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Purification of EPO from urine retentates prior to IEF detection

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INTRODUCTION

The implementation of the method for EPO detection, as developed by Lasne et al (2000; 2002) is one of the biggest breakthroughs in the antidoping fight in the recent years. The test relies upon differences in the isoelectric patterns between the endogenously produced EPO (huEPO) and the recombinant EPO (rEPO). The correct interpretation of a profile requires a correct assignment and numbering of the bands, which in some cases of arc shaped bands for example, may be difficult. These observations suggest that some urine contains either particular molecules or an excess protein content that impair the uniform electrophoretic migration of the isoforms, by creating steric hindrance or uneven current along the gel. The improvement of the sample preparation prior to isoelectric focusing was pointed as essential by Peltre and Thormann (2003) in their report on the evaluation of the EPO method. It is obvious that a better purification of EPO prior to IEF, resulting in non-perturbed isoform profile will increase the overall quality of the gel, improve the confidence in the densitometric analysis and thus, facilitate profile interpretation.

The experimental strategy consisted in developing both an anion exchange chromatographic fractionation and an immunoprecipitation process in order to purify indiscriminately EPO isoforms from urine retentates.

EXPERIMENTAL

Urine retentates were prepared according to Lasne et al (2000) and were further fractionated by chromatography on a anion exchange resin (BioSep-DEAE 75*7.8mm, Phenomenex) using a loading buffer of 50 mM TRIS-HCl pH 7.4 and a second buffer composed of the

loading buffer with 500 mM NaCl. A step gradient of 150 mM NaCl was used to elute the EPO isoforms and a subsequent washing step with 500 mM NaCl was included. The flow rate was 1 mL/min and 10 mL fractions were collected and concentrated over 30 KDa centricons (Millipore).

Retentates were also purified by immunoprecipitation using α -EPO MAb clones 16/26 and protein G-Sepharose beads. EPO was eluted from Ab-beads complex with 7M urea, 2M thiourea, 0.5% ampholytes and 1% tween-80 solution and immediately loaded on IEF gel. EPO detection method was performed according to Lasne et al. (2000).

RESULTS

Fractionation by anion exchange

Urine retentates were fractionated in 3 fractions as presented in Figure 1. About 50% of the total material absorbing in UV at 280 nm is removed in the loading and the washing fractions. The integrity of the isoforms is preserved in the eluting fraction.



Figure 1: Fractionation of a representative urine retentate on DEAE column and EPO IEF profiles of the collected fractions 1, 2 and 3, compared to the original retentate.

Fractionation of various urine retentates appears to improve the profile resolution and facilitate the densitometric quantitation (Figure 2). For example, the densitometric profile of sample D is significantly improved after fractionation.



Figure 2: Urine samples non treated (NT) and after fractionation (F) of DEAE.

Isolation by Immunoprecipitation

Specific EPO isolation by immunoaffinity technique appears to be more selective and also to greatly improve the resolution of urine samples (Figure 3), as illustrated with the densitometric profile of sample 2. Whereas the bad quality of the image obtained without IP would have prevented any interpretation of the result, the pattern is very clear after IP.



Figure 3: IEF profiles of urine samples submitted to immunoprecipitation (IP) compared with the untreated retentates (NT).

Purification of reference standards in urine

Urine retentates presenting a smeared IEF migration were spiked with excess rEPO and NESP without apparent isoform discrimination using both methods (Figure 4).



Figure 4: IEF profiles of urine retentate spiked with standards and submitted to anion exchange fractionation and immunoprecipitation, with comparison to pure standards of NESP and BRP.

CONCLUSION

Removing interfering material from the urine samples results in a more uniform migration pattern, avoiding arc-shaped, smeared or diffused bands, and hence facilitates the integration and the quantitation of isoelectric profiles. The non selective fractionation by anion exchange reduces the protein concentration of the retentates by around 50% which is shown to be efficient in removing interfering particles. Immunoaffinity is very selective and does not discriminate the isoforms and, as expected, also increases significantly the resolution of the profiles. Future work on combining these two procedures will be conducted to increase resolution of the actual detection test. We intend to employ such a strategy of sample purification for the development of any complementary detection method, such as SDS-PAGE or mass spectrometry.

REFERENCE

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