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Individual Reference Ranges for serum Erythropoietin (sEPO) – A Possible Approach to Detect Misuse

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1 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 [1, 2]. This makes recombinant human erythropoietin (rhEPO) a potential doping agent for endurance athletes [3]. 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.

Spontaneous serum erythropoietin (sEPO) levels have been determined in multiple blood samples withdrawn from 179 athletes preparing for a marathon run at various dates over a 8 month period in 1997 and in single blood samples collected from 72 elite athletes from track and field events and cross-country skiing. Daytime variation and the influence of a single subcutaneous (s.c.) application of 100 I.U. rhEPO per kg BW on sEPO levels have been studied with 10 untrained healthy individuals in Jan. 1998.

2 Materials and Methods

2.1 Erythropoietin application

Appropriate volumes of RECORMON 10000 were injected subcutaneously into the abdominal wall of 10 (6 males/ 4 females) healthy subjects. RECORMON 10000 was provided courtesy of BOEHRINGER MANNHEIM, Germany. Two days and one day before EPO application altogether 11 or 12 blood samples were withdrawn from each subject every two hours between 7am and 6pm. 100 I.U. rhEPO per kg bodyweight were given to each

subject around 8am of application day. Just before and 6, 12, 24, 48 and 72 h after application blood was collected.

2.2 Reagents

Serum erythropoietin levels were determined with a chemiluminescence erythropoietin immunoassay from NICHOLS INSTITUTE DIAGNOSTIC, Germany. The assays were carried out according to the manual except that each standard solution was measured in triplicate and all unknown samples were measured once. Earlier results showed that measuring the unknows in duplicate didn't improve precision substantially. The reproducibility for a QC sample with a mean concentration of 29.2 I.U./L was 12% RSD determined in 13 runs of 6 different reagent lots. The computation methods used (see 2.4 below) calculated 95% confidence intervals for the results of the unknown samples based on the variation of the standard solutions.

2.3 Blood samples

Blood samples were withdrawn from a forearm vein and collected in CORVAC Integrated Serum Separator Tubes, SHERWOOD MEDICAL. The blood was allowed to clot and after centrifugation serum was withdrawn and stored in two aliquotes at -70°C.

2.4 Calculations

The statistik software package "S-PLUS 3.4" from "MATHSOFT, INC." running on an IBM RS6000-990 under AIX 4 was used for calculations. A four-parametric logistic model was fitted to the calibration data using the S–Plus function "`Calibration`" from O'Connell, M.A. et al. [4]. The concentrations of the unknown samples were calculated using the S–Plus function "`Multi.Calib`" from Robinson–Cox, J.F. [5]. These functions are available from the StatLib–Server (`http://lib.stat.cmu.edu`).

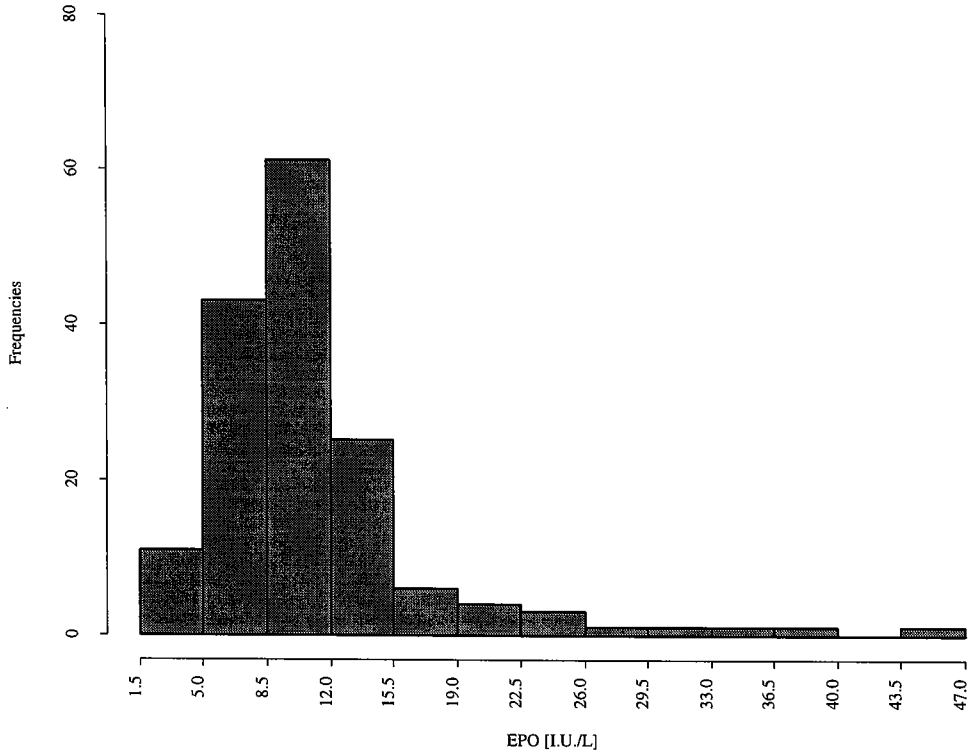

Figure 1: Distribution of sEPO concentrations of healthy athletes preparing for their first marathon run

3 Results

3.1 Marathon data

Of the 179 athletes preparing for their first marathon 158 showed at least one concentration above the limit of quantitation (LOQ). Figure 1 shows the distribution of the sEPO concentrations of those athletes with a median of 9.7 I.U./L and an inter-quartile range of 4.9 I.U./L (min 2.7, max 46.3, 25% 7.3, 75% 12.2). The distribution is skewed as can be expected from a biological parameter like EPO.

24 out of the 179 athletes had three blood samples taken in the months March, May and September. The concentrations found in these samples are plotted in figure 2. The ratios of individual maximum to minimum levels were from 1.1 to 4.9 with a median of 2.0 and an inter-quartile range of 1.0 .

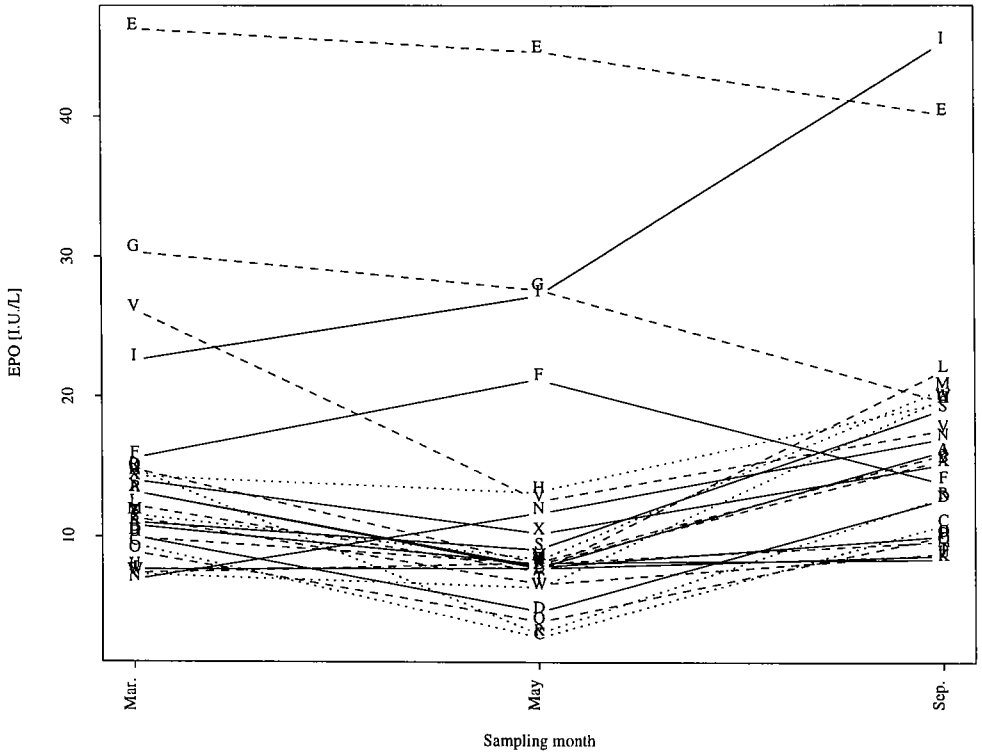

Figure 2: Individual variation of sEPO concentrations of 24 athletes (A-X) preparing for their first marathon over a period of 6 months

3.2 Elite athletes data

Figure 3 shows the distribution of sEPO concentrations in samples withdrawn from 72 elite athletes, 17 from the German Athletic Federation and 55 cross-country skiers from the German Skiing Federation. This distribution is also skewed with a median of 10.4 I.U./L and an inter-quartile range of 4.5 I.U./L (min 5.2, max 29.4, 25% 8.7, 75% 13.2). Of the 72 samples 64 showed concentrations above the LOQ.

3.3 EPO application data

The daytime variation between 7am and 6pm for two consecutive days and the time course of rhEPO elimination after s.c. injection into the abdominal wall of 10 subjects is shown in figure 5. Without EPO application the ratios of individual maximum to minimum sEPO levels range from 1.3 to 2.3 with a median of 1.8 and an inter-quartile range of 0.73 . There is no evidence (figure 4) that a circadian rhythm of endogenous sEPO levels exists.

48h after application the individual sEPO levels were from 2.6× to 5.9×higher than the

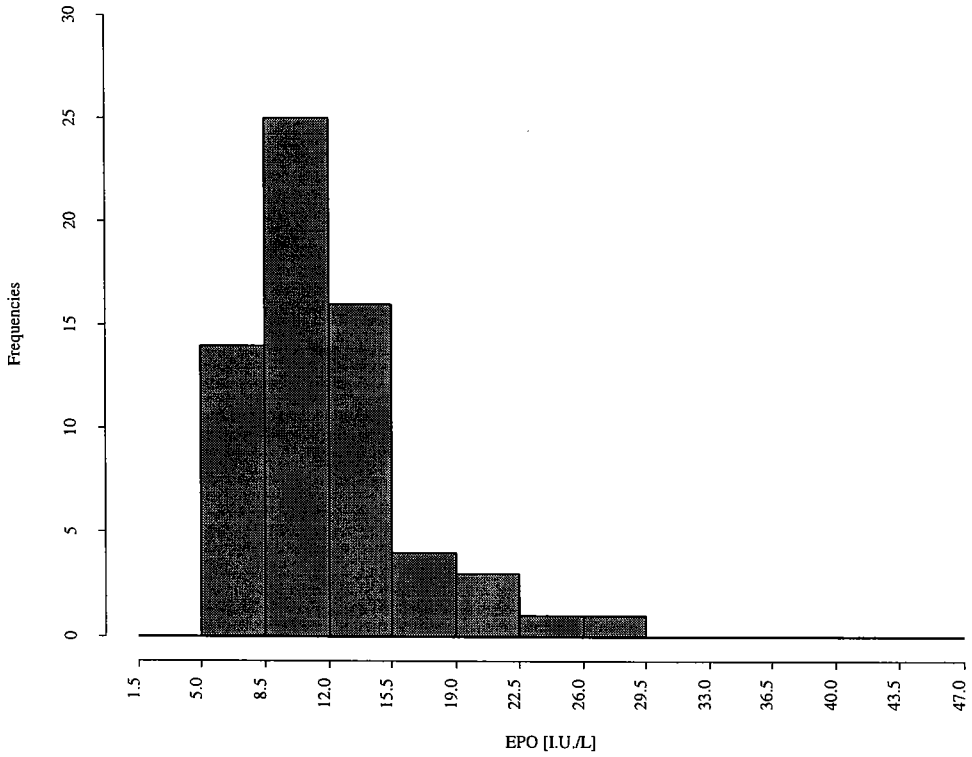

Figure 3: Distribution of sEPO concentrations of elite athletes from athletics and cross-country skiing

respective individual minimum levels with a median of 4.5 and an inter-quartile range of 1.2 . For all subjects the sEPO levels 48h after application were significantly elevated since the lower limit of the 95% confidence interval for that value was greater than the upper limit of the 95% confidence interval for the respective maximum value without application. The sEPO levels 72h after application were not significantly elevated anymore.

4 Discussion

As yet there is no established method to detect misuse of erythropoietin. Usually the IOC and the international sport federations ask for direct evidence of misuse of a forbidden substance by gaschromatographic separation and massspectometric detection (GC/MS). That was easily accomplished since almost all of those substances were of exogenous origin. Exceptions from that are the peptide-, protein- and glycoproteinhormones and testosterone. Until recently a misuse of testosterone was only detectable through a reference range of its ratio to Epitestosterone since there was no analytical measure to differenti-

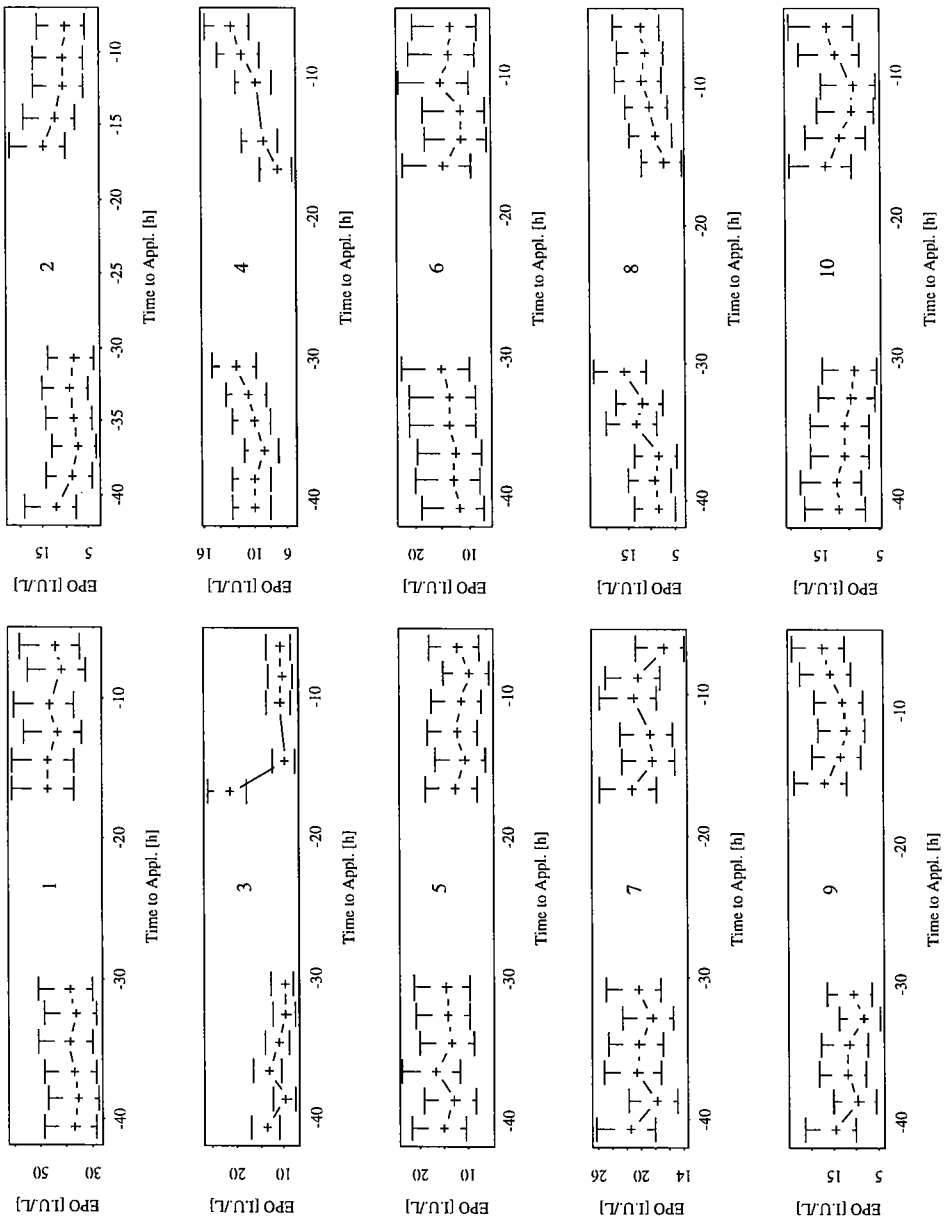

Figure 4: Daytime variation of sEPO levels with 95% confidence intervals

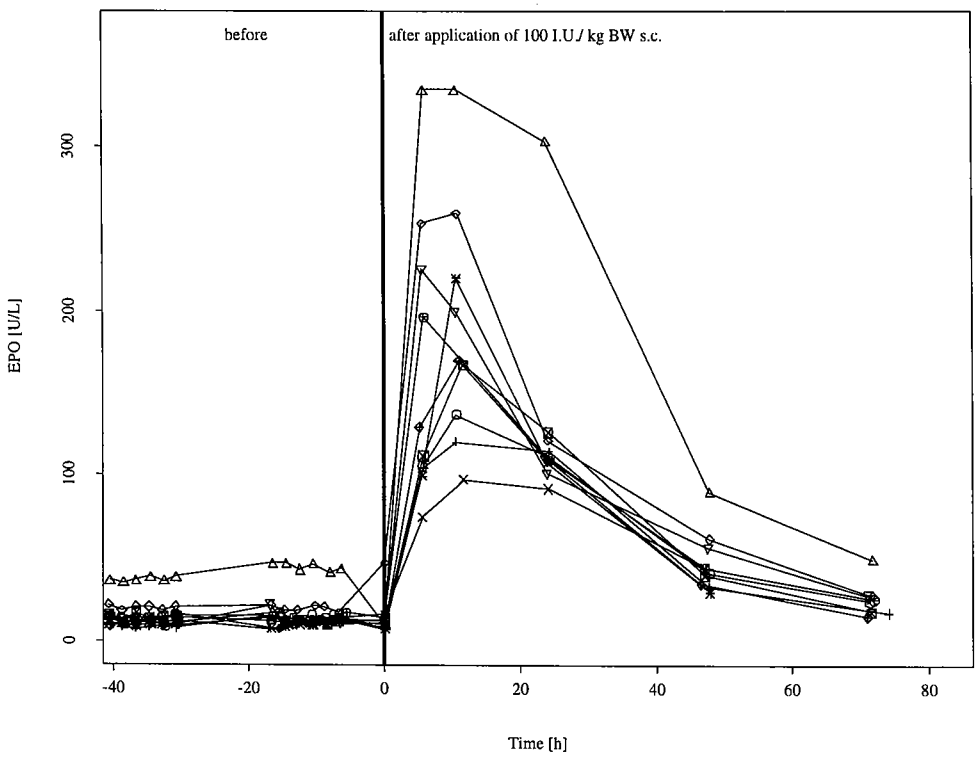

Figure 5: sEPO concentrations of 10 healthy subjects before and after application of rhEPO

ate between exogenous and endogenous testosterone. Same goes for recombinant human erythropietin and endogenous human erythropoietin which can not be differentiated with appropriate confidence by analytical means.

An approach as was used with testosterone employing population- and individual-based reference ranges could be used to detect a misuse. The results show that individual variation of endogenous sEPO levels is rather small compared to the changes that occur after a single s.c. application of 100 I.U. rhEPO per kg BW.

The boxplots in figure 6 show that the populations of marathon runners (Section 3.1) and elite athletes (Section 3.2) are very well comparable, the medians and the inter-quartile ranges are closely related. Just there are more extreme values in the high range of the population of the marathon runners. The one value above 43.5 I.U./L in figure 1 would clearly fall outside the upper limit of a reference range based on the populations shown here (Sec. 3.1 + Sec. 3.2).

That value belongs to a male subject who is denoted with open triangles in figure 5 and with "E" in figure 2. It can be seen that for all sampling dates the sEPO levels of

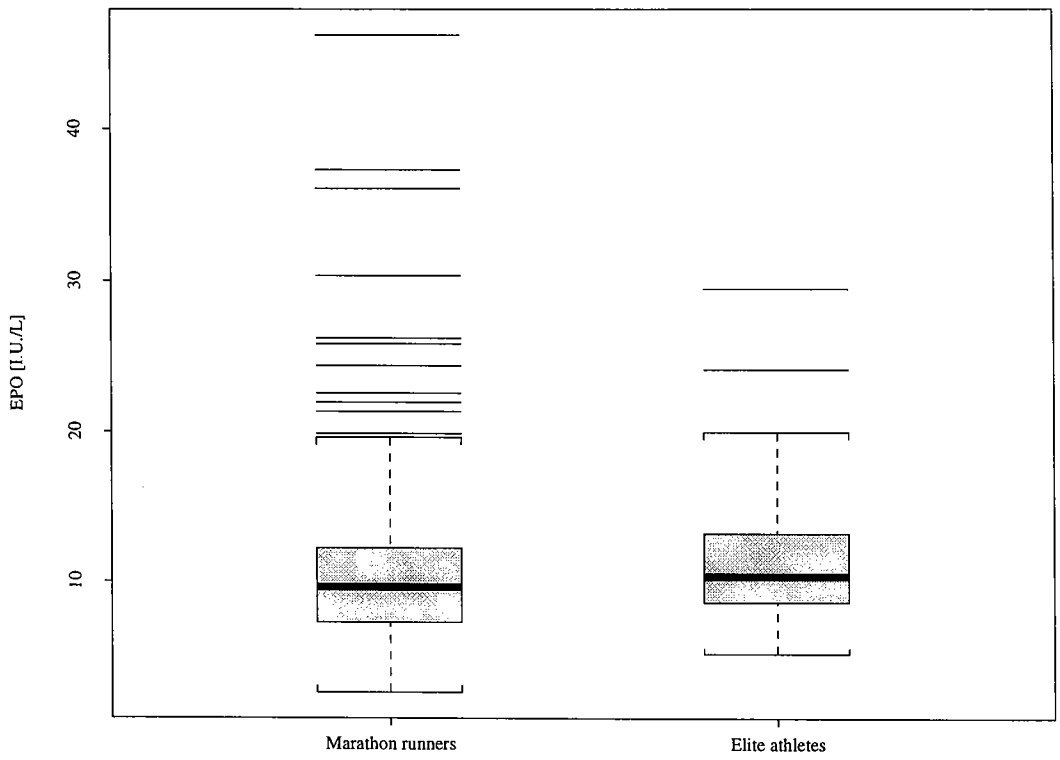

Figure 6: Boxplots of sEPO concentrations of 158 marathon runners and 64 elite athletes

this subject were around 40 I.U./L and well above the majority of the endogenous sEPO levels. The high values are caused by a naturally elevated sEPO level. After application this subject also shows the highest response to the applied dose (figure 5; the drop at 0 h is most likely a sample mixup).

If that value belonged to an athlete that athlete would have to be followed up. If the high value was caused by a naturally elevated sEPO level the following samples would also show high values. If the following samples showed much lower values or a wide variation it could be an indication of EPO misuse.

Elevated values due to EPO application decrease rapidly (see fig. 5 and [6, 7, 8]). For our 10 subjects 72h after a single application there is no significant difference to baseline values anymore (Sec. 3.3). An earlier study with two subjects has shown that multiple s.c. applications every second day didn't lead to accumulation of sEPO (data not shown). There is no evidence of a circadian rhythm of sEPO levels as was described by Wide et al. [9] for 27 in-patients, by Grünenfelder [10] for 6 healthy subjects and by Klausen et al. [11] for 9 healthy subjects at sea level and high altitude. Roberts et al. [12] measured sEPO levels in 26 healthy men and also couldn't find a significant circadian variation.

As with testosterone misuse an athlete in question should be invited to participate in an endocrinological study over two to three days under supervision. Each day several blood samples would be collected, evaluated for sEPO and other parameters and the results would be used to set up an individual refernce range. From that further evidence could be obtained as to wether there was a misuse of EPO.

Of course, to make this possible first of all blood sampling would have to be introduced to anti-doping control, especially to out-of-competition control. Two international sport federations, the UCI and the FIS, have already introduced competition blood sampling to measure haematocrit and haemoglobin respectivly.

Furthermore more data must be collected regarding factors that could influence endogenous sEPO levels like hypoxia, physical strain, diet and ethnic background to name a few. The influence of EPO applications on sEPO levels must be studied with a larger population.

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