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Active urine and detection of recombinant erythropoietin

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Abstract

The detection of recombinant human erythropoietin (rHuEPO) in urine is based on the differentiation of its isoelectric profile from that of the corresponding natural hormone.
Correct interpretation of a result is thus dependent on the ability to ensure that no interference has distorted the investigated profile.
The term "active urine" refers to urine samples in which the structure of EPO (natural or recombinant). has been altered by some factor.
In most of these active urine samples, the EPO has been digested and thus immunoblotting of the hormone results in a blank image and a report of "undetectable EPO". In some rare samples, however, a particular activity leads to a shift in the EPO pattern toward the basic values of the pH gradient. In these cases, the isoforms are located in the basic area used to characterize Epoetin alfa and beta. Fortunately, a clear difference in the distribution and location of their isoforms permits the differentiation of shifted natural EPO and rHuEPO profiles. Furthermore, a "stability test" was developed to determine whether shifting activity has interfered with the analysed profile, and the test is systematically performed before reporting an adverse analytical finding for EPO.

Experimental

The isoelectric profiles of EPO in urine were monitored through the isoelectric focusing (IEF) and "double blotting" method previously described (1). Briefly, urine samples (20 mL) were submitted to ultrafiltration using membranes with a molecular weight cut-off of 30,000 Da in order to concentrate the hormone in the retentate. This retentate was assayed for its EPO level by ELISA (human EPO Quantikine IVD from R&D Systems) and submitted to isoelectric

focusing (IEF) (pH gradient of 2 –6). The EPO isoforms were specifically revealed by the double-blotting method using monoclonal anti-human EPO AE7A5 from R&D Systems (2,3). The final result was a chemiluminescent image of these isoforms.
For the stability test, pepstatin (60µM final concentration) and 1%(v/v) Complete (Roche) were added to a small volume of urine (0.5 mL) before and after diafiltration (dialysis of the sample without concentration) with 50 mM acetate buffer, pH 5, using a Microcon MWCO: 30,000 Da (Millipore). Erythropoietin Biological Reference Preparation (BRP) (European Pharmacopoeia Commission) and Darbepoetin alfa (Amgen) were then added to a final concentration 1.5 x those used for the reference preparations used in IEF. After one night of incubation at 37°C, 20 µL of this sample was heated at 80°C for 3 minutes and then submitted to IEF and double-blotting of EPO.

Results

Broadly speaking, "activity" refers to the different processes that may affect the structure of the EPO molecules present in a urine sample. However, only one of them, described here as "shifting activity", requires special attention when interpreting an EPO test result.

I Detection of active urine samples

Three different cases are observed.

In the first case, ELISA indicates that the retentate obtained from ultrafiltration of urine in the preparative step of the test is devoid of EPO. This situation may, of course, correspond to a true low secretion and excretion of this hormone in urine. However, in some of these cases, EPO is in fact digested by the proteases that are present in urine (in particular acidic proteases). This situation results in a blank image when analyzed by IEF.

In the second case, ELISA indicates sufficient concentration of EPO in the retentate to be visualized by IEF. However, a blank image is obtained as in the previous case. The explanation of this discrepancy between ELISA and IEF will be given by the following case.

In the third case, the IEF reveals unusual patterns of EPO composed of isoforms located in the basic part of the pH gradient. However, these patterns are clearly different from those of Epoetin alfa or beta and are thus differentiable from urine samples positive for these substances. Indeed, whereas the most intense bands are numbers 1, 2 or 3 in the cases positive for Epoetin, they are in a more basic position in active urine samples (Fig.1). These basic bands in fact correspond to EPO isoforms shifted by the enzymatic removal of negative electrical charges. In this situation, it is thus impossible to know whether the EPO was initially natural or recombinant and the result of the test must be "unclassifiable result, degraded urine sample".

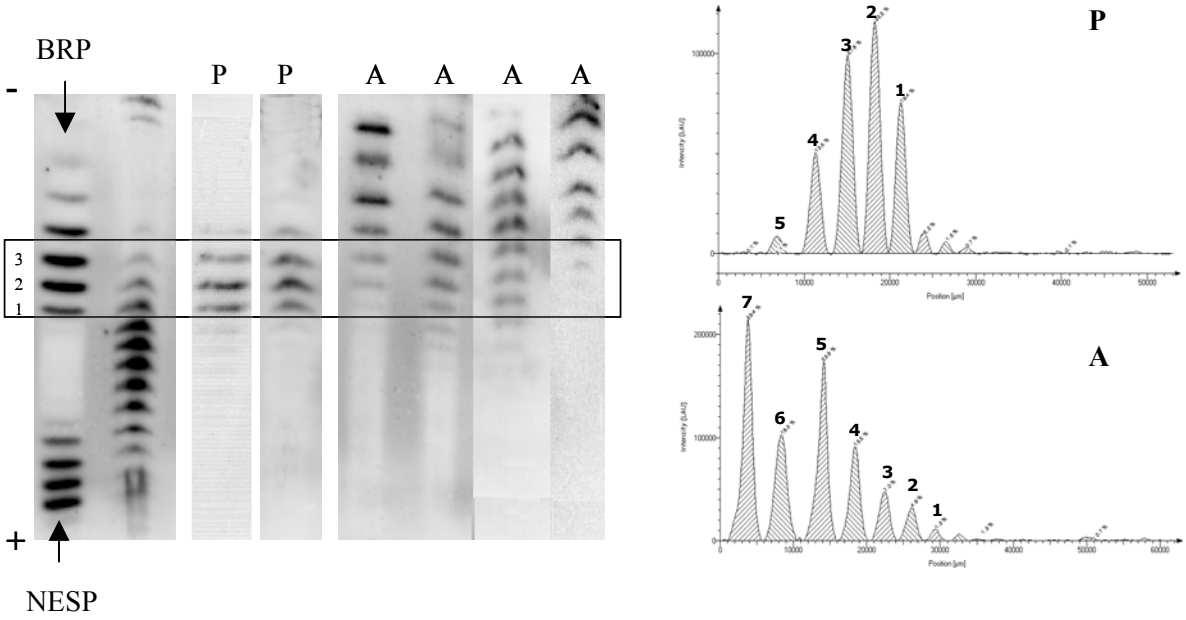

Figure 1. Third case: active urine samples giving rise to shifted EPO patterns. Note the different distribution of the basic bands in case of active urine samples (A) in comparison with cases positive for Epoetin (P).

The second case described above (discrepancy between the ELISA result and the absence of an IEF image) corresponds to high shifting activity causing the EPO isoforms to be located outside the 2-6 pH gradient used for the analysis. When a broader pH gradient (2-8) is used, these shifted isoforms are detected (Fig.2).

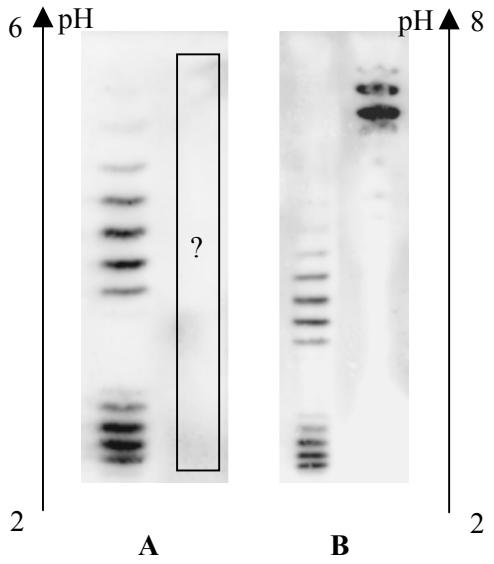

Figure 2. Second case: in spite of a sufficient EPO level in the retentate, no EPO bands are detected after IEF in the usual pH gradient 2-6 (A). These bands appear if the pH gradient is extended toward the basic values (2-8) (B).

Whereas the first two cases give rise to an unambiguous EPO test result of "undetectable EPO", it is very important to differentiate the third case (shifting activity within the pH gradient 2-6) from a true positive case for Epoetin.

In addition to the unusual positions and distributions of the EPO bands, another indicator of activity must be taken in account: since shifting activity is very likely enzymatic, the EPO IEF pattern is unstable and the bands are progressively moved toward the cathode with the duration of sample storage. Thus, any significant change in the pattern between screening and confirmation should signal the possibility of an active urine sample.

The third indicator is a clear demonstration of shifting activity with the stability test (ST) in which the shifting activity of urine is tested toward recombinant EPO. For this, the tested sample is incubated with recombinant EPO (BRP and Darbepoetin alfa) before IEF and double-blotting of this hormone. Since the sample is directly submitted to IEF without any concentration by ultrafiltration, its endogenous EPO is not detected. The result permits the assessment of any shift in the position of the added recombinant EPO (Fig. 3). If any shift is observed, the sample is considered as "unstable" and this rules out any interpretation of its own EPO. The final conclusion of the analysis therefore is "unclassifiable result, degraded sample".

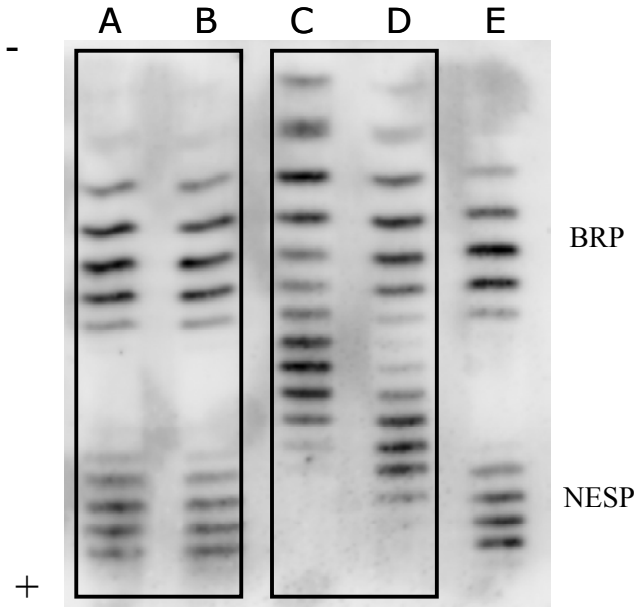

Figure 3. Stability test: by comparison with the band positions of references BRP and NESP not incubated in urine (E), stable urine samples (A, B) that do not modify the patterns of incubated BRP and NESP can be differentiated from unstable urine samples (active urine) that shift these patterns toward the cathode (C, D). Note that in sample D, the shifting activity is more easily evaluated on NESP than on BRP.

Some technical points of the stability test are important: in the acidic conditions of the test, the addition of pepstatin is crucial since it protects both the references and the enzyme responsible for activity from digestion by acidic proteases that may be present in the urine sample. In particular, the dissolution of pepstatin in methanol in the stock solution must be checked before use.

The use of Darbepoetin alfa is convenient since shifting activity is more dramatic on this reference than on BRP.

II Characterization of shifting activity: preliminary experimental results and observations

We assumed that shifting activity was an enzymatic process and thus tried to characterize it.

The molecular weight of this enzyme appeared greater than 30,000 Da since no activity was recovered in the filtrate from ultra-filtrated active urine.

Though the activity was still detectable in neutral and alkaline pH conditions, the optimal pH was acidic (pH tested: 5, 7.5, 8, 9.2). The enzyme appeared to be very thermostable: 30 min of heating at 95°C were necessary to inactivate it and six freeze-thawing cycles of urine did not affect it. However, activity was completely neutralized by adding 0.5 M NaCl to urine; it

is thus important to diafiltrate urine with 50mM acetate buffer for the stability test and not to add a more concentrated buffer that might inhibit activity due to higher ionic strength. Neither Complete, a mixture of anti-thiol, anti-metallo and anti-serine proteases, nor pepstatin, an anti-acidic proteases, inhibited an active urine sample. This indicated that the enzyme responsible for shifting activity was not a proteolytic enzyme. On the contrary, 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid (DANA) inhibited active urine samples, indicating that neuraminidases were implicated in this activity and that the observed rise in the pI of the EPO isoforms was due to removal of negatively charged sialic acid residues. However, some active urine samples were inhibited by 4-methoxyphenylsulfamate, an inhibitor of glycosulfatases, as well. It is thus difficult to conclude definitely on the involved enzymes at the present time.
The possibility of a bacterial origin of activity was envisaged since many of the active urine samples presented a bacterial proliferation of *Enterococcus faecalis,* though not all did.
Inoculation of different strains of *E. faecalis* into non-active urine samples showed that activity could be induced, depending on the strain used. Furthermore, the same strain induced activity in some samples but not in others. These results led to the hypothesis that the enzyme responsible for activity is expressed by certain strains of bacteria in specific surrounding conditions.

III Frequency of active urine samples

Active urine samples are rare. Out of 577 samples analyzed in our laboratory in 2003, 8 gave rise to shifted EPO profiles and 8 to EPO profiles outside of the pH gradient 2-6 (Fig. 4).

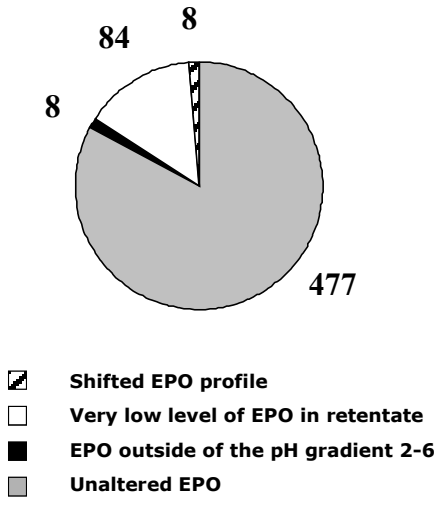

Figure 4 : Results of EPO testing in 2003.

Because bacterial proliferation appeared to be implicated in the activity, 348 urine samples analyzed for EPO from January to August 2004, were submitted to direct microscopic examination. Since many of these samples had been frozen immediately after collection, only 56 of them presented bacterial proliferation.

As can be seen in Fig. 5, the proportion of samples with altered EPO was clearly greater in these 56 samples.

These observations point out the importance of preventing bacterial proliferation from collection to analysis of urine samples.

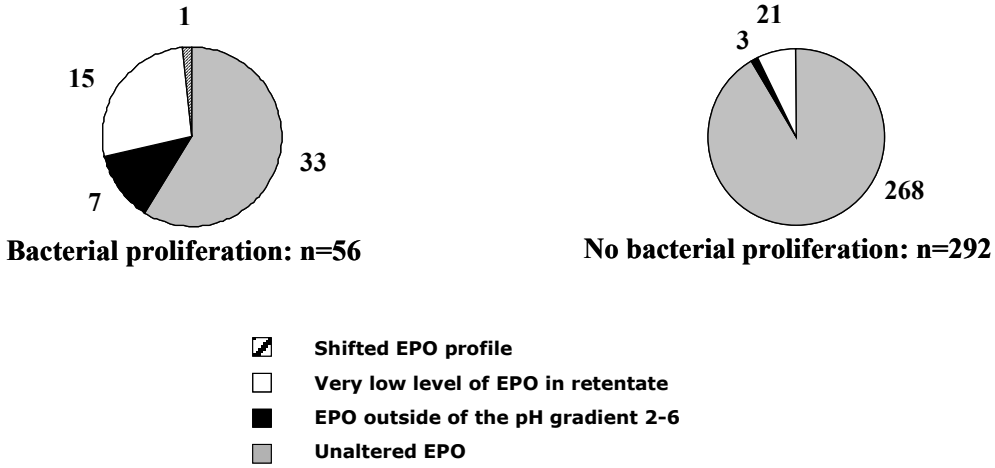

Figure 5: Results of EPO testing from January to August 2004.

Conclusion

In addition to the cases of activity that give rise to the absence of EPO on the interpreted image and the report of "undetectable EPO", shifting activity (within the pH gradient 2-6) requires more attention during interpretation of a result. The characteristics of a shifted EPO profile and stability testing can be used to clearly identify urine samples presenting this activity, which leads to the report of "unclassifiable result, degraded sample".

Though probably not exclusive, a bacterial origin was highly suggested by our observations and experiments. In order to prevent such situations, we strongly recommend freezing the urine samples immediately after collection.

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