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W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

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A. BREIDBACH, W. SCHÄNZER:
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Andreas Breidbach and Wilhelm Schänzer

Fast and Accurate Determination of Urinary Human Erythropoietin with Nichols Institute Diagnostics Chemiluminescence Immunoassay for Erythropoietin after Gelfiltration

Institute of Biochemistry, German Sport University, Cologne

Introduction

Erythropoietin (EPO) is a sialoglycoprotein with 165 AA, three N-linked glycans and one O-linked glycan, which promotes the formation of red blood cells and therefore influences the oxygen carrying capacity of blood. This makes recombinant human Erythropoietin (rhEPO) a potential doping agent for endurance athletes (1). Available as a recombinant, chinese hamster ovary (CHO) cell expressed product since 1988, the International Olympic Commitee put recombinant human Erythropoietin on its "list of banned substances" in 1990.

Up to this time there is no established method to control this ban, and therefore athletes can misuse rhEPO without having to fear sanctions. A possible approach to detect a misuse is the establishment of reference ranges. These could be ranges for parameters influenced by EPO, like certain blood parameters (2) or fibrinogen degradation products in urine (3), or ranges for EPO itsself in blood or urine. To sanction an athlete for a misuse, strong evidence has to be provided. The determination of EPO itsself is stronger evidence than the determination of some EPO influenced parameters. In doping analysis, especially in out-of-competition control, blood sampling at this time is not legal and is associated with many problems (4), so urine is collected. For blood many immunological assays (ELISA, IRMA, ILMA) are commercially available for the direct determination of EPO, but to date only Wide et al. (5) and Souillard et al. (6) were able to determine urinary EPO. These two methods involved immunoradiometry and the one of Wide et al. was very time consuming.

As in routine doping control the volume of a urine sample is limited and high numbers of samples have to be tested within a limited period of time, a method for the determination of urinary EPO has to be fast and use only a small volume of urine. Here we present a fast and accurate method for the direct determination of EPO in urine using gelfiltration as a clean-up step with 2.0 mL of urine and a cycle time for one sample of ca. 5 min.. NID's chemiluminometric immunoassay is used for quantification with an incubation time of 15 h . This method is not only interesting for the detection of EPO misuse but also for the determination of excreted EPO in pharmacological studies.

Materials and Method

Chemicals and reagents

Disodium hydrogenphosphate dodecahydrate (Na_2HPO_4*12 H_2O), sodium chloride, potassium chloride and sodium azide (NaN_3) were of analytical grade and purchased from MERCK, Germany. Bovine serum albumin (BSA), heat-shock-fractionate fraction V, was purchased from SIGMA, Germany, the chemiluminescence erythropoietin immunoassay and the trigger solution set from NICHOLS INSTITUTE DIAGNOSTIC, Germany. The rhEPO (EPREX 2000 IU/ 0.5 mL) was provided courtesy of NICHOLS INSTITUTE DIAGNOSTIC, Netherlands.

The wash buffer was prepared by dissolving 10 mmol (3.08 g) Na_2HPO_4*12 H_2O, 120 mmol (7.01 g) NaCl, 2.47 mmol (0.2 g) KCl and 0.5 g NaN_3 in 1000 mL of bidestilled water. The pH was adjusted to 7.4 by adding 120 mmol/L HCl. The elution buffer was prepared by dissolving 1 g BSA in 1000 mL of wash buffer. The pH was adjusted to 7.4 by adding 120 mmol/L HCl. Before use, the buffers were filtered over a hydrophillic durapore filter (pore size 0.45 mm; MILLIPORE, Germany) and degased.

EPO stock solution

The stock solution was prepared by adding elution buffer to 100 µL of a solution of EPREX 2000 IU/ 0.5 mL to bring the volume up to 100.0 mL. 3 mL aliquotes were stored at -20 °C. This stock solution was diluted tenfold and calibrated against the NICHOLS EPO-Kit standards following the regular assay protocol.

Calibration solutions

For the calibration solutions, 25, 75, 125, 175, 375, 625 and 1000 µL aliquots of the stock solution were each brought up to a volume of 100.0 mL with elution buffer. 3 mL aliquots of these solutions were stored at -20 °C.

Quality control

The urine of an endurance athlete, collected over a period of 12 h during daytime, was stored in 3 mL aliquots at - 20 °C. This urine is used as a quality control (QC).

Gelfiltration

Two HiTrap Desalting columns (PHARMACIA BIOTECH, Germany), each containing 5 mL of Sephadex G-25 Superfine gel , were connected in series. New columns were treated with wash buffer according to the manufacturers instructions. Each sample preparation cycle started with the application of 15 mL wash buffer. We used a programmable peristaltic pump (MS-Reglo Digital 4/8;ISMATEC, Germany) to apply buffers and samples to the twin column. The column was then equilibrated with 15 mL of elution buffer. In the third step, 2.0 mL of calibration standard or urine were applied to the column. Finally elution buffer was again applied to the column. In our set up the first 2.0 mL of elution buffer were discarded

and the next 3.0 mL were collected in 5 mL polystyrol test tubes (75*12 mm; SARSTEDT, Germany). The exact elution volume for EPO has to be determined for each set up used. The manufacturer suggests a maximum flow rate of 10 mL/min. for these desalting columns. So one preparation cycle takes less than 5 min. After use the column was washed with wash buffer and stored at 5°C.

Immunoassay

The standard assay procedure has been changed to the following: 400 µL of the gelfiltration eluat containing EPO, 100 µL of the zero standard and 100 µL of the combined antibody reagent were vortex mixed in 5 mL polystyrol test tubes. Then one avidin coated bead was added to each test tube. Zero standard, antibody reagents, avidin coated beads and the wash solution are supplied in the NID kit. The test tubes were then incubated for 15 h at room temperature on a rotator at 180±10 rpm. After the incubation the test tubes were washed according to assay instruction. The chemiluminescence of each test tube was counted for 1 s in a luminometer (BERTHOLD, Germany) according to manufacturer's instructions. The signals are given in RLUs (**R**elative **L**ight **U**nits).

Results

Calibration curve

To obtain the calibration curve for this method, the seven calibration solutions and a blank (wash buffer) were centrifuged for 10 min. at 1500 ***g**, gelfiltrated and assayed in triplicate as described above. The data was evaluated using a four parameter logistic (FPL) fit with the statistic software »S-Plus 3.4« on a UNIX workstation IBM RS/6000 990 with AIX 3.2.5. The functions used are available from the STATLIB server (http://lib.stat.cmu.edu) with the archive name »Calibration«(7). The limit of quantification (LOQ) has been calculated from the FPL fit as the lowest concentration that can be determined with a relative standard deviation of 0.2 or smaller.

Accuracy

To determine the accuracy of this method we spiked rhEPO from our stock solution into urine. We prepared the spiked urines by adding 500 µL of elution buffer containing different concentrations of EPO to 2500 µL urine to yield final concentrations of an additional 0, 2.8, 4.7, 6.6, 14.2, 23.7 and 37.9 IU /L. The seven spiked urines were centrifuged for 10 min. at 1500 ***g**, gelfiltrated and assayed in duplicate as described above. The mean of the signals was used to calculate the concentrations of these urins. The concentration of the urine without added EPO was substracted from the calculated concentrations of the other six samples. Figure 1 shows a plot of the calculated concentrations versus the added concentrations of the spiked urines. The error bars show the 95% confidence interval for the

calculated concentrations as obtained from the confidence band around the calibration curve and the uncertainty of obtaining a new signal from an unknown sample.

Precision

The QC urine was prepared and determined ten times, five times, four times and three times on a total of 4 different occasions.

Date	Mean	Stdev.	RSD	N
15.11.96	9.7	0.4	4.0	4
13.12.96	11.9	0.3	2.8	3
09.07.97	9.0	0.4	5.0	10
16.07.97	10.8	1.1	10.0	5
Total:	10.3	1.3	12.4	4

The intra-assay variation ranges between 2.8 and 10 % relative standard deviation (RSD). The inter-assay variation totals to 12.4 % RSD for 4 different days.

Specificity

NID has tested the antibodies for cross reactivity. No cross reactivities with other serum proteins or with triglycerides were found (see assay manual).

Spontaneous urinary EPO concentrations

The spontaneous urinary EPO concentrations of 34 different, freshly collected urine samples of healthy athletes and nonathletes were determined following the presented method. 16 of them contained EPO concentrations below the minimum detectable concentration and 12 concentrations were above the limit of quantification (LOQ). The concentration range of these urines was 1.6 to 6.1 IU/ L with a median of 2.6 IU/ L, a first quartil of 2.2 IU/ L and a third quartil of 5.0 IU/ L. 6 urines contained detectable EPO concentrations which could not be determined with sufficient precision.

Discussion

The presented method is able to determine urinary EPO concentrations in the range 1.2 to 37.9 IU/L. There is no evidence for a systematic error. The calculated concentrations were within a range of 71-110% of the added concentrations. This suggests that this method is sufficiently accurate within the tested range. Precision (intra-assay variation) with a range of 2.8-10 % and reproducibility (inter-assay variation) with a value of 12.4 % are also acceptable for a immunoassay of biological samples(8).

The distribution of the spontaneous EPO concentrations shows a marked skewness as is expected for biological parameters. Therefore the distribution is characterized by the median and the first and third quartil. That only 12 of 34 tested urine samples contained EPO concentrations above the limit of quantification (LOQ), suggest that more sensitive methods

should be developed. The sensitivity could be increased by concentrating the urine using gel chromatography instead of diluting it by gelfiltration. But gel chromatography would essentially prolong the preparation time. Increasing the volume of applied urine to the gel filtration columns would lead to incomplete clean up. Adding one or more of the desalting columns would solve that problem, but would again prolong the time needed for washing and equilibrating the gel filtration system. On the other hand, after application of EPO a raise in urinary concentration is expected (5,6) and the rational behind this method is to provide a means of filtering out urine samples with elevated EPO levels for further investigation so only the upper limit of a reference range is of interest. Because of that, and the restrictions in sample volume and analysis time that apply to doping analysis, this method has to be looked at as a best suited compromise.

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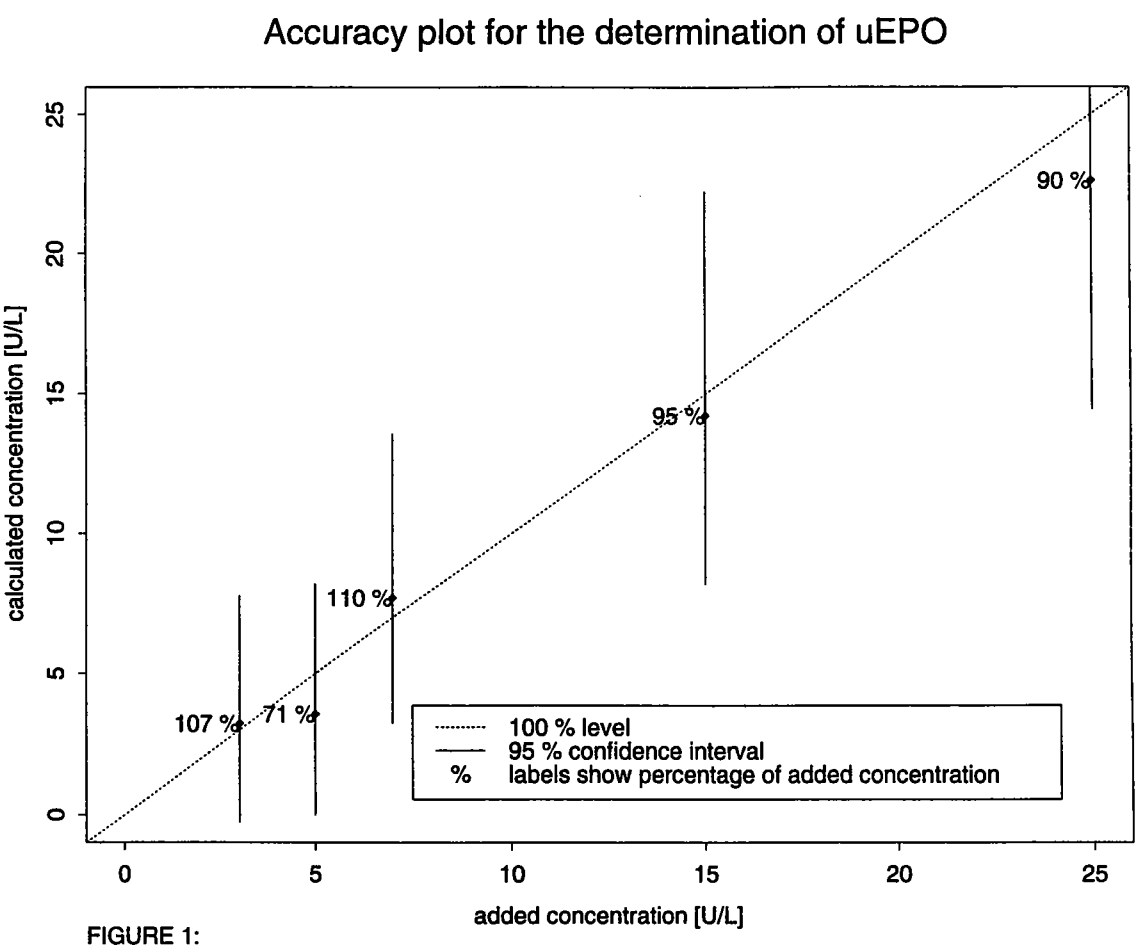
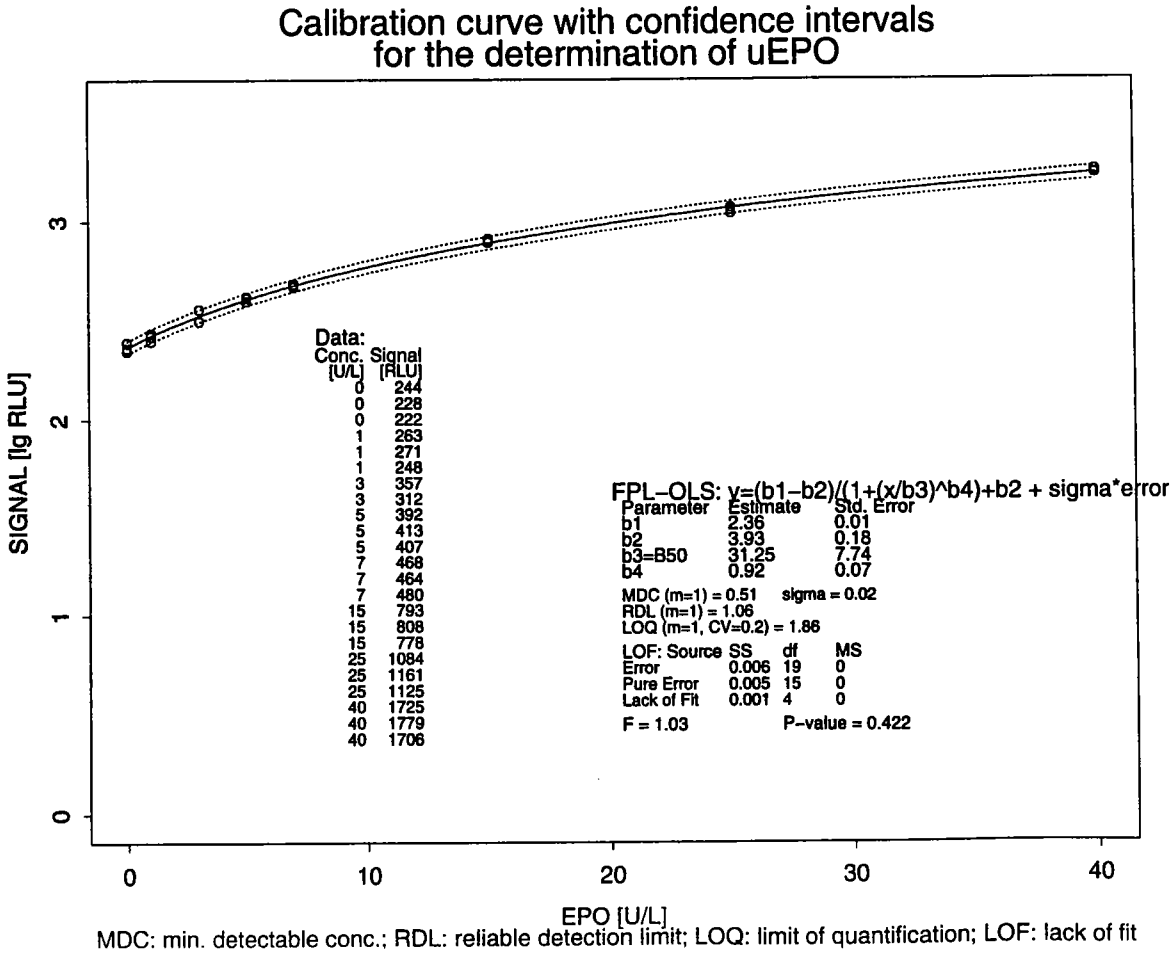


FIGURE 1:



MDC: min. detectable conc.; RDL: reliable detection limit; LOQ: limit of quantification; LOF: lack of fit

Observation on Hematology and Biochemistry of Six Chinese following rhEPO Administration

S.M Yang, X.B Wang , Z.L Fang , Sh Wang , Y. Qin , M.Q YI, H.Gao, B.H Xu , L.L Zhou, F.X Zeng, J.J Guo, Zh. Y Yang ,J. Deng, M.H Xie, Z.Y Yang, Ch.J Zhang

Doping Control Centre of China, Beijing

ABSTRACT

Six male volunteers (age 19 to 23) were subscutaneously adiminstered by rhEPO with a dosage of 30 IU/kg bwt, three times a week for 4 weeks. The observation was made on 6 hematological (RBC, HGB, HCT, MCV, MCH and MCHC) and 2 biochemical indices (EPO, TfR) following the administration. Compared with 4 individuals in control group(age 19 to 23) RBC, HGB and HCT were elevated by the injection (4.65±0.33 *vs* 5.07±0.38 t/l, 134.50±8.2 *vs* 150.10±10.1 g/l, 41.76±1.93 % *vs* 46.42±2.83 %; P<0.05). The serum concentrations of TfR and EPO were found significantly increased following the multiple injections of rhEPO (3.42±0.60ug/ml *vs* 4.56±0.88 ug/ml, 9.96±8.30 mIU/ml *vs* 19.94±10.50 mIU/ml ; P<0.05).

key words

recombinant erythropoietin ; ELISA; Chinese; hematology; transferrin soluble receptor

INTRODUCTION

Erythropoietin (EPO) is the principle hormone regulating the mammalian erythrocyte diffrentiation . It is produced by the cells adjacent to the proximal renal tubules in response to signals from the renal oxygen-sensing device. Since DNA-recombinant products (rhEPO) is available on the market it has been used as replacement agent in the patients with impaired production of EPO. It was also demonstrated that EPO increases the erythrocyte production and dramatically improve the sense of well-being and quality of life in the patients with chronic renal disease [1]

In the study involving healthy individuals it has been reported that rhEPO administration will increase the haemoglobin concentration with a related increase in VO_{2max} and improve the performance during a standard maximal treadmill run [2,3] . Because of the obviousness as a threat to the fair competition in sports and the health of athlets, the Medical Subcommission of International Olympic Committee has put rhEPO into the list of banned dugs in 1990 in spite of no effective method used in doping test. Since then, many work has been proceeded for the methodological development. In these studies, hematological observation and immunoassay in blood did take an important part. Hematological indices (such as HCT, RBC, HGB) and the blood levels of EPO and TfR exhibit a significant elevation after the administration of rhEPO to normal individuals. These elevation would be used in the screening for the EPO-suspect case.

Unfortunately, the dope analyst have to be faced with a challenge, that is, since the hypoxia can dramatically stimulate secretion of EPO altitude training would play a role in the elevation of blood EPO and in the consequent changes of hematology and biochemistry[4] .

To discriminate the exogenous injection from the endogenous EPO an important proceeding has been successfully made with electrophoresis isolation based on the different median charge possessed by the endogenous EPO and rhEPO[5]. In this work an index in terms of mAMU (albumin mobility units) was presented, which is the electrophoretic mobility of the activity in relation to that of human serum albumin. If the mAMU gotten in serum analysis by electrophoresis is smaller than 670 it would be an indication of rhEPO's existance . More conveniently, this method could be used in urine analysis although it should be further evaluated.

As an initial study to demonstrate the feasiblility and validity of immunoassay in EPO test we conducted this observation. 10 volunteers participated in the experiment, 6 hematological and 2 biochemical indices were observed.

METHODS

Subjecs

10 male volunteers (aged 19 to 23yr, mean 21yr) were random divided into two groups: control group N=4 and rhEPO-treated group N=6. For clinic diagnosis they are healthy and in a moderate training and labour work. The volunteers gave their written consent to participate. The study was approved and sponsored by Anti-Doping Commission of China.

rhEPO treatment and sampling

rhEPO-β, (Recomon 2000, Boehringer Mannheim GmBH, Germany) was subscutaneously administered to the 6 volunteers in rhEPO-treated group, 30 IU/kg bwt, three times a week for 4 weeks. Ferric fumarate (200mgx3/day) and folic acid (5mgx3/day) were orally administered to both the control group and the rhEPO-treated group.

10ml of blood was taken at 8:00 to 10:00 am, added with heparin the blood was centrifuged, the serum and plasma was kept at -70°C.

Sampling was begun from day 1 before injection and continued to day 3, day 9 and day 11 after the first injection; from day 15, every two days till day 29 after the first injection. The last two sampling was scheduled on day 34 and day 40 after the first injection. Totally 140 specimens were gotten.

Morning urine was collected in a volume of 100ml for the urinary assay of fibrin and fibrinogen degradation products (the result was not included in this paper).

Hematological observation

6 indices including haematocrit (HCT), red blood cell count (RBC) and concentration of haemoglobin(HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were determined using Automatic Hemology Analyzer (Cell-Dyn 1400, Abbott Co. U.S.A). Statistics was made to get average, standard deviation and, Student's T-test (One-tailed) adopted for checking the difference.

Biochemical measurement

Serum EPO and serum TfR were measured using ELISA (enzyme linked immunosorbent assasy) (R&D Systems, Inc. Minnespolis, U.S.A.) for the all 140 specimens. According to the manufactures' specification, the raw data was gotten on a Microplate Autoreader (EL-311, Bio-Tek Instruments , Winooski, VT, U.S.A).

RESULT

Hematological indices

As shown in Tab 1, out of the 6 indices, RBC, HGB and HCT of the rhEPO-treated group were increased by rhEPO injection compared with the control group ($P<0.05$), although the averages of the levels of both the control group and the rhEPO-treated group are still within the normal range (RBC: 4.00-5.50 t/l, HGB: 120-160 g/l, HCT: 40-51%, refered from a clinic statistics in An Zhen Hospital , Anding Road 11, Beijing). The levels of MCV, MCH and MCHC were not shown with a significant difference by this study.

Tab 1 Summary of Hematological Measurements

index	unit	control group N=4	rhEPO-treated group N=6
RBC	10^{12}/l	4.65±0.33	5.07±0.38*
HGB	g/l	134.50±8.2	150.10±10.1*
HCT	%	41.76±1.93	46.42±2.83*
MCV	fl	90.15±4.22	91.86±4.45**
MCH	pg	29.14±1.42	29.90±1.46**
MCHC	g/l	323.65±6.64	325.45±5.08**

*P<0.05 **n.s

Biochemical measurements

As shown in Tab 2, the assays indicates that the serum level of EPO and TfR could be appearently elevated by rhEPO injection. The level of the control group is 9.96±8.30 mIU/ml for EPO and 3.42±0.60 ug/ml for TfR. The former can hit the normal range

provided by the manufature's specification (EPO: 3.3-16.6 mIU/ml, from 123 normal individual)[6] , the latter is slightly higher than the upper limit of normal range (0.85-3.05 ug/ml, from 1000 healthy individuals) [7] . However, the levels of EPO and TfR in the rhEPO-treated group give out a significant difference compared with the respective control group (P<0.05).

Tab 2 Summary of Biochemical Assays

index	unit	sample	control N=4	rhEPO-treated N=6
EPO	mIU/ml	serum	9.96±8.30	19.94±10.51*
TfR	ug/ml	serum	3.42±0.60	4.56±0.88*

* P<0.05

DISCUSSION

1. Within the rhEPO-treated group a longitudinal change of the individuals could be seen in the plot of the concentration of EPO *vs* time in day (Fig.1,Fig.2) with a big promotion on the third day after the first injection but, without an appearent drawing back after the injection stopped (sampling points of 4 days and 10 days after the *last* injection). This is baffling since the detectable duration of EPO reported in blood is only 24 h [8,9] . The reason might be the inaccuracy of the assay or some other things left unknown.

The plot of TfR concentration *vs* time exhibits a smooth increase as the volunteers multiply injected by rhEPO. A big promotion appeared on the 9th day after the first injection and an obvious drawing back could be seen after the injection stopped (Fig.3, Fig.4). As reported by many researches the promotion of TfR in blood after rhEPO injection will appear with a hysteresis relatively to the secretion of reticulocyte and its getting into the circulation [10] and therefore, a prolonged drawing back should be seen on the plotting. While in our study we failed to demonstrate the hysteresis after the injection stopped, for which we considered it appears after the EPO's drawing back. Obviouslly, it is difficult to show the hysteresis with a large interval of sampling and in such small population and, a study special for the longitudinal investigation should be performed in a large-enough polulation before TfR could be actually used.

2. The measurement of the hematological changes provides a supplementary index for the rhEPO's possible existance. Together with the analysis of EPO and TfR in serum and/or in plasma a screening method could be formed for EPO dope, which might be economic and acceptable. Although some hematological indices (MCV, MCH and MCHC) did not exhibit a promoted level in our experiment (but did in some other work) HCT, RBC and HGB might be enough as a supplement in the screening, which are reported by many experts as a confirmable utility in their study.

A problem probably encountered in the exploitation of these indices is that in our study, the subjects in rhEPO-treated group are not demonstrated by any significantly higher value of RBC, HGB and HCT than the normal range of the male population (RBC :4.00-5.50 t/l, HGB:120.0-160.0 g/l, HCT: 40-51 %, provided by the statistics in An Zhen Hospital, Beijing). Obviously, this is due to that the dosage used in our experiment is only a clinic recommendation for proventing the volunteers from disaster. If the abuser of rhEPO adopted the recommended dosage and satisfied with their promoted performance the hematological determination would be useless for doping test.

3. As a practical-used procedure it is necessary to distinguish the promoted EPO level by exogenous injection from "the exogenous induction" implying the altitude training. At this moment no evidence has been presented that by biochemical and hematological analysis, altitude training and exogenous rhEPO injection could be told by their difference. Fortunately, the different median charge possessed by endogenous EPO and the recombinant has highlighted the proceeding of EPO test. Either a capillary electrophoresis or its coupling with mass spectrometer would be a outlet for the test, provided that the detection of glycoprotein using electrospray or time of flight mass spectrometer is successful.

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