Australian Government Analytical Laboratories

Progress Report

on

Improved Method for the Detection of Erythropoietin Isoforms in Urine

Prepared for

The Department of Communications Information Technology and the Arts (DCITA)

5 December 2003

Summary of Project

To simplify the methodology used so that the French EPO urine test can be applied to more samples at substantially lower cost. This will take place in a number of stages:

1. In collaboration with Proteome Systems develop a robust immobilised pH gradient (IPG) gel that can be used in all IOC laboratories to improve reproducibility of results, particularly between laboratories, obtained using the gel electrophoresis test for recombinant EPO developed by the French IOC laboratory. The gel developed will also have the capability to resolve the isoforms of the new EPO replacement Novel Erythrocyte Stimulating Peptide (Egrie and Browne 2001);

2. Investigate means of selectively extracting recombinant EPO from urine to improve the sensitivity of the current method and remove the need for the complicated double blotting process.

3. Investigate whether improved the extraction methodology can lead to the measurement of EPO isoforms in blood.

4. Investigate how mass spectrometry can be applied to the analysis of the purified extracts for recombinant EPO.

Project Milestones

Achievement Date	**Milestones/Activities**
5 December 2003	• IPG gel developed for EPO urine test • Conditions optimised for use of IPG gel • Testing of complete IPG method • Clean up methods selected • Clean up methods optimised for urine
16 April 2004	• Clean up methods optimised for urine (continued) • Clean up methods optimised for blood • Evaluation of data • ADRP reports
30 June 2004	• Final report to Anti Doping Research Program Panel

Progress to date

The progress to date has been within expected timeframes but with some rescheduling of priorities. There has been a delay in ASDTL working with the IPG gels because of delays from Proteome Systems Limited. They had promised to obtain these for ASDTL but so far there has been no word on delivery.
In the meantime several sample purification procedures have been investigated.

Milestone 1 – IPG gel developed for EPO urine test.

- Proteome Systems Limited (PSL) have successfully developed an immobilised pH gradient gel (IPG) which covers a low pH range. This gel has been shown to sharply resolve the isoforms of both recombinant erythropoietin and darbepoietin (NESP).

Milestone 2 – Conditions optimised for use of IPG gel.

- The running conditions for the low pH range IPG are typical of the gel type, requiring an overnight exposure to an electric field, to a total of about 36,000 volt hours. This produces sharp separation of standard preparations of EPO and NESP.

Milestone 3 – Testing of complete IPG method.

- Conditions for the transfer of separated EPO or NESP from the IPG to membranes (Western blotting) for antibody visualisation have been established and demonstrated using standard preparations.
- Transfer from samples containing a urine matrix has not been successful. It is not clear what the interfering substance or process is.
- ASDTL staff are in discussion with PSL with the aim of obtaining a supply of IPGs for trials within the doping laboratory environment. To date, sample separation has occurred at PSL with the rest of the procedure performed at ASDTL. ASDTL is currently awaiting from gels from PSL.
- In the interim, the ability of the current method to separate and identify the highly acidic isoforms of NESP has been improved, mainly through the optimisation of sample loading procedures.

Milestone 4 – Clean up methods selected.

- Clean up can be achieved by removing identified interfering substances, specifically albumin and immunoglobulins.
    - Cibacron Blue affinity gel, a mixed-mode affinity medium, has been widely used to remove unwanted abundant proteins from sample matrices prior to specific analysis for a less abundant target analyte. EPO proved to have a very low affinity for Cibacron blue, and was therefore separated from the more abundant proteins with very good recovery.
- Clean up can also be achieved by selectively extracting EPO from the matrix.
    - Immunoaffinity methods are widely used for this purpose. The poor availability of sufficient quantities of a suitable immobilised antibody preparation has made progress difficult. ASDTL is investigating the production of a suitable immobilised antibody with the Australian Racing Forensic Laboratory and Charles Sturt University, under a grant from the World Anti Doping Agency.
    - An immobilised preparation of the lectin Wheat Germ Agglutinin (WGA) has a high affinity for the terminal sialic acid residues on EPO. We have shown that WGA extracts EPO from complex matrices with reasonably good efficiency.

Milestone 5 – Clean up methods optimised for urine.

- When Cibacron blue gel is used to extract EPO from urine, the EPO resides in the flow-through portion, and other proteins are retained. This sample will still require extensive concentration before application to the isoelectric focussing procedure. Much of the trial work has been done in urine and so the recovery of EPO from urine is known to be good (to 80%). Reproducibility is still to be demonstrated. It is still to be determined whether this method extracts a representative set of EPO isoforms, compared to ultrafiltration.
- Urine samples for treatment with WGA are partly concentrated by ultrafiltration, and buffered. The EPO is released from the column using an elution buffer. This produces a slightly concentrated preparation that is substantially free of major interfering proteins. Recoveries under slightly varied conditions varied from 22 to 55%. It is also still to be determined whether this method extracts a representative set of isoforms.
- The final choice of method may differ for urine and serum, as the compositions of the two matrices differ considerably.
- The choice of method will also be guided by cost-effectiveness. All of the proposed methods require expensive reagents. They can be regenerated and re-used, and so the per-sample cost will depend on the number of cycles which can be obtained from each reagent or combination. The method in current use requires expensive single-use filtration devices, and improvements in the per-sample preparation costs are very desirable.

Estimated Project Expenditure

Cost Item (detailed breakdown)	Total Approved for Item/Category (Excluding GST)	Amount Spent in Reporting period	Cumulative Amount Spent to Date
Salary, Scientific personnel 10% of SPOA plus on costs 50% of SPOC plus on costs	$30,000	$16,814	$16,814
Salary, technical personnel 100% of technician plus on costs	$45,000	$12,403	$12,403
Consumables Approximately 25 IEF gels at$1,800 per gel dye affinity, lectin and ion exchange columns, visualisation reagents	$45,000	$32,400	$32,400
EPO test kits and reference standards	$10,000	$2,137	$2,137
Overheads: (indirect costs including support infrastructure, equipment maintenance and repairs, services etc.)	$23,641	>$23,641	>$23,641
Sub-totals	$153,641	>$87,395	>$87,395