PROJECT REVIEW

"Optimization of the method for the detection of erythropoietin and its analogues "

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Erythropoietin (EPO) is an hormone regulating the synthesis of red blood cells, thus increasing the ability of blood to carry oxygen. Its analogues could be used by athletes in endurance sport and therefore appear in the WADA list of banned substances. Nowadays there are direct and indirect methods for the detection of erythropoietin.

The direct method developed by Lasne, is divided into four steps: urine concentration, isoelectric focusing separation, double blotting and detection by chemiluminescence. The development of other forms of recombinant EPO, and the need to increase the window of detectability of EPO administration, led to the need of updating the Lasne method to ensure the highest efficacy and efficiency of the anti-doping analysis. For instance, due to the differences in the apparent molecular mass of recombinant and endogenous erythropoietins, the administration of some recombinant EPOs can also be detected by SDS PAGE, another tool to clarify the profile of the suspicious samples.

The overall aim of the project is to study the effect of some specific modifications of the Lasne method, in order to (i) reduce the overall time of the analysis, (ii) simplify the sample treatment process, (iii) improve the limit of detection, and (iv) increase the robustness of the results. Particularly, we plan to evaluate the effect of some modification at the level of (i) the preparation of the gel, (ii) the incubation steps respective to the blotting process, and (iii) the antibody incubations following the second blotting step. The effectiveness of the proposed solutions will be tested on a significant number of reference samples, of spiked urines and of negative and positive samples coming from our routine activity.

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Results and Conclusions

In the first part of this project we focused on the possibility of reducing the overall analytical times of the method for the detection of erythropoietins (EPOs), possibly completing the procedure in less than one working day from the start of the analytical process. The method is basically the same originally proposed by Lasne et al. and presently implemented by the WADA accredited laboratories, with few significant modifications involving the use of a novel blotting system based on vacuum technologies (namely, the SNAP i.d.® system). The system allows in principle a significant shortening of the time required for blocking, washing and antibody incubations, and we have considered its potential effectiveness if used after the first blotting step of the isoelectrofocusing (IEF) procedure. By applying a vacuum to actively drive reagents through the blotting membrane, the time of incubation for the blocking step and the washing step are indeed drastically shortened.

Furthermore, since the differences between the apparent molecular masses of endogenous and recombinant erythropoietins and analogues make possible to discriminate the endogenous and some exogenous EPOs by SDS-PAGE, the utility of the SNAP i.d.® system was verified also for this procedure. Finally, due to the recent developments in the field of electrophoretic EPOs detection, the utility for the SDS/Sarcosyl-PAGE procedure was also investigated. The alternative procedures involving the SNAP i.d.® system in the IEF and SDS/Sarcosyl-PAGE methods were validated in terms of sensitivity, specificity and robustness using blank urine samples spiked with biological reference preparation of exogenous erythropoietin (BRP, rEpo) or darbepoetin α (NESP) or epoetin δ (Dynepo) or CERA and urine samples from antidoping controls found to be negative during the screening analysis. The effectiveness of the newly developed approach has been also evaluated analyzing urine samples from excretion studies.

The results showed that even if the time of each step is drastically reduced (the use of SNAP i.d.® system reduces the time for the complete analysis five to six hours) the limits of detection, the specificity and the robustness of the newly developed procedure are very similar to those obtained by the method currently used by the WADA accredited laboratory of Rome. We have validated both the IEF method and the SDS/Sarcosyl-PAGE method with the use of the SNAP i.d.® system. The shortening of the time required to complete the procedure may reveal particularly valuable especially on the occasion of major international events, where the analytical workload drastically increase and, in parallel, the reporting times are very compressed.

In the second part of the study we have evaluated the effect of some specific modifications of the procedure, in particular different reagents: a secondary antibody goat anti-mouse IgG (H+L) biotin conjugated different from the one currently in use in our laboratory purchased from Pierce/Thermo, two different streptavidin-peroxidase adducts

(one complexed and one conjugated) and a specific monoclonal antibody anti-hEPO from mouse (Clone 23H2) on a gelatinous support inside a column to purify the urine samples were tested. The results obtained showed no significant advantage with respect to the reference procedure, although the results obtained the monoclonal antibody anti-hEPO used for immunopurification step is not good to purify urine samples but we have preliminarily obtained good results using it as primary antibody.