Department of Communications, Information Technology and the Arts

Anti-Doping Research Program

Progress Report

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Project: Improved Method for the Detection of Erythropoietin Isoforms in Urine.

Investigators

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SUMMARY

This report outlines the progress so far in our attempts to simplify and improve the existing urine test for recombinant human erythropoietin (EPO). The test is based on using gel electrophoresis to separate the isoforms of EPO which are more acidic in endogenous urinary EPO than in recombinant EPO. The test, though reliable, is expensive not only because of the cost of the reagents and consumables but also because of the multistage complex process which requires a high degree of skill and expertise to perform. We have been collaborating with Proteome Systems on this project because of their high level of expertise in the development of gel technology for protein separation. It was originally intended to proceed in a stepwise fashion to simplify and improve the ruggedness of the EPO test. The first step was to replace the ampholyte gels currently used with commercial immobilised pH gradient (IPG) gels of the appropriate (unusual for normal protein separation) pH range. A considerable amount of work has been performed on this aspect of the project which was initially anticipated to be fairly straightforward. Unfortunately it has been found that the replacement of the laboratory-prepared ampholyte gels with commercial IPG gels is not simple. Suitable pH range IPG gels have been prepared by Proteome Systems and, whilst they have been shown to very effectively resolve the isofoms of recombinant EPO, the effective transfer of EPO to the double blotting membranes needed for the detection of low levels of EPO in urine has not been achieved. However during the course of the work we have modified our ampholyte gel methodology so that we can routinely detect the new Amgen EPO variant Aranesp. Similarly, Proteome Systems have produced a more acidic IPG gel which can resolve both EPO and Aransesp. In addition Proteome Systems have almost completed the commercial development of the gel electrophoresis and blotting system that is required to use the IPG gel technology.

Because of the unexpected problems with replacing the ampholyte gels in the existing method a different approach has been tried. Proteome Systems have used the IPG gels in combination with their 2D gel electrophoresis technology to develop a method, with our assistance, which has the potential to completely replace the existing double blotting method with considerable cost savings. A joint paper on this new method has just been presented to the 22nd Cologne workshop on dope analysis (abstract attached). This is an exciting development which has the potential not only to simplify testing for recombinant EPO but also to increase the robustness and specificity of the test. The remainder of this year's activities will be concentrated on developing this method for routine use.

The program for 2004-2005 will include the full validation of the 2D gel method, the further development of specific clean up methods, and the application of electrospray mass spectrometry to the characterisation of extracted EPO.

BACKGROUND

The current method used to confirm doping with human recombinant EPO is based on the fact that recombinant EPO is significantly less acidic than urinary EPO despite the recombinant product being produced from mammalian cells containing the human gene. This difference in acidity arises from the fact that the recombinant product is less heavily glycosylated and has fewer sialic acid residues than normal urinary EPO. The test for recombinant EPO in urine developed by our colleagues at LNDD in Paris (Lasne and de Ceaurriz 2000), whilst able to detect the abuse of EPO, has a number of significant limitations. They all relate to the complexity of the method, which means that:

- the production of results is slow and cannot always be guaranteed
- the reproducibility between laboratories is not as high as desired
- the cost of the method is so high that it is impractical to test as many samples as required.

A more robust and simpler method is needed. The year one program is the first stage in a series of steps to simplify and automate the methodology so that it can be economically applied to high volume screening. The first step in this long process is to obtain a reliable source of IPG gels that can be used in the first stage of the method. Proteome Systems have obtained a Biotechnology Innovation Fund grant to develop a gel or gels specially designed for the detection of recombinant EPO. ASDTL is providing the knowledge and skills needed to test the gels provided and incorporate them into the urine test for recombinant EPO but are not funded to do so. Proteome Systems have now developed a commercial IPG gel that can resolve the isoforms of recombinant EPO. The resolution and reproducibility is superior to the ampholyte gels currently used. The availability of such a reliable commercial gel will not only significantly improve the robustness of the method but will also reduce the overall cost and complexity of the method.

The double blotting process used in the method is a major source of the method's complexity and cost. Alternative means of separating the recombinant EPO from other proteins will be examined. The methods which have shown the most promise so far are extraction methods using dye affinity chromatography and another using lectin affinity chromatography (Trout 2002). Dye affinity chromatography preferentially removes albumen (Gianazza and Arnaud, 1982), which is the main protein present in urine and thus has the potential to remove much of the interfering protein. Lectins react with glycoproteins including EPO and thus can be used to retain only the glycoproteins whilst discarding all other proteins. The use of ion-exchange media will also be investigated (Morimoto et al., 1996). The aim is to remove more than 90% of the interfering proteins whilst retaining at least 50% of the EPO. If this approach is successful it will not only simplify the method but also mean that it could be applied to blood as well as to urine. It is known that serum EPO concentrations correlate much more closely with EPO use than do urinary EPO concentrations.

Once a purified EPO can be extracted from urine or blood then detection will be simplified. It should also be possible to use mass spectrometry (MS) to more specifically characterise the extracted EPO. At present the confirmation of the presence of peptide hormones and other large bioactive molecules is done using techniques that rely on specific antibody reactions to large molecules. All banned drugs that are detected in IOC laboratories, other than peptide hormones, must be confirmed by the use of MS. The reason the peptide hormones were excluded from this requirement was that it was not practicable to attempt mass spectrometric analysis of large bio-molecules both because of their high molecular weight and because of the very low concentrations found in blood and urine. However with the ever increasing demands of proteomics research the use and capabilities of MS using electrospray and liquid chromatography for the analysis of bio-molecules has increased dramatically in the last few years and will continue to do so. As electrospray MS has already been used in this laboratory for the confirmation of the presence of haemoglobin based oxygen carriers (Trout, 2002a) and it is planned to extend the technology for the detection and confirmation of recombinant EPO. This activity is combined with another mass spectrometry project which has been funded by WADA and for which the appreciation of the Australian dollar relative to the US dollar is having profound budgetary implications.

AIMS

The primary objective is to simplify the methodology used for urinary EPO isoform measurement so that the test can be applied to more samples at a substantially lower cost per sample. It is proposed that 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 Aranesp or 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.

Experimental Methods

The method currently used to detect recombinant EPO in urine is a variation of the original Lasne method (Lasne et al 2002). 20mL of each urine was concentrated to 30uL using two ultrafiltration devices. The concentrated samples underwent isoelectric focussing on an ampholyte gel and then were transferred with two Western blotting steps. The membrane was visualised using Pierce SuperSignal West Femto Maximum Sensitivity Substrate. The signals were recorded with a Fuji LAS-1000 camera and quantitated with Fuji Image Guage v3.41 software. It is this accepted method against which all improvements and modifications must be measured. The IPG gels were produced using proprietary technology by Proteome Systems. For the evaluation of the IPG gels the procedures used for sample preparation and double blotting were essentially identical to those used in the original method.

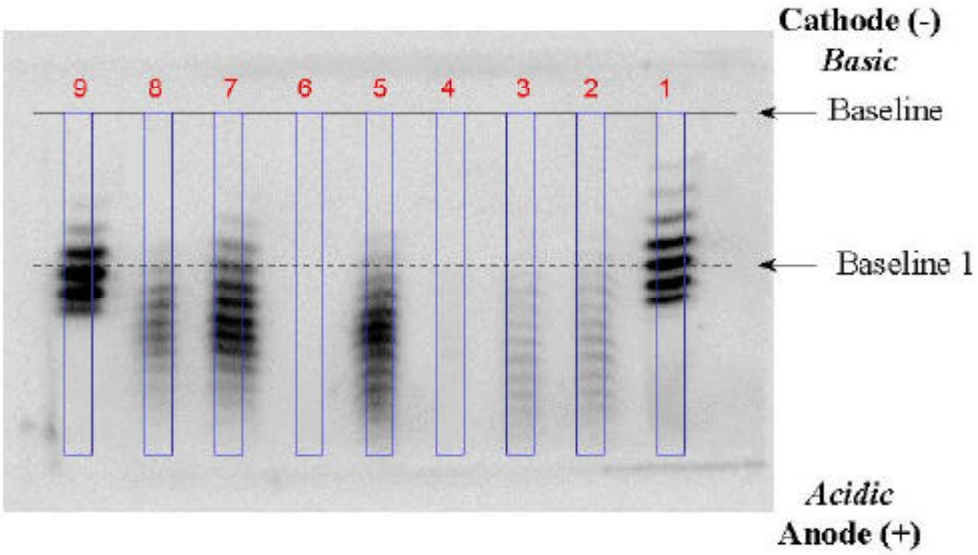
Results

Proteome Systems have prepared IPG gels with suitable pH ranges for the efficient separation of both recombinant EPO and the new more acidic variant Aranesp. As expected the resolution of the isoforms is superior to that obtained with the current ampholyte gels. However when real urine samples spiked with recombinant EPO were run on the system the results were disappointing. Even at relatively high concentrations of EPO the resolution seen in standards was not achieved and it appears that the efficiency of transfer of EPO from the IPG gel to the double blotting membranes was poor. Many variations of the method were tried but none achieved a result that could be regarded as suitable. Thus the first part of the project, replacing the ampholyte gel with a more reproducible and better resolving IPG gel, has not been achieved. This was thought to be relatively simple but it is now apparent that factors such as sample preparation and double blotting, which have been optimised in the current method, cannot be readily transferred to IPG gels.

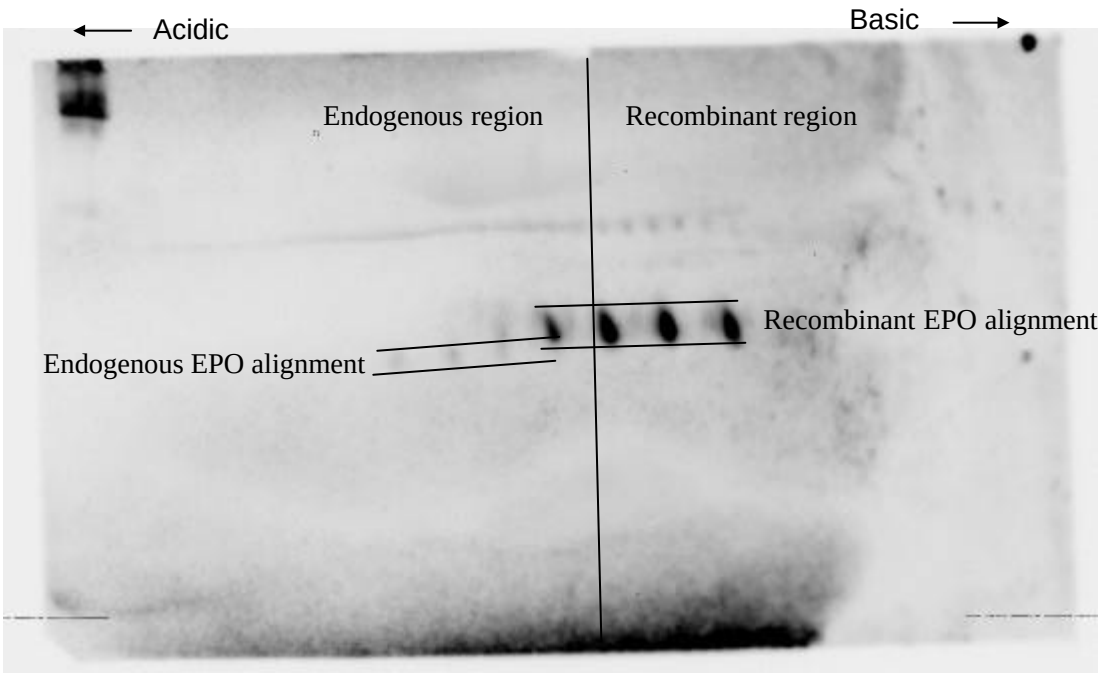
During the course of this work our routine ampholyte gel method was successfully modified so that it can now resolve the isoforms of Aranesp as well as those of EPO.

A completely new approach was tried in which the sample preparation procedure was altered to a more conventional protein precipitation procedure, the samples were run individually on IPG strips of the appropriate pH, and then separated in the second dimension using PAGE gels. Using a conventional Western blot the separated spots were transferred to a membrane and visualised. The method uses the automated gel electrophoresis and Western blotting equipment developed by Proteome Systems. Shown below are pictures of the current 1D gel separation for several normal urines and the new 2D gel method for a normal urine spiked with recombinant EPO. With the existing method several extracts are run on one gel (lanes 2 to 8) with recombinant EPO standards run at each end (lanes 1 and 9). The separation based on the PI of the proteins occurs in the vertical axis. The identification of doping is based on the presence of isoforms above the baseline. With the 2D gel method only one

sample is run per gel. In the picture the separation based on PI runs horizontally whilst the separation based on size runs vertically. It can be seen that the resolution of the bands of EPO is significantly better using IPG gel technology. In addition there is partial vertical resolution based on apparent size which means that separation of recombinant and natural urinary EPO is much more distinct than with the existing method.



Existing 1D gel method



New 2D gel method using IPG gels

Report on Progress

Summary Table

Activity	Estimated completion in proposal	Progress thus far	On schedule Y/N
IPG gel developed for EPO urine test	July 2003	IPG gel now available with suitable pH range for both EPO and Aranesp.	Y
Conditions optimised for use of IPG gel	Dec 2003	Failure to achieve desired sensitivity when using the IPG gel as a replacement for ampholyte gel. New method being evaluated.	N
Testing of complete IPG method	February 2004	The new method has been given its first blind trail using spiked urine samples. Refinement and comparative testing of the method will occur over the next four months.	N
Method available for IOC labs	Mar 2004	The completely new method should be available within 6 to 9 months. Method reported at Cologne Doping Workshop Mar 2004.	Y*
Cleanup methods selected	November 2003	Three methods selected.	Y
Cleanup methods optimised for urine	April 2004	The immunoaffinity approach shows the most promise but more antibody material is needed to continue the work.	N**
Cleanup methods optimised for blood	June 2004	No work has yet begun on blood due to delays with antibodies.	
Evaluation of data	Jan 2004 and June 2004	Data reviewed.	Y
Report to ADRP	January 2004	Report requested in November 2003. Preliminary report submitted in December. Second report requested in March 2004.	Y

*The major difficulties encountered in optimising an IPG gel for use with the existing double blotting technology have completely changed the course of this project. It was believed both by Proteome Systems and ASDTL that this should be relatively simple and achievable by December 2003. This has not been found to be the case. Thus the incremental method improvement approach has been abandoned. The new 2D method outlined in the results section above has the potential to be the new simpler EPO method, which was to be developed in the second year of this project with an estimated completion in February 2005. Assuming that the sensitivity can be further improved and some problems with band identification are resolved this target should be readily achievable. The method is significantly less expensive to perform having fewer steps and requiring less skilled staff. The results obtained so far also indicate that the differentiation of urinary and recombinant EPO is superior to the existing method which will result in improved selectivity.

****** Immobilised antibodies for this aspect of the work are to be produced by the Australian Racing Forensic Laboratory and Charles Sturt University with funding provided by the World Anti-Doping Agency (WADA). Some immobilised antibody columns have been evaluated but

delays in signing the contract with WADA have meant that the larger quantities needed for the work to continue are not yet available. It is anticipated that the antibody project will commence within the next month.

For the period up to June 30 it is anticipated that the project will concentrate on the extensive evaluation and validation of the new 2D EPO method. This will require technology transfer and demonstration of the robustness of the method in routine laboratory use. The validation will require numerous samples to be run on both old and new methods. As the method is so different new criteria will need to be developed for defining a positive doping case. Samples of urine from a statistically significant number of elite athletes will need to be run to support the new criteria.

EXPENDITURE

The funds expended for the period from July 2003 to the end of February 2004 are set out in the table below:

Expense Category	**Amount Approved**	**Amount Spent**
Salary, scientific personnel	$30,000	$18,000
Salary, technical personnel	$45,000	$21,000*
Consumables	$55,000	$38,000**
Overheads	$22,282	$19,000
Totals	$152,282	$96,000

* Because of the delays encountered in the period from September to December 2003 less gels than planned have been run and hence less technical staff time has been used. This activity will increase significantly from March to June.

** Some of the consumables needed for the completion of this year's project have already been purchased.

Proposal for Year 2

The original proposal was for a two year program with similar expenditures in each year of the program. Because of developments in the first year the activities to be performed in the second year have changed. Some of the expenditure on new method validation which was not to occur until 2004-2005 will now take place in year one of the project whilst further development of cleanup methodology will now take place in year 2.

The major objectives for Year 2 are –

- Complete the validation for the new 2D gel EPO method including setting new criteria for the detection of doping with recombinant EPO. Estimated completion is December 2004..
- Develop cleanup methods for extracting EPO from urine which have sufficient selectivity and efficiency to enable EPO to be detected without the need for double blotting. Estimated completion is December 2004.
- Develop cleanup methods for extracting EPO from blood with sufficient selectivity to enable the measurement of EPO isoform distributions in serum or plasma. Estimated completion June 2004.
- Develop mass spectral methods for the detection of recombinant EPO. Estimated completion February 2004.
- Demonstrate that mass spectrometry can be used to detect recombinant EPO added to urine. Estimated completion June 2004.

Abstract submitted to the 22nd Cologne Workshop on Dope Analysis 7th till 12th March 2004.

Title of the paper: "Detection of recombinant erythropoietin in urine by two-dimensional gel electrophoresis"

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Abstract

The International Olympic committee (IOC) banned the use of recombinant human erythropoietin (rHuEPO) in 1990. There has been a growing need to develop a sensitive, reproducible and less complex technique than the currently used method to identify illegal use of banned protein drugs. We present a two-dimensional gel electrophoresis (2D-PAGE)-Western method for the detection of natural human erythropoietin (HuEPO) and rHuEPO (epoetin alfa) in urine. This method involves a high throughput sample preparation of the EPO, 2D-PAGE, a single electro-blot followed by chemiluminescent immunodetection. The method is cost effective, sensitive and easy to use.

As well as differing in the iso-electric point (pI) of the isoforms, natural erythropoietin separates at a lower apparent molecular mass from recombinant erythropoietin in the second dimension gel. This allows the 2D-PAGE system to separate natural and recombinant erythropoietin distinctly using both isoelectric profile and molecular mass separation. This approach has the potential to also detect other protein drugs in other human body.

References

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