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Measurement of urinary erythropoietin levels in athletes

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Introduction

Detection of the misuse of erythropoietin (EPO) by athletes remains a major challenge for the scientific community involved in sports drug testing. EPO is a naturally-occurring hormone produced by the kidney in response to lowered oxygen levels in the blood. It regulates the concentration of circulating red blood cells and has been used by some endurance athletes to increase their aerobic capacity and, thus, enhance performance.

Two approaches have been used to develop tests for detecting EPO abuse in athletes. The first involves the measurement of indirect markers, such as the concentration of soluble transferrin receptor (1,2), which change as a result of EPO administration. The second approach aims to develop a method which would distinguish recombinant EPO from the endogenous, naturally-occurring form. Whilst the amino acid backbone in the endogenous and recombinant forms of EPO are identical, there are subtle differences in the glycosylation pattern of the protein when produced by recombinant technology (3,4). These differences in the glycosylation pattern form the basis for studies aimed at distinguishing endogenous EPO from recombinant EPO (5).

Methods for directly measuring recombinant EPO will require a significant amount of the protein. Whilst serum has a higher concentration of EPO than urine, much larger volumes of urine can be obtained and, in addition, only two sports, cycling and skiing, currently collect blood samples from athletes. For these reasons, an assay for measuring EPO in urine was developed. As there is little information available on urinary EPO levels in athletes, a study was also undertaken on a large number of athletes in order to establish reference intervals. The data was used to determine if variables such as sex of athlete, type of sport, urinary pH and specific gravity of urine influence the levels recorded.

Materials and Methods

Urine samples

Samples were collected between 10th October 1998 and 26th November 1998 and comprised 11 SOCOG (Sydney Organising Committee for the Olympic Games), 22 New Zealand, 1 Papua New Guinea and 172 ASDA (Australian Sports Drug Agency) samples from a variety of sports (Table I). The total number of sports examined was 28. The pH of the urine was measured 'in the field' by the Sports Drug Agency just after collection of the urine specimen using pH indicator paper and the specific gravity (SG) of the urine was measured in the laboratory on the day of receipt using a Paar Model DMA48 Digital Density Meter (Paar Scientific Ltd, London, UK).

Table I. Breakdown of samples into different sports

Sport	<i>Male</i>	<i>Female</i>
Athletics	10	6
Bodybuilding/ Powerlifting	10	2
Boxing	13	
Cycling	67	7
Rugby/Football	13	
Swimming	9	14
Triathlon/ Ironperson	5	5
Other	24	23
Total	149	57

Preparation of urine samples

An aliquot of each urine sample (5 ml) was dispensed on the day of receipt and frozen at -20°C till required. On the day of assay, the aliquot was thawed, mixed thoroughly and then centrifuged at 4000 rpm (~3000 g) for 10 min at 10°C. Supernatant (4.5 ml) was dispensed into a pre-weighed ultrafiltration unit (Millipore, Bedford, USA) with a nominal molecular weight cut-off of 10,000. The ultrafiltration unit was re-weighed and then the urine samples concentrated to less than 200 µl by centrifuging at 4000 rpm for 30 minutes at 10°C. Urine samples that had not concentrated to 200 µl after 30 minutes were re-centrifuged till the

volume was reduced to less than 200 μ l. The concentrated urine was washed twice with 500 μ l PBS (2mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 8 mM Na_2HPO_4 , 150 mM NaCl), pH 7.4, by centrifuging in the ultrafiltration unit to a final volume of less than 200 μ l each time. The retentate was made to a final volume of 300 μ l with PBS, pH 7.4, and the ultrafiltration unit reweighed. The concentrated urine samples were then stored at 4°C overnight.

Analysis of urinary EPO levels

EPO levels in the concentrated urine samples were measured using a 'Human EPO Quantikine IVD ELISA kit' (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Multicalc software (Wallac Oy, Turku, Finland) was used to generate a standard curve and analyse data. The concentration factor for the urine was estimated using the weights recorded for the ultrafiltration unit alone, the unit containing urine and the unit containing concentrated urine. This factor was then used to calculate the concentration of EPO in the original urine. Urinary EPO controls were prepared by spiking urine with four different concentrations of recombinant EPO (Eprex 10 000, Janssen-Cilag, Australia) to give final concentrations of 5, 27, 53 and 107 mU/ml. Aliquots of the spiked urines were stored at -80°C till analysis. The control spiked at 5 mU/ml was used to monitor the concentration process. The inter-assay coefficient of variation for this control was 13.6 % (mean 5.4 mU/ml; n=4). The inter-assay coefficients of variation for the remaining three controls which were used to monitor the performance of the ELISA were 5.1, 13.1 and 10.1 %, respectively (n = 8 or 9 assays). The average minimal detectable concentration (MDC) is defined as follows: $\text{MDC} = \text{conc at } [Y(0) + 3\Delta Y]$ where $Y(0)$ is the fitted raw response at 0-conc, and ΔY is the estimated error of $Y(0)$. The MDC of EPO was 2.2 mU/ml (n = 9 assays) which equates to 0.2 mU/ml in the unconcentrated urine.

Statistical analysis

Statistical significance was tested at the $p < 0.01$ level using the non-parametric Mann-Whitney U test (Statistica Release 4.5, StatSoft Inc., Tulsa, OK).

Results and Discussion

The effect of ultrafiltration on recovery of EPO in urine

To determine the recovery of EPO in urine, 8 urine samples (with no detectable concentration of endogenous EPO) were centrifuged and then spiked with recombinant EPO (Eprex 10 000)

at a final concentration of 8.2 mU/ml. A portion of each sample was concentrated using the standard ultrafiltration protocol whilst the remainder was retained for analysis without further treatment. The recovery of spiked EPO in the neat, unconcentrated urine and the ultrafiltered, concentrated urine was 55.7 ± 19.5 and 85.5 ± 11.0 %, respectively, suggesting that there is an inhibitory substance in the urine which is removed during ultrafiltration. A small number of samples were ultrafiltered but not washed and the recovery of EPO in these samples was very similar to that obtained in neat urine (data not shown). These results suggest that the washing step after ultrafiltration is important in order to maximise the recovery of EPO in urine and that assay of neat urine will lead to an underestimate of the actual level of EPO in urine.

Urinary EPO levels in athletes

The urinary EPO levels in both male and female athletes showed a very skewed distribution (Fig. 1a). The maximum levels of urinary EPO measured in males and females were 13.5 and 7.7 mU/ml, respectively, whilst the median levels were 1.28 and 0.25 mU/ml, respectively (Fig. 1b). Urinary EPO levels were significantly higher in males than in females ($p < 0.01$) and this trend was similar amongst different sports (Figs. 2a and b). The urinary EPO levels fell below the MDC in 35% of female samples and 15 % of male samples.

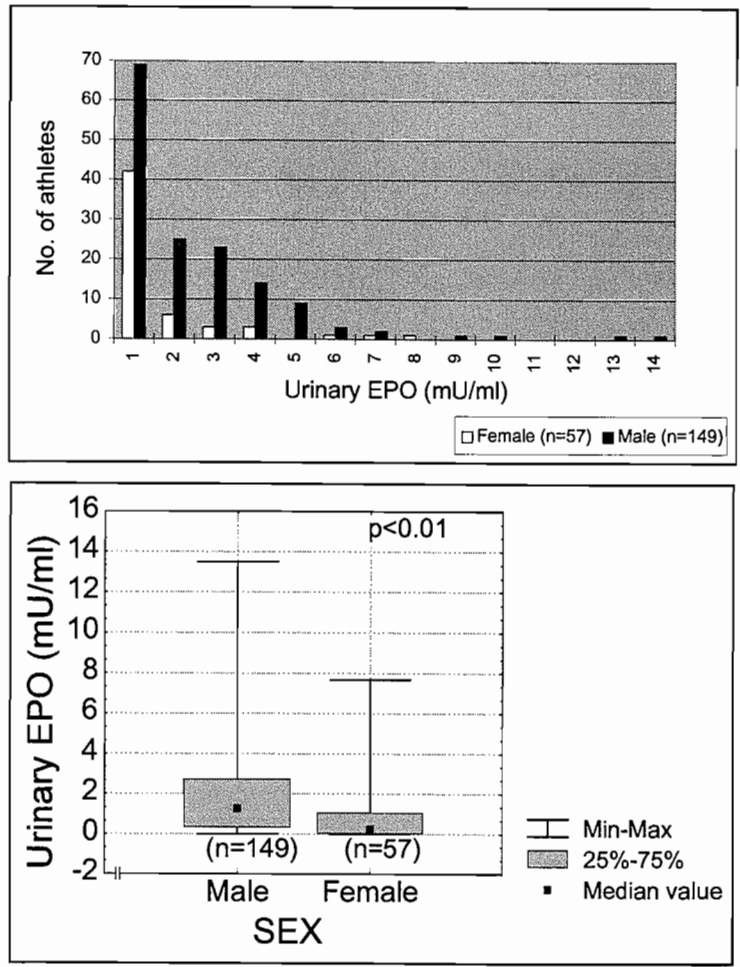


Fig. 1. Frequency distribution (a) and box- and whisker-plot (b) of urinary EPO in male and female athletes.

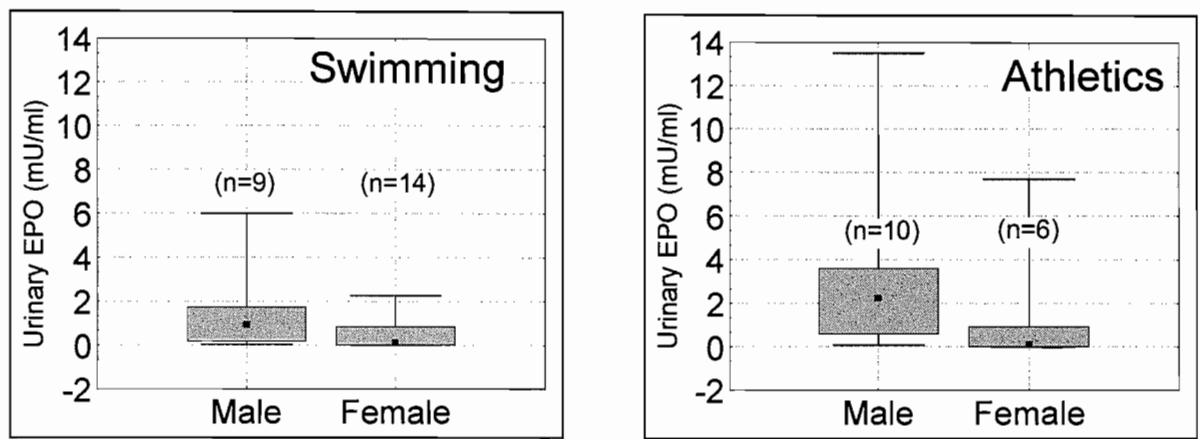


Fig. 2. Box- and whisker-plots of urinary EPO in male and female swimmers (a) and athletes (b). The legend is the same as shown in Fig. 1(b).

For comparison, serum EPO levels were determined in a second group of athletes based at the Australian Institute of Sport (AIS) (Fig. 3). The mean \pm S.D. for serum EPO levels in AIS male ($n = 43$) and female ($n = 18$) athletes were 7.9 ± 3.1 and 5.9 ± 2.8 mU/ml, respectively which is 4-6 times higher than the levels measured in urine. The distribution of the serum EPO values was much less skewed than the distribution for the urine values. The results indicate that EPO levels in both urine and serum tend to be higher in males than in females although a larger population of athletes would need to be examined to confirm this result.

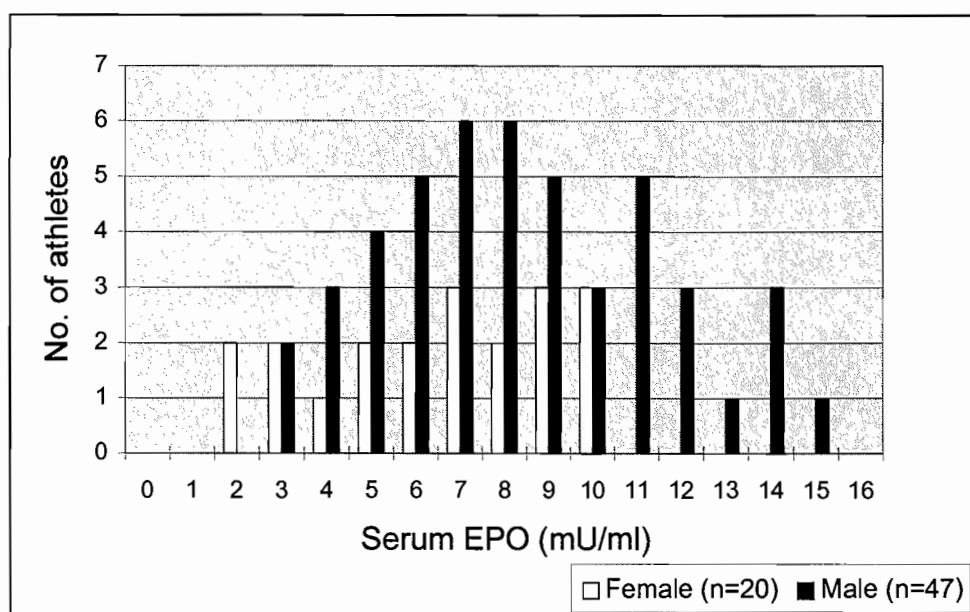
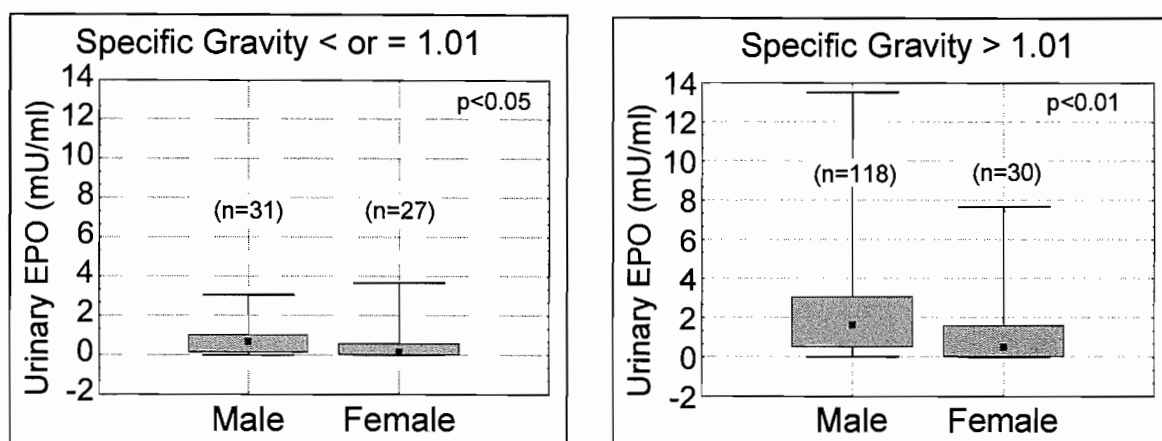


Fig. 3. Frequency distribution of serum EPO levels in athletes

Relationship between specific gravity (SG) and urinary EPO levels

The level of EPO measured in urine was significantly lower in samples with a $SG \leq 1.01$ (Fig. 4a and b). This was clearly apparent in male athletes where the number of samples was higher than for female athletes. In this study, female urines tended to have a lower SG than male urine so it was possible that the difference between values for males and females was simply due to differences in SG. However, a breakdown of the male and female urines by SG showed that SG alone does not account for the difference between urinary EPO levels in males and females (Fig. 4).



Figs. 4(a) and (b). The influence of specific gravity on urinary EPO levels. The legend is the same as shown in Fig. 1(b).

The effect of urinary pH on levels of urinary EPO

The effect of urinary pH on levels of EPO measured in male urine was determined by comparing levels in urine with a pH of 5 with levels in urine of pH > 5. Since the measurement of pH in this study was performed ‘in the field’ using pH indicator paper it was not possible to categorise the urine samples into more than two groups. To account for differences in SG, the urinary EPO levels were corrected for SG using the Levine-Fahy equation as previously described (6). The level of EPO (corrected for SG) was significantly lower in urine samples with a pH 5 than in urine with pH > 5 (Figs. 5a and b). To determine if the lower EPO levels in urine of pH 5 was due to increased degradation of EPO in the urine, 8 urine samples (with no detectable concentration of endogenous EPO) were centrifuged and then spiked with recombinant EPO (Eprex 10 000) at a final concentration of 9.1 mU/ml. Two portions of each samples were concentrated using the standard ultrafiltration protocol and then the concentrated urine was washed with either PBS, pH 7.4, (standard protocol) or with 50 mM sodium acetate buffer, pH 5.8. The recoveries of spiked EPO in the urine after washing with pH 7.4 and pH 5.8 buffers were $96.5 \pm 10.2\%$ and $63.1 \pm 23.9\%$, respectively. The recovery of spiked EPO in the urine was thus dependent on the pH of the wash buffer. Further studies using a more accurate measurement for pH will be important in order to evaluate the effect of pH on urinary EPO levels in more detail.

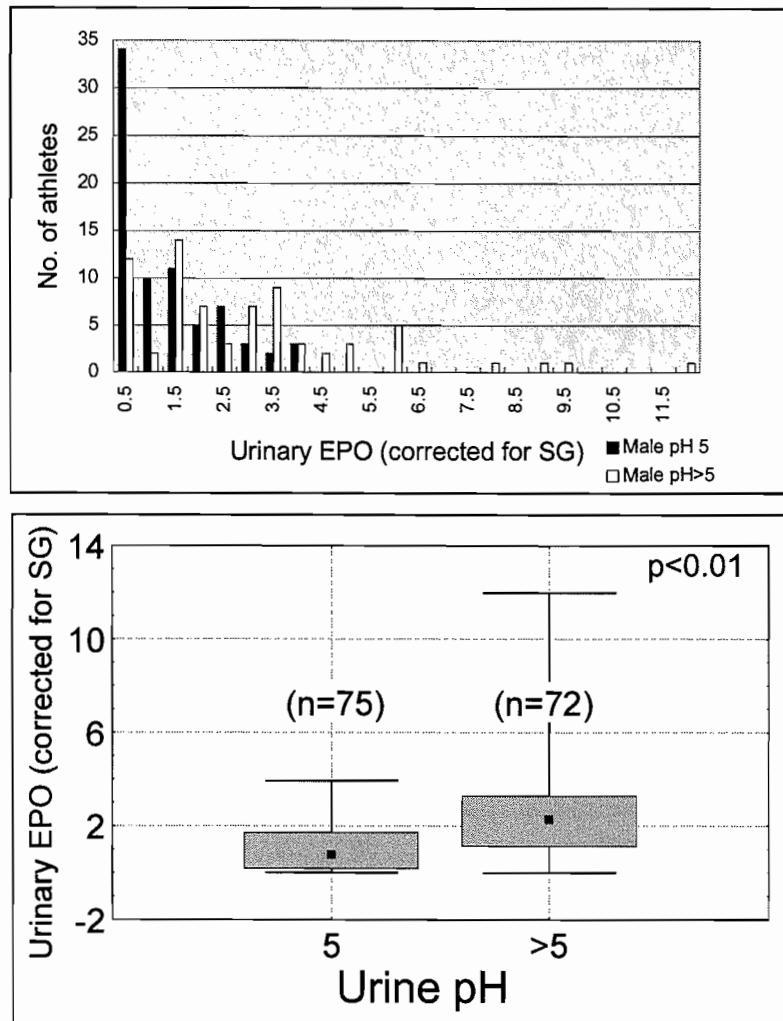


Fig. 5. Comparison of the frequency distribution (a) and box- and whisker-plot (b) for urinary EPO (corrected for SG) in male urine with pH = 5 and pH > 5. The legend for the box- and whisker-plot is the same as shown in Fig. 1(b).

Summary

A method has been developed for measuring EPO levels in urine follow ultrafiltration of the urine specimen. Analysis of 206 urine samples collected from athletes through Sports Drug Agencies has demonstrated that urinary EPO levels are significantly higher in males than in females. Both the specific gravity and the pH of the urine influenced the levels of urinary EPO. The results suggest that EPO is either more susceptible to degradation in urine at pH 5 or it undergoes a change in conformation at this pH giving rise to a reduced affinity for the antibodies used in the ELISA.

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