"Preparation and characterisation of new immunopurified urinary and plasmatic EPO standards. Acronym: REFEPOS"

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Project Summary

The analytical strategy of doping control relies on the availability of reference standards against which compare the results obtained after the application of any procedure. The situation with the endogenous urinary EPO standard (NIBSC 2nd international reference preparation) needs careful attention:

- the urinary standard (uEPO) from NIBSC is coming to an end and according to the correspondence held with NIBSC, there are no plans to produce a new batch.

- At this stage, only 2 vials (20 IU) per laboratory and year will be provided.

- The NIBSC uEPO standard was produced by unspecific protein concentration from urine of anaemic patients. Hence it contains very high concentrations of proteins other than uEPO.

- Despite all efforts devoted so far, there has not been an appropriate characterisation of the origin of the charges responsible for its IEF behaviour.

- The comparison between any standard obtained from patients and a standard obtained from healthy volunteers has to be performed.

- An appropriate analytical standard of serum or plasma EPO is needed and has to be developed.

The technology for EPO immunopurification has already been developed and the members of the research team have first-hand experience in the field. However, its up-scaling for large volume processing (e.g. ten litres or urine per day) has to be developed tested properly.

With this background, the aim of the project is:

- developing the methodology for producing a urinary EPO standard by processing urine from patients and healthy volunteers.

- producing immunopurified urinary and serum EPO standards, available for doping control purposes.

As secondary objectives, the daily variation of their urinary EPO profiles will be studied and, using EPO from patients producing elevated concentrations of the structural characterisation of EPO will be approached.

Results and Conclusions

The general aim of the project was the development of a large scale inmunopurification procedure and its application for the preparation of a relatively large scale batch of endogenous EPO standard in urine and, if feasible, plasma from healthy volunteers and patients producing large amounts of this glycoprotein.

The immunopurification procedure developed implied a sample pretreatment includingdissolution under alcaline conditions of the sediment/precipitates present in urine, followed by a thorough multi-step filtration (first, a filtration through a triple glass fibre filter AP25 (Millipore), 2 µm AP20 (Millipore) and 0.5 µm RW06 (Millipore); second a 0.22 µm Millipak filter unit).

Immunopurification of urinary volumes between 1.5 and 2 L was achieved using 2 mL Sepharose columns containing as much as 12 mg of anti-EPO monoclonal antibody. Columns needed to be used at 4ºC, overnight at a 2-3 mL/min flow rate. Recovery, during the development phase showed to be roughly around 50% for solutions of up to 80 IU/L.

However when real urine samples were treated, it soon became evident that the urinary matrix was able to block the columns, even after substantial filtration. Tamm-Horsfall protein, the most abundant glycoprotein in urine, could have been part of the problem.

Extensive washing of the immunoaffinity columns, changing of filtering procedures as well as heating the urine samples seemed to delay the problem, but did not solve it.

On the other hand, it was impossible to find proper patients or volunteers with elevated EPO excretion. Patients from clinical hematology, pneumology or oncology resulted in depleted EPO concentrations. Finally it was decided to use urine from healthy volunteers with serious implications regarding the amount of EPO that could be reached. Immunopurified EPO was very sensitive to preparation conditions with significant losses due to irreversible adsorption on glass or plastic vials.

Overall, recovery was below 5% and only a very small, symbolic, batch of 15 vials of 1.6 IU/vial of liophylized material could be obtained in a buffer composition compatible with electrophoretic or mass spectrometric methods.

Obtaining reasonable amounts of this glycoprotein standard, and at a reasonable cost, would imply an international approach with access to a number of high EPO excreting individuals under proper long term conditions.