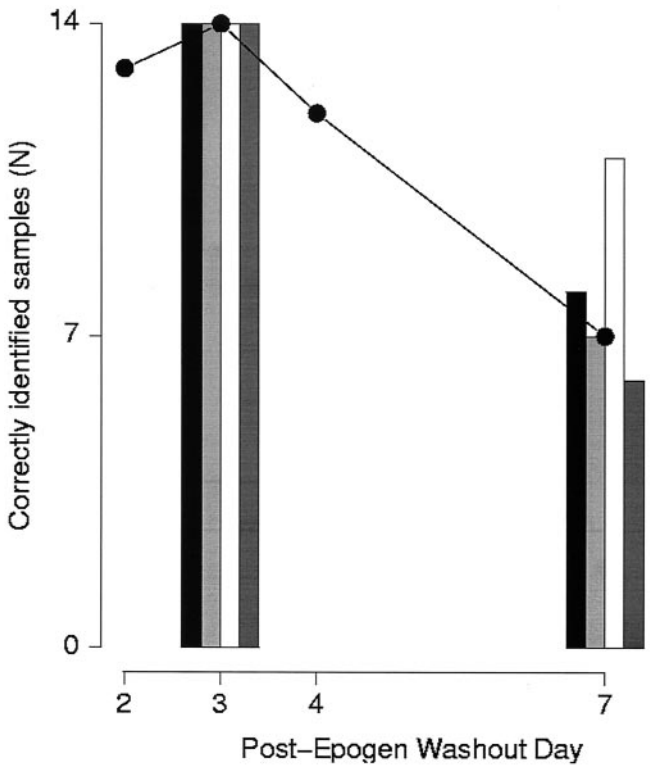

Fig. 4. Number of individuals found to have rHuEPO in their urine 2, 3, 4, and 7 days after the last dose of epoetin alfa.

The *solid lines* connect data points (●) corresponding to the TBR, and the *columns* represent the data determined by visual inspection of the IEF image. The data for the four visual data readers are indicated by *column shading*.

COMPARISON OF THE TBR AND VISUAL METHODS OF DATA ANALYSIS

Shown in Fig. 4 are the differences between the TBR and the visual method of data analysis; Fig, 4 also provides data on the time course of detection of rHuEPO in urine. On post-epoetin alfa days 2, 3, 4, and 7, the TBR properly identified 13, 14 (all), 12, and 7 of the 14 samples, respectively. The visual readers properly identified all the samples on post-epoetin alfa day 3 and between 6 and 11 of the 14 samples on day 7. The percentages of positive results for the two methods of data analysis were identical for the post-epoetin alfa day 3 data and very similar on day 7.

Discussion

The pattern of urinary isoforms of rHuEPO differs from that of endogenous EPO. The former are clustered into four or five bands in the most basic portion of the gel, whereas the latter, which include as many as 14 bands, overlap with and are parallel to the rHuEPO bands in the basic region but are also found in the more acidic region of the gel. Furthermore, after several days of epoetin alfa administration, the endogenous bands become less dense and eventually disappear. These results confirm the findings of Lasne and de Ceaurriz *(6)* and further establish that the method is suitable for detecting rHuEPO in urine.

Our results pertain to epoetin alfa, the drug adminis-

tered; however, the results would be very similar if epoetin beta were administered. Epoetin beta has been shown to migrate like epoetin alfa, and it has one more band in the basic region *(6)*. It is not possible to comment on the applicability of the method to epoetin omega *(15)* because its pattern of urinary isoforms has not been published.

When rHuEPO is administered to healthy or diseased individuals, it increases the hematocrit, reticulocytes, macrocytes, serum EPO concentration, and sTfr concentration *(16)*. In this study, the increase in the weighted erythropoietic index had reached statistical significance vs the placebo group by day 2, and it continued to increase until it plateaued about day 12. The weighted erythropoietic index did not begin to decrease until the second day after rHuEPO was discontinued and thereafter decreased rapidly but was still greater than the index for the placebo group on day 7 postadministration. This confirms that an indirect index of erythropoietic activity, such as the on-score *(10)*, is a valid indicator of rHuEPO activity.

The presence of rHuEPO in urine followed a similar course. Although endogenous HuEPO contains isoforms that focus in the same area as rHuEPO, there is a significant difference between epoetin alfa and placebo groups in the urinary EPO isoform patterns with respect to the density of the bands within one lane *(6)*. The main difference is where the darkest bands are located. The time course of detection of urinary rHuEPO (Fig. 4) revealed that nearly all of the samples collected on days 2, 3, and 4 after epoetin alfa was discontinued contained rHuEPO. Thus, rHuEPO was highly detectable in urine for up to 4 days after we discontinued epoetin alfa that had been given nine times in 18 days. Between days 3 and 7 after the discontinuation of epoetin alfa, the number of cases detected decreased to ~50%. Therefore, as with many performance-enhancing drugs, the most effective use of this test is in out-of-competition testing. In the urines of individuals B and C, there was hardly any detectable endogenous EPO. The reason for that could be down-regulation of endogenous EPO production *(17, 18)*. It could also be explained by the presence of overwhelming amounts of urinary rHuEPO, which led to the need to dilute the retentate, which in turn would make endogenous EPO harder to detect.

In 1995, Wide et al. *(5)* first reported detecting rHuEPO in urine by electrophoresis. Their assay was sufficient to detect rHuEPO in the urine of 11 of 11 individuals in samples collected up to 24 h after rHuEPO was administered (20 IU/kg three times per week) for 7–9 weeks. The detection rate decreased to 75% at 48 h. Urinary EPO has also been detected by RIA during rHuEPO administration (200 IU/kg on 5 of 10 days) but not 4 days after the last dose *(8)*. In the current study, the last collection day was day 7 postadministration, and approximately one-half of the participants still had detectable urinary rHuEPO.

The visual and numerical methods for evaluating IEF data produced very similar results (Fig. 4). Four different data readers, using the three visual criteria, correctly identified the 14 individuals in the epoetin alfa group and the 10 individuals in the placebo group on samples collected 3 days after the last dose of epoetin alfa. On day 7 postadministration, when the amount of rHuEPO was expected to be decreasing and the serum concentrations of EPO had decreased *(16)*, the readers still detected rHuEPO in ~50% of the samples, although with a lesser degree of concordance.

Densitometry converts band patterns into numerical values that can be readily combined into various scores or ratios. Because the isoforms of HuEPO and rHuEPO focus in overlapping areas, whatever scores or ratios are used must account for the usual variation of HuEPO isoform patterns. We selected the TBR as our preferred method of numerical analysis after evaluating various other ratios that could be used to characterize urinary rHuEPO. Compared with numerical methods that require analyzing all of the bands, the advantages of the TBR method include simplicity (only four bands need to be analyzed), speed (the analysis and calculation times are considerably reduced), fewer lanes declared invalid because of interfering spots, and easier accommodation of lanes that are not in perfect vertical alignment. In addition, there is no ambiguity regarding assignment of band 0.

In our procedure, the isoform pattern is visually inspected and the three criteria described here are applied. Samples that do not meet these criteria are declared "no detectable rHuEPO". If the criteria are met but some bands overlap or have interfering spots, the samples are reanalyzed. The TBR is then determined by lane densitometry. If both the visual and numerical criteria are met, it is highly likely that the sample contains rHuEPO. From the perspective of statistical limitations, at this time our numerical data are sufficient to determine the 99th percentile. The 99.9th percentile or higher margins of safety require a larger number of samples. Accordingly, we believe it is advisable to accumulate more data and to participate in interlaboratory studies designed to enhance the criteria for stating that a sample contains rHuEPO.

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