AGAL

Final Report
(AGL02/110)

on

Activities and Outcomes from Research into Drug Detection Procedures.

Prepared for

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

31 July 2002

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Introduction

This report details the activities and outcomes achieved from the research, conducted by Australian Government Analytical Laboratories (AGAL) through the Australian Sports Drug Testing Laboratory (ASDTL), in relation to drug detection procedures. This work was funded by the Department of Communications Information Technology and the Arts (DCITA) under the Commonwealth Government's sports policy: Backing Australia's Sporting Ability (BASA).

The format of this report has been chosen to mirror the research plan provided to DCITA and as such reports each sub-project area and its results separately.

P2002-01: Robust Test for Growth Hormone – Defining Interactions Between Anabolic and Peptide Hormones

This collaborative project, with the principal investigator located at the Garvan Institute of Medical Research, is a three year program and approximately two thirds of the funding required has been provided by WADA ($400,000 USD for Year 1; $400,000 USD for Years 2 and 3 dependent on progress).

Summary of Project Purpose and Aims:

The major thrust of this project is to develop a robust test(s) for growth hormone (GH) doping in sport. The specific aims are to:

1. Investigate interactions between GH, EPO and androgenic steroids, which may be relevant to implementation of doping tests.
2. Continue the evaluation of molecular isoforms of GH (22k, 20k, and 17k) as a direct test for GH doping.
3. Define the reference range of potentially useful markers of GH in elite athletes utilising an existing databank of more than 3000 samples collected from elite athletes of diverse ethnic origins.

This is a three-year program and approximately two thirds of the funding required is to be provided by WADA. Additional funding from BASA was sought in order to complete the program.

However, WADA funding for this project is insufficient to undertake all the work elements proposed. In addition, the project collaborators believe that the prioritisation of work components requested by WADA will result in long delays between different facets of the research. Additional funding through BASA will allow complete, well integrated planning of the project, greatly enhancing the chance of a test for GH being available for the 2004 Olympic Games.

The project plan developed for year one is set out below:

#	*Objective*	*Target*
1	Key positions appointed	November 2001
2	EPO administration studies sorted	February 2002
3	Database established	February 2002
4	20 and 22k GH assayed	May 2002
5	GH markers assayed	May 2002
6	Statistical analysis completed	July 2002
7	Databank samples sent to appropriate laboratories	June 2002
8	Monoclonal antibody screen completed	June 2002

#	*Objective*	*Target*
9	Report to WADA on EPO administration study samples	October 2002
10	Recruitment started for GH/testosterone administration study	September 2002
11	17k ELISA assay established	October 2002

Report on Progress

Because no WADA funds were received in 2001/2002 the plan has obviously not been completed. However with the funds provided by BASA it has been possible to begin the project concentrating on the development of an indirect test for GH abuse. The indirect approach relies on the measurement of relatively long lasting markers of GH such as IGF-1. However in order to apply such an indirect test the normal range of these markers must be established by measuring levels in a large population of elite athletes from different countries. ASDTL has stored samples from just such a population of elite athletes collected during the EPO2000 study. There were 1152 (739 males, 413 females) state/national level athletes from 12 countries recruited as volunteers to provide blood samples and urine samples. Approval has been obtained from the subjects for such analyses to be conducted. The BASA funds were used to initiate the measurement of the concentrations of IGF-1 in these blood samples. These measurements are the first step in determining whether the natural variation in IGF-1 values is sufficiently small to enable the rise in IGF-1 concentration that occurs with GH doping to be detected.

The BASA funds were sufficient for approximately 3000 samples to have their IGF-1 concentrations measured. The assays were carried by Professor Rob Baxter's group at the Kolling Institute who are members of the consortium set up for this project. This sample set is sufficiently large to determine if factors such as age, sex, sport, and ethnic background correlate with changes in IGF-1. It is already known from normal clinical studies that IGF-1 levels are affected by age.

Randomly selected samples were re-extracted and assayed to obtain data on the uncertainty of the whole assay procedure. The mean %CV of the assay at low (<150 ng/mL), medium (150-300 ng/mL) and high (>300ng/mL) are 5.0, 4.1 and 4.4% respectively.

The IGF-I data were subjected to a number of preliminary analyses. The data set was divided according to sex. The male and female sets were sorted by age, sport, ethnic group and country of origin. All error bars on figures are standard deviation.

IGF-1 concentrations (ng/mL)

Overall Characteristics – Male		Overall Characteristics – Female	
Mean	153.92	Mean	161.28
Standard Deviation	50.79	Standard Deviation	58.02
Mean - 2SD	52.34	Mean - 2SD	45.24
Mean + 2SD	255.49	Mean + 2SD	277.31
Kurtosis	4.63	Kurtosis	4.18
Skewness	1.50	Skewness	1.58
Count	1887	Count	1071
Median	147	Median	151.00
Norm IQR	41.5128	Norm IQR	46.70
Lower robust 95%	63.97	Lower robust 95%	57.60
Upper robust 95%	230.03	Upper robust 95%	244.40
Minimum	49	Minimum	41.00
Maximum	489	Maximum	475.00

The overall distributions of the male and female data sets are similar, but differ in detail, particularly during adolescence. The data are non-normally distributed, being both positively skewed, and having a narrow distribution compared to the normal distribution. This is most obvious by inspection of Figures 1 and 2 below.

IGF-I concentrations were examined across age groups, yearly from 14 to 25 years, and above 25 years. It has long been established that there is a peak in circulating IGF-I concentrations during adolescence, and that the peak in girls is earlier than that in boys.. This distribution was also observed here. IGF-I levels (mean ± SD) in males peaked in 16 year old males at 199±63 ng/mL, declining to 127±41 ng/mL in the group over 25 years. IGF-I levels in females was maximal at 269±109 ng/mL in 14 year olds, declining to 129±40 ng/mL in the group over 25 years.

Figure 1: Frequency distribution of IGF-I concentrations in male athletes.

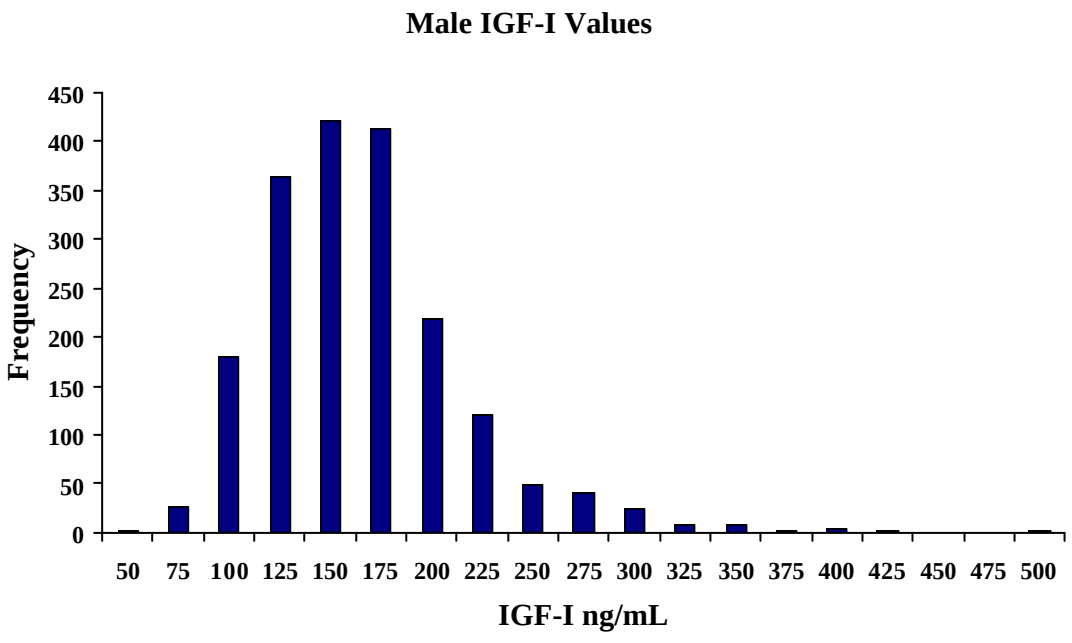
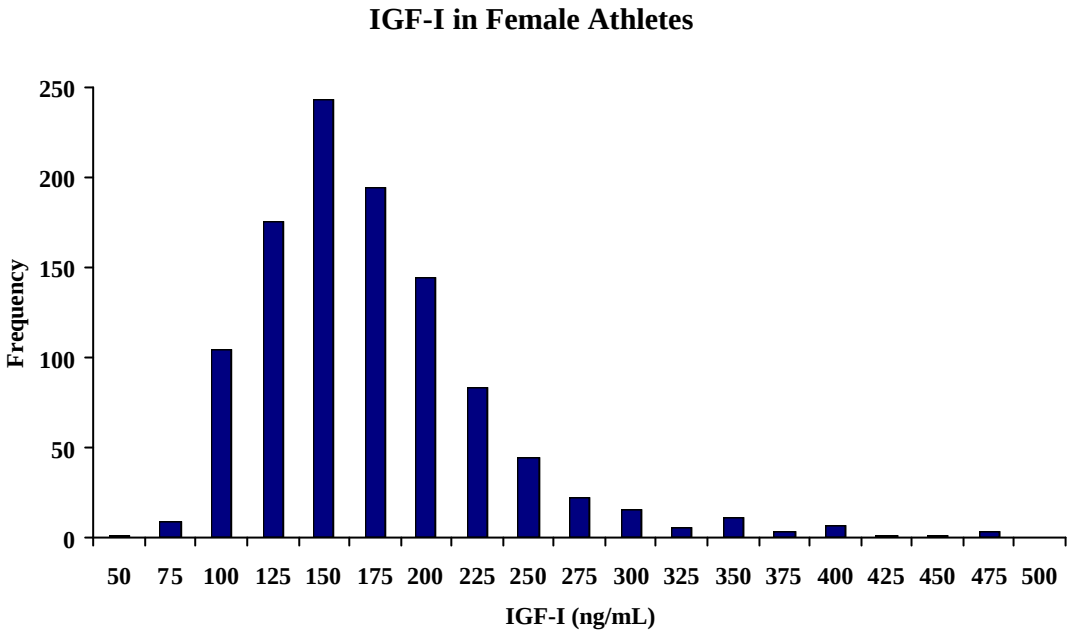
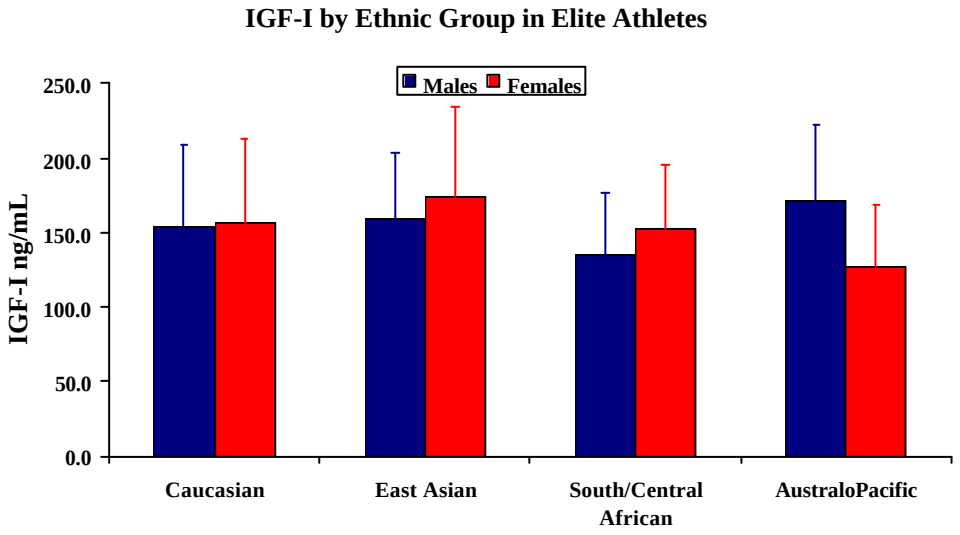


Figure 2: Frequency distribution of IGF-I concentrations in female athletes.



The original collection of demographic information yielded data on the broad ethnic origin of the athletes, who were mainly divided into Caucasian, East Asian, South/Central African and Australo-Pacific (Australian Aboriginal, Melanesian, Polynesian). There are some apparent differences among the groups as may be seen from Figure 3, however it should be recalled that there is considerable variation within the groups as well. The ranking of the groups with respect to concentration differ by sex. This may reflect other factors such as the range of sport types being played.

Figure 3: IGF-I by ethnic group and sex in elite athletes.



Although there was a strong association between country of origin and ethnic group, placing the athletes' IGF-I concentrations in country groupings distinguished particular countries with respect to others whose athletes were in the same ethnic group. In particular, in both males and females, athletes from Hong Kong had IGF-I concentrations of 224.0±66.5 and 267.8±108.4 ng/mL in males and females, respectively, were much higher than in Beijing, China, where the IGF-I concentrations were 147.4±27.9 and 169.6±37.8 ng/mL in males and females, respectively. Similarly, athletes from the USA had noticeably higher IGF-I concentrations than those from Australia, New Zealand, Mexico or France. The significance of these observations requires further investigation.

Differences in IGF-I concentrations between sport types in males and females were not marked. The sports were reported according to the following divisions: Power, Power/Endurance, Endurance, Aesthetic/ Skill, Athletics, Combat Sports, Court Ball Games, Field Sports, and Other (unstated, or multiple varied sports). The only apparent substantial difference between groups lay with the Power/Endurance classification, where IGF-I concentrations appeared slightly higher than other groups.

Conclusions and Further Study

The IGF-I data presented here suggest that the elite athlete population are similar to the general population in the distribution of IGF-I concentrations, especially with respect to age.

A number of differences between other groups were noted. Of particular interest were observations of country differences, such as the higher IGF-I concentrations observed in Hong Kong compared to Beijing in China. Similarly, the IGF-I results from the USA were higher than those from other countries where the population was predominantly Caucasian.

It must be remembered that these are simple preliminary analyses of the data. More sophisticated multivariate analysis may provide some indication of the sources of the variations observed among the different components of this population, as has already been done with the markers of EPO abuse.

It will be appropriate to apply more elaborate statistical analyses when the next phase of the work is performed, and the sample set is analysed for the other candidate analytes proposed as markers for hGH abuse. These data are not presently available from any other source, nor is any similar set of samples from elite athletes likely to be available in the near future. These IGF-1 measurements are likely to be the cornerstone used to determine whether an indirect method for the detection of growth hormone doping is statistically defensible.

The objectives achieved with the BASA funding are:

2	EPO administration studies sorted	February 2002
3	Database established	February 2002
5	GH markers assayed (IGF-1 only)	June 2002

P2002-02: Mass Spectrometry of Peptide Hormones

No BASA funds were allocated to this project in 2001/2002.

P2002-03 Recombinant EPO in urine

This project aims to simplify the methodology used in the current EPO urine test (the French isoelectrophoresis test as used at Sydney 2000 Olympic Games as part of the EPO 'Sydney protocol' test) so that it can be applied to more samples at substantially lower cost.

Summary of Project Purpose and Aims:

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;
2. Expand the method so that it includes the new EPO replacement NESP (Novel Erythrocyte Stimulating Peptide);
3. Investigate means of selectively extracting recombinant EPO from both urine and blood. This should have the dual benefit of removing the need for the complicated double blotting process and enabling the test to be applied to blood; and
4. Investigate the use of mass spectrometry to detect and confirm the presence of recombinant EPO. This links with Project P2002-02.

The project plan developed for year one is set out below:

#	*Objective*	*Target*
1	First IPG gel tested	October 2001
2	Correct pH range gel chosen	December 2001
3	Validation of EPO method with IPG gel	April 2002
4	Extension of method to include NESP	June 2002
5	Paper presented at Cologne workshop	March 2002
6	Commercial gels made routinely available to other IOC laboratories	July 2002
7	Paper submitted for publication	August 2002

Report on Progress

The discovery at the 2002 Winter Olympics that NESP is already being abused by athletes, has caused the priorities in the project to be modified. A commercial IPG gel has been developed that gives far superior resolution of the isoforms of EPO and can satisfactorily resolve recombinant and human urinary EPO. However this gel is not

suitable for NESP and it has been necessary to attempt the development of a stable IPG gel which will also resolve the more acidic NESP isoforms, before completing the validation of the EPO method with the IPG gel.

Below is a summary on the status of the work carried out so far by Proteome Systems:

Prototyping of pH 3-5 slab IPG gels manufactured in the Boston faciltity.
The IPG gels are now being manufactured using computer controlled gradient pourers which will give highly reproducible results over time. The pH 3-5 gels will soon become commercial products of Proteome Systems and work is continuing on the pH 2.5-5 gels.

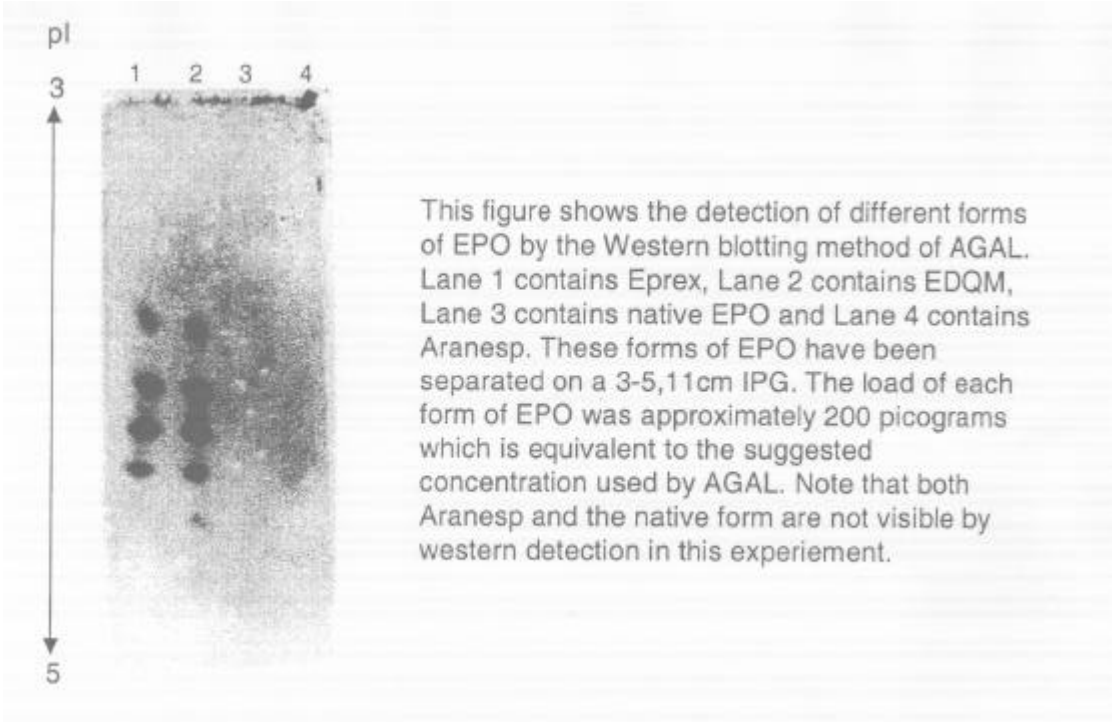
Optimisation of the separation of recombinant EPO on IPG slabs.
Both the pH 3-5 and the pH 2.5-5 gels have been tested with recombinant EPO (Eprex). The results show a superior separation to that currently achievable using the existing ampholyte gels. Some early results were presented at the 20th Cologne Workshop on Dope Analysis in March 2002. The latest results show that the Eprex isoforms are resolved on both the pH 2-5-5 and pH 3-5 gels but that the separation is optimal on the 3-5 gel.

Good separation of Aransep from Eprex EPO but little separation of Aranesp isoforms.
Using the pH 3-5 gel Aranesp is clearly resolved from Eprex however the isoforms of Aranesp are bunched together at the acidic end of the gel. This is why the pH 2.5-5 gel is being investigated as means of improving the resolution of the Aranesp isoforms.

Blotting protocol established for Western antibody detection.
Whilst it is clearly important to obtain optimal separation of EPO and Aranesp isoforms on the gel it is also vital to be able to transfer the EPO to a membane for detection by western blotting antibody interactions. An extensive investigation of transfer conditions and buffers has established that the Khyse-Anderson semi-dry transfer system is the best choice. The results obtained from western blotting antibody detection are shown below:

Future developments.
The optimisation of the resolution of Aranesp is to be investigated by alkylating the cysteine residues and giving the EPO and Aranesp more basic properties. The reason for this is that it is difficult to formulate IPG gels with a pH below 2.5.

Western blotting antibody detection of the various forms of EPO on narrow IPGs



This figure shows the detection of different forms of EPO by the Western blotting method of AGAL. Lane 1 contains Eprex, Lane 2 contains EDQM, Lane 3 contains native EPO and Lane 4 contains Aranesp. These forms of EPO have been separated on a 3-5,11cm IPG. The load of each form of EPO was approximately 200 picograms which is equivalent to the suggested concentration used by AGAL. Note that both Aranesp and the native form are not visible by western detection in this experiement.

Apart from the development of IPG gel technology being carried out by Proteome Systems with assistance from ASDTL (project aims 1 and 2), a significant part of the program at ASDTL has been concerned with the development of means of selectively extracting recombinant EPO from both urine and blood.

The French-developed test for the detection of recombinant human EPO (rhEPO) in urine relies on the extraction of EPO from a comparatively large volume of urine by ultrafiltration , to obtain sufficient hormone for analysis. This procedure retains undesirable proteins which interfere in the isoelectric focussing process. Extra steps are required to circumvent this difficulty, including the use of expensive protease inhibitors and a secondary blotting step.

A number of alternate methods for sample cleanup were found in the literature. Two were trialled as possible extraction methods one using dye affinity chromatography and the other using lectin affinity chromatography.

1. Dye affinity chromatography with agarose-immobilised Cibacron Blue F3GA uses a mixed-mode medium which can be manipulated to retain various serum proteins, and which is particularly useful for removing albumin which is a very abundant protein in serum, and hence (relatively) in urine.

    - Reuseable, prepacked cartridges containing agarose-immobilised Cibacron Blue F3GA were used for intial studies. Samples of 20 mL urine, spiked with

recombinant EPO (rhEPO) were loaded to the cartridge. The majority of protein was eluted using an increasing salt gradient, then the EPO-containing fraction was removed with 0.5M NaSCN in a small volume. This was concentrated to final volume in a suitable buffer with a small 30,000 MWCO ultrafilter. This procedure removed 94±6% of protein from the sample while concentrating 45±17% of the EPO in the retained fraction.

- Samples spiked with rhEPO and samples containing only native EPO were successfully prepared, separated by isoelectric focussing (IEF) and visualised using Western blotting.

2. Wheat germ agglutinin (WGA) is a lectin with an affinity for N-acetylglucosamine (GlcNAc), with preferential binding to dimers and trimers of this sugar. The lectin therefore binds to the carbohydrate moiety of EPO, and can be displaced using free GlcNAc. Agarose-immobilised WGA was mixed with 20mL buffered urine for 16 hours at 4ºC. The suspended gel was buffer-washed, and the EPO eluted with a solution of 0.1M GlcNAc. The eluate was concentrated and washed with a small 30,000 MWCO ultrafilter, as for the dye-binding procedure. This procedure removed >99% of the protein in the urine sample, while recovering 39±15% of the added EPO. Again, the procedure produced extracts which were successfully separated and visualised using the EPO Western blotting method.

EPO cannot be recovered from serum by ultrafiltration due to the high overall serum protein concentration. Serum was spiked with rhEPO and subjected in sequence to dye-binding affinity chromatography, then WGA affinity preparation. Most of the protein (>99%) was successfully removed. Sufficient of the added EPO (60.7±16%) was recovered to allow visualisation by IEF and Western blotting.

Both methods show considerable promise as preparation methods for urinary and serum EPO for isoelectric focussing and Western blotting. The methods require refinement, as evidenced by the variable recovery.

Owing both to delays in provision of funds to Proteome Systems and to changes in drug testing requirements, the project is not to schedule. The objectives that have been achieved are:

1	First IPG gel tested	January 2002
2	Correct pH range gel chosen	May 2002
5	Paper presented at Cologne workshop	March 2002

In addition the aim of having commercial IPG gels available for the detection of recombinant EPO has been achieved however the new requirement to resolve Aranesp as well as EPO has prevented their routine use.

The development of alternative methods of sample pre-treatment prior to analysis by gel electrophoresis is showing considerable promise and may lead to a simplified

method for urinary EPO analysis and enable the electrophoretic method to be extended to serum.

P2002-04: Extension of Statistical Profiling

Several of the substances banned by the IOC are produced as endogenous compounds in the human body. This requires parameters to be established whereby it is possible to distinguish between administered drug and natural production. This requires measurements from many samples collected over a period in order to determine population statistics and monitor normal distributions amongst various population groups. With the addition of endogenous compounds such as EPO to the range of compounds that can be detected it is now necessary to have more information relating to the natural levels found in urine and blood where available. With time this will also enable the monitoring of individual athletes over time.

Summary of Project Purpose and Aims:

To extend the database of endogenous substances to enable constant monitoring of the normal distributions amongst various population groups. With the addition of endogenous compounds such as EPO to the range of compounds that can be detected it is now necessary to have more information relating to the natural levels found in urine and blood, where available.

The project plan developed for year one is set out below:

#	*Objective*	*Target*
1	Measurement of EPO levels in routine ASDA samples started	September 2001
2	Commencement of routine running of one gel per week to establish EPO basicity variation	October 2001
3	Athlete data entered into database	August 2002
4	Preliminary report for Cologne workshop	March 2002
5	Paper prepared for publication	August 2002
6	Completion of 2000 EPO in urine measurements	August 2002

Report on Progress

Prior to the commencement of this project there was a major review of the source of the samples to be used in the study. The original plan called for the measurement of EPO levels in routine ASDA urine samples. However there were several problems with this approach.

- Without a corresponding blood sample it would not be possible to correlate blood and urine EPO levels.
- ASDA sample group is by definition a suspect group.
- The Australian athlete population is not sufficiently ethnically diverse.

- It is difficult with ASDA samples to find out when the same subject has been retested.

All these problems have been overcome by analysing the urine samples collected for the EPO2000 study. However due to the very cost of carrying the EPO testing the budget for this particular project has been significantly exceeded. However the data generated is of considerably more value than that from the original project.

Analysis of Serum and Urinary EPO, and Urinary Isoform Distribution.

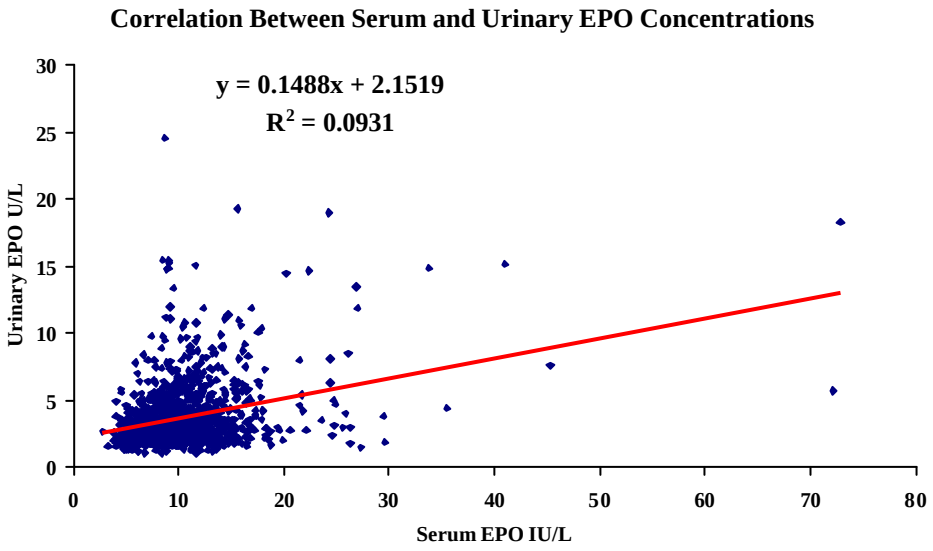
Sera were analysed for EPO concentration by immunoassay on the DPC Immulite analyser, using proprietary reagents. Similarly, urinary EPO concentrations were estimated using the same assay, after the sample had been buffer-washed over a 30,000 MWCO ultrafiltration membrane to remove low MW interferences.

The urinary isoform pattern was estimated in those samples where sufficient EPO was deemed to be present (a urinary EPO concentration above about 4 U/L). The samples were prepared by concentration over an ultrafiltration membrane with a 30,000 MWCO, and analysed by isoelectric focussing followed by Western blotting according to the standard method in use in the laboratory.

Initial Analyses.

Serum EPO data on these samples, which were collected as the samples arrived at ASDTL, have been extensively analysed for publication in support of the multivariate models developed to infer the misuse of recombinant EPO (rhEPO). A total of 1168 urine samples were analysed for total urinary EPO concentration. These data confirm that there is no substantial correlation between the concentration of EPO in simulataneously collected serum and urine samples (Figure 1). The slope of the regression like is significantly greater than zero, but the correlation is very low and so for any individual sample pair the relation between the two matrices is very loose.

Figure 1: Correlation between EPO levels in serum and simultaneously collected urine samples.



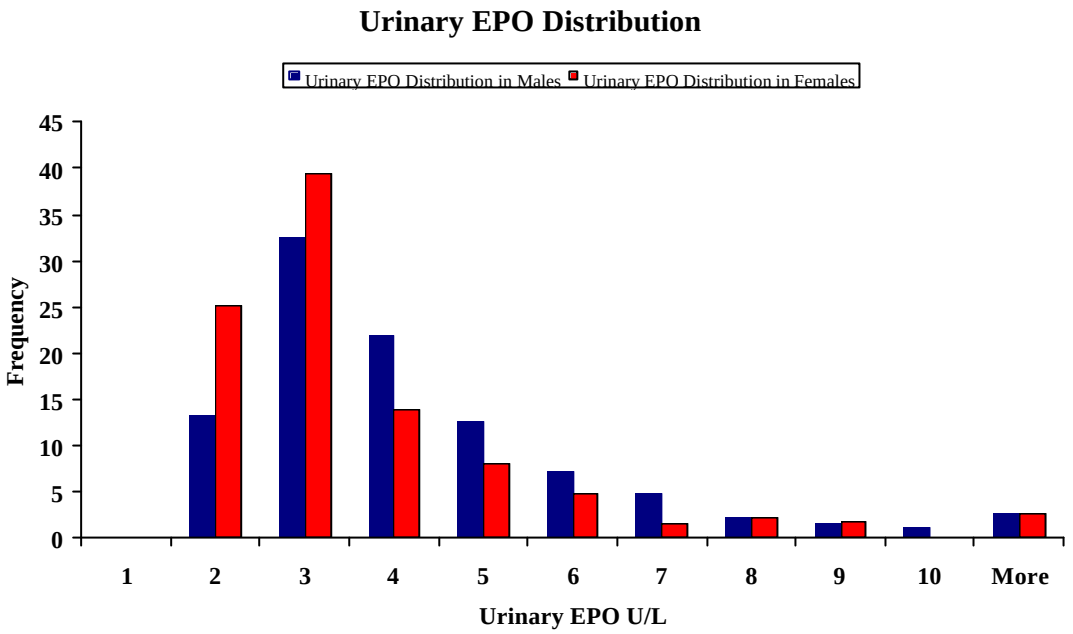
The basic descriptive statistics for EPO levels in urine and serum in males and females are shown below. As has been noted before, serum EPO in females is slightly

higher than in males. However, urinary EPO is slightly lower in females than in males. When the detailed distribution (Figure 2) is examined it can be seen that 78.8% of females, but only 67.7% of males, have a urinary EPO of less than 4 U/L. As the analysis of urinary isoform patterns relies on the presence of a sufficient amount of EPO, typically more than 4 U/L, to produce a satisfactory isoform analysis, this finding suggests that it will be slightly more difficult to obtain satisfactory results from female athletes. This may lead to them being slightly less liable to full testing in normal operations.

Female Statistics			
Serum EPO U/L		*Urinary EPO U/L*	
Mean	11.71	Mean	3.34
Standard Deviation	6.86	Standard Deviation	2.34
Mean + 2SD	25.43	Mean + 2SD	8.02
Kurtosis	34.87	Kurtosis	14.92
Skewness	4.71	Skewness	3.38
Median	10.35	Median	2.60
Minimum	3.80	Minimum	1.20
Maximum	72.90	Maximum	19.00
Count	372	Count	372
Male Statistics			
Serum EPO U/L		*Urinary EPO U/L*	
Mean	9.90	Mean	3.89
Standard Deviation	3.49	Standard Deviation	2.39
Mean + 2SD	16.87	Mean + 2SD	8.67
Kurtosis	4.09	Kurtosis	13.27
Skewness	1.36	Skewness	2.85
Median	9.40	Median	3.20
Minimum	2.80	Minimum	1.10
Maximum	29.50	Maximum	24.60
Count	795	Count	795

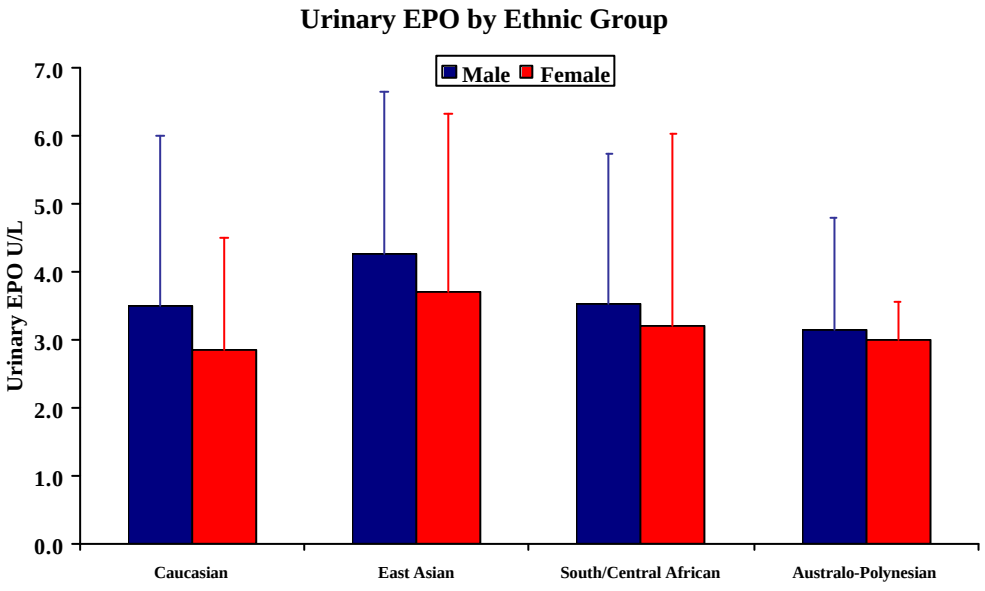
The overall distribution of the urinary data is non-normal and is heavily positively skewed in both sexes.

Figure 2 - Normalised distribution of urinary EPO concentrations in male and female elite athletes.



Samples were divided into groups by ethnic origin, as shown in Figure 3 below. The groups for each sex were analysed by one-way ANOVA. The results should be treated with some caution, as the data violate some assumptions for this analysis, but it appears that both male and female athletes of East Asian origin returned significantly higher urinary EPO concentrations than the other groups. The actual difference is quite small, and does not bear on the final result of the EPO test, rather on the selection of samples for further study as already noted.

Figure 3: Urinary EPO concentrations in male and female athletes



Urinary EPO Test Results - Percentage Basic Isoforms

Urine samples were selected by screening for EPO content, with samples with urinary EPO >4 U/L progressing to the Western blotting procedure.

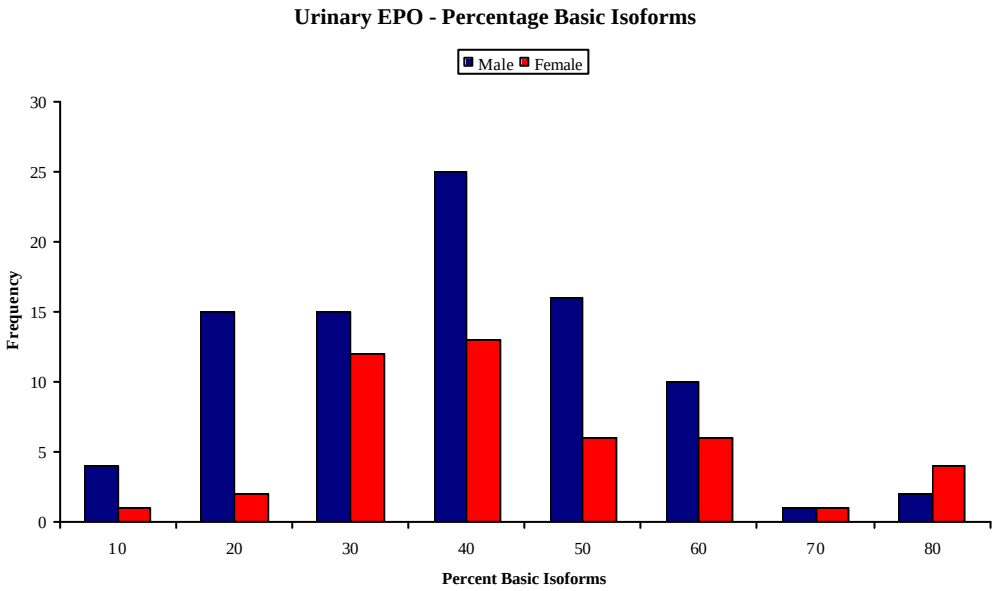
After the Western blot image is assessed under a number of qualitative criteria to ensure the image is of good quality, analysis of urinary EPO isoform structure by IEF and Western blotting yields a numeric result, referred to as the percentage of basic isoforms. This is obtained by reference to a standard recombinant EPO preparation.

It was not previously thought that there is a relationship between the EPO content and the percentage of basic isoforms, and this is confirmed here by regression analysis of the data.

There is also no difference in percentage basic isoforms between males and females, by Student's t test.

Urinary EPO, Percentage Basic Isoforms	Male	Female
Mean	35.1	39.6
SD	15.4	16.9
Count	88	45
Median	35.5	37.0
Minimum	6.0	6.0
Maximum	79.0	77.0
5th Percentile	11.35	19.8
95th Percentile	59.65	73.6

Figure 4: Distribution of percentage basic EPO isoforms in males and females.

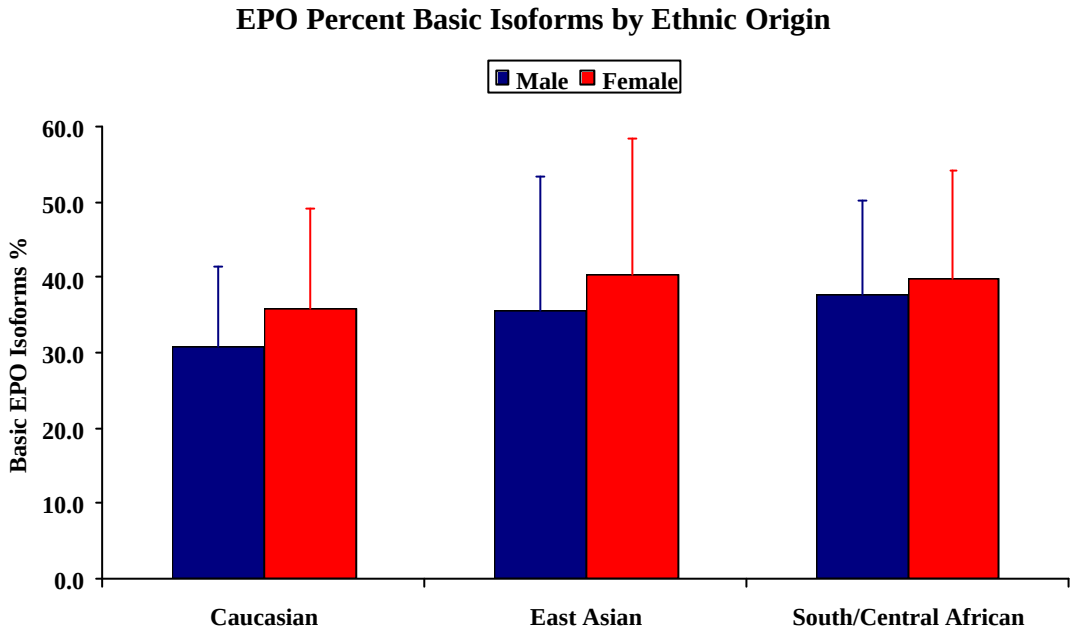


The distribution of the percentage of basic isoforms more closely approaches a normal distribution than other data. A number of samples cluster between 70 and 80%, but none exceed 80%.

The Effect of Ethnic Origin on Percentage Basic Isoforms.

The percentage of basic isoforms of EPO in urine is not significantly affected by the ethnic origin of the subjects. This can be seen from Figure 5, especially in the large standard deviations of the data. Australo-Pacific subjects were present in small numbers in this analysis and are not shown.

Figure 5: EPO percentage of basic isoforms - effect of ethnic origin.



Conclusions.

The analysis of EPO in urine proceeds in two stages. First the amount of EPO in the sample must be determined in order to decide whether sufficient is present to obtain a useful result from the Western blotting procedure. Serum EPO analysis is no guide to this, as it does not correlate with urinary EPO concentration. In those samples where sufficient EPO is present (about 20% of females and 30% of males), the full isoform analysis can be run.

No samples analysed by this procedure had isoform distributions with >80% basic isoforms by the definition of the procedure. A number were relatively high by this critierion, and the overall data set will be useful to assess other criteria for positivity which have been proposed.

In addition to the samples collected during the EPO2000 study a subsidiary project has been collecting samples from volunteers who have been subjected to varying degrees of exercise stress. This project will not only provide data on whether exercise stress has any effect on the distribution of urinary EPO isoforms but will also provide information as to whether the distribution of isoforms in an individual is stable over time. Both pieces of information are necessary to support the urinary EPO test as a

stand-alone proof of doping. Over 400 urine samples have been collected and analysed and the data interpretation has begun. A full report on the results is not expected before December 2002. When both aspects of the project are completed a paper will be prepared for peer reviewed publication. At present there is no published data on the effect of ethnicity and exercise from a large population of athletes.

P2002-07: Analysis of Sports Supplements

The increase in positive drug samples due to the presence of metabolites of nandrolone is largely due to the presence of compounds such as norandrostenedione or norandrostenediol in commercially available dietary supplements. The compounds norandrostenedione and norandrostenediol, whilst banned in sport by the IOC, are freely available as health food products in the USA and there have been several cases of dietary supplements being contaminated with these compounds. If such a supplement is taken it is likely that the athlete consuming it will test positive to the metabolites of nandrolone, as the metabolites of norandrostenedione and norandrostenediol are the same as those of nandrolone.

Summary of Project Purpose and Aims:

To determine the extent to which dietary supplements freely available in Australia are likely to cause positive drug tests in athletes. This will be a survey and is not intended to provide guarantees for any product on the market but to provide a general overview of the current situation as it stands in Australia. Various products (with input from ASDA, AIS and a general random selection) will be purchased and checked for contamination by IOC banned steroids. The results of the analytical work will be published as a general survey of products obtained in Australia.

The project plan developed for year one is set out below:

#	*Objective*	*Target*
1	Method developed and validated for the detection of contaminants in dietary supplements	November 2001
2	Samples purchased for survey	November 2001
3	Analysis completed for 30 supplements	February 2002
4	Preliminary report to ASDA and ASC	February 2002
5	Preliminary results presented at Cologne Workshop	March 2002
6	Further 100 analyses performed and paper presented for publication	August 2002

Report on Progress

The methodology used in the analysis of the nutritional suplements was adapted from the procedure of Geyer, H., et.al.(Institute of Biochenistry, German Sports University, Cologne, Germany). The method for the analysis of prohormones has been validated in our laboratory and applied to the analysis of 43 dietary supplement products purchased in the Australian market. The products were screened for 19-nor-4-androstene-3ß,17ß-diol, 19-nor-5-androstene-3ß,17ß-diol, DHEA, 4-androstene-3ß,17ß-diol, 19-nor-4-androstene-3,17-dione, 5-androstene-3ß,17ß-diol, 19-nortestosterone, 4-androstene-3,17-dione and Testosterone.

The supplements tested are listed in Table 1 below. Of these supplements two have been found to contain small amounts of the prohormones DHEA, 4-androstene-3,17-dione and testosterone. Both were HMB products. Whilst the levels detected were low and unlikely to lead to a positive urine drug test the problem with contamination found in overseas studies is confirmed.

Owing to the late commitment of funds to this project it has not been possible to complete the analysis of the number of supplements specified and expenditure has been correspondingly lower. The objectives achieved are:

1	Method developed and validated for the detection of contaminants in dietary supplements	April 2002
2	Samples purchased for survey	April 2002
3	Analysis completed for 30 supplements	May 2002
6	Further 13 analyses performed	June 2002

The project will be continued so that more supplements which are readily available on the Australian market can be tested. Of particular importance will be any detection of the 19-nor prohormones, whose presence at even trace levels can lead to positive doping tests. Excretion studies are also in progress in an attempt to detect the contaminants in the urines of volunteers who ingest the dietary supplements. Nine of these studies have been completed.

Table 1: P2002-07 Analysis of Sports Supplements.

Manufacturer	Product	Type
Aminoactive Australia	Max's HMB	Beta-hydroxy-beta-methylbutyrate
	Max's Muscle Builder	Branched chain amino acids
	Max's Bio-Engineered Wpi	Whey protein isolate
	Max's Bio-Engineered Rapid Weight Gain	Whey protein isolate
	Max's BCAAS	Branched chain amino acids
	Max's L-Glutamine	L-glutamine
Aussie Bodies Pty Ltd (Aust)	Wp1 Whey Protein Supplement	Whey protein isolate
	Ugf Deluxe	Whey protein isolate
	Colostrum Muscle Gainer	Whey protein concentrate
	Colostriod	Growth factors igf-1, igf-2
	Micronised Creatine Monohydrate	Creatine monohydrate
	Perfect Protein	Whey protein supplement
Body Ripped Sports Nutrition Pty. Ltd. (Aust)	Body Ripped	Anti – catabolic mass muscle low fat formula whey peptide fortified
Experimental and Applied Sciences (EAS) USA.	Phosphagen Hp	Creatine
	Hp Betalean	Herbal extracts
	Neurogain	Amino acids & vitamins
	Phosphagain 2	Amino acids & vitamins
	Hmb	Beta-hydroxy-beta-methylbutyrate mnohydrate
	Betagen Orange	HMB & creatine
	Myoplex Lite	Whey Protein isolatesoy Protein Isolate

Manufacturer	Product	Type
Horleys Health (NZ)	Horleys The Power To Perform	Whey protein
Musashi Pty Ltd (Aust).	L – Ornithine	Amino acids
	Lysine Hudrochloride – Usp	Amino acids
	Mega Tribulus Terrestris	Herbal extract
	Muscle Bcaa's	Amino acids
	HMB 500mg	Hmb
	100 % Pure Creatine Monohydrate	Creatine monohydrate
	Growling Dog Creatine	Creatine
	Growling Dog Creatine	Carbohydrate transport blend micronised creatine
	The Growling Dog Energy Mix	Atp/creatine/inosine
	Huan – The Dispersion	Amino acids
	Kuan – The Creative	Amino acids
Horleys Health (NZ)	Horleys The Power To Perform	Whey protein
Muscle Tech R&D, Inc. (USA).	Advanced Thermogenic Formula Hydroxycut	Hydroxagen Chromium picolinate l – carnitine
Next Proteins International. (USA)	Designer Whey	Protein Supplement
Power Foods International –(Aust	Trim Fat Shield	Trim fat shield fibre-tr3
Power Foods International Pty Ltd	Vital Strength	L-carnitine
Prolab Nutrition (USA).	Pro Lab Nutrition Pure Whey	Whey protein
Sport AST Science (USA)	Ny – Tro Pro – 40	Whey protein

Manufacturer	**Product**	**Type**
	Vp 2	Whey protein isolate
Synergistic Sports Science	Gh Stack	L-arginine-2-pyrrolidone-5-carbohydrate L-lysine hydrochloride Niacin
	Power Stack	Creatine

P2002-09: Carbon Isotope Ratio Mass Spectrometry Inter-laboratory Study

Detection of endogenous steroid doping using carbon isotope mass spectrometry is a recent addition to the range of tests used in the IOC accredited laboratories. Despite having been used at the Nagano Winter Games (1998) and the Sydney 2000 Summer Games, the test is still somewhat experimental, with most of the laboratories that employ the technique working independently of each other to develop required criteria. In order to avoid the possibility of conflicting results issued from different laboratories, a more cohesive approach to carbon isotope testing is required.

Summary of Project Purpose and Aims:

This is an ongoing project started in January 2001 with the ultimate goal of achieving agreement among IOC-accredited laboratories on a CIR method for the detection of administered endogenous substances, and with IOC/WADA on the use of CIR.

The project plan developed for year one is set out below:

#	*Objective*	*Target*
1	Analysis of Stage 1 samples completed	August 2001
2	All Stage 1 results received from participating laboratories	September 2001
3	Interim report on data sent to participating laboratories	December 2001
4	Final results of Stage 1 presented and discussed at Cologne anti-doping workshop	March 2002
5	Design of Stage 2 of study based on results and discussions with Stage 1 participants	May 2002
6	Preparation of standard materials for Stage 2 started	June 2002

Report on Progress

Fifteen sets of samples for Stage 1 of the study were distributed at the Cologne Doping Workshop in March 2001. As some laboratories use keto steroids and others diols for their CIR measurements the sample set included both as well as hydrocarbon standards for checking instrument calibration. The sample set comprised two hydrocarbon standards, two mixed keto steroid standards, two diol standards, and three urine samples. Results were received from five laboratories two of which used Finnigan Delta Plus instruments and three of which used Micromass Isoprime instruments.

The results from the hydrocarbon analyses showed that one laboratory was an outlier with delta values significantly more negative than expected. Results from the analysis

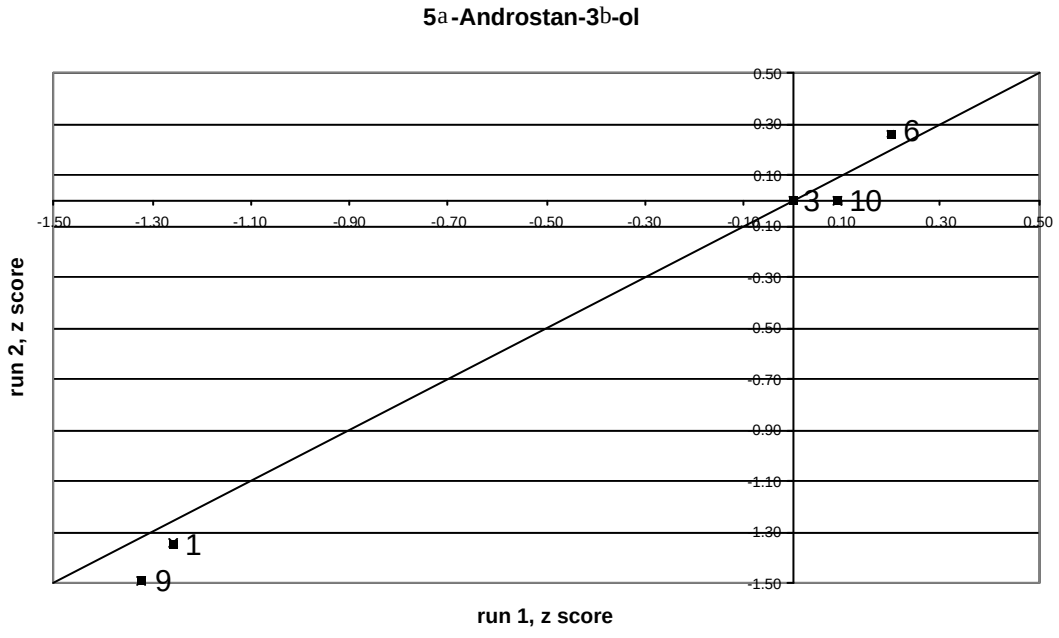
of the steroid standards showed that there was a significant and repeatable difference in the delta values obtained from the laboratories using Finnigan instruments compared to those using Micromass instruments. The results for the analysis of 5a-androstan-3b-ol are presented below as an example. The Youden diagram shows the results falling into two groups, laboratories 1 and 9, the Finnigan users in one group and the other group consisting of Micromass users. This observation was particularly evident in the between laboratory variation, suggesting that at least in the case of 5a-androstanol measurements, an instrument bias exists. The within laboratory variation was not related to instrument type.

5a-Androstanol Results and Summary Statistics

lab number	data		robust z score		between lab z-score	within lab z score
	run 1	run 2	run 1	run 2		
1	-36.94	-36.86	-1.26	-1.35	-1.30	0.00
3	-33.45	-33.37	0.00	0.00	0.00	0.00
6	-32.90	-32.70	0.20	0.26	0.23	0.81
9	-37.12	-37.24	-1.32	-1.49	-1.41	-1.35
10	-33.20	-33.38	0.09	0.00	0.04	-1.75

number of results	5.00	5.00
median	-33.45	-33.38
normIQR	2.77	2.59
robust CV (%)	-8.29	-7.75
minimum	-37.12	-37.24
maximum	-32.90	-32.70
range	4.22	4.54

Youden Diagram of Robust Z-Scores from 5a-Androstan-3ß-ol Results



It is probable that the instrument bias is due to the different methods that are used by each manufacturer to calibrate the reference gas. It is interesting to note that the Micromass instruments give values that are closer to those obtained from off line combustion analysis and hence are apparently more accurate.. It might be expected that this instrument bias would be reflected in the measuring the urine samples provided but this was not the case. The reason is that in interpreting CIR data from samples comparative measurements are always used. Values are compared to the laboratory's reference range or to endogenous reference compounds found in the same urine. The results reported for the urine samples were in agreement and did not show any instrument bias. However there is a clear need to confirm that Micromass and Finnigan instruments do give different numerical values for the delta values and establish the exact cause for this difference. The existence of different numbers for the same sample is likely to cause confusion in tribunals even though the conclusions reached as to the synthetic or natural origin of the steroid are the same.

Owing to the fact that only five laboratories completed the study it has been decided not to proceed with Stage 2 of the project at this stage. Since this project was begun the World Association of Anti-Doping Scientists (WAADS) has been formed. One of the main aims of WAADS is to carry out inter-laboratory studies to improve the quality and comparability of analyses carried out in IOC laboratories. Owing to the very interesting results obtained from Stage 1 it is proposed to carry another study similar to this with the samples circulated by WAADS. This should ensure a much higher participation rate, confirm the instrument bias, and enable Stage 2 to be carried out later with more meaningful results.

Stage 1 of this project has been completed on schedule. The objectives achieved are:

- Analysis of Stage 1 samples completed — September 2001
- All Stage 1 results received from participating laboratories — October 2001
- Interim report on data sent to participating laboratories — January 2002
- Final results of Stage 1 presented to participants at Cologne anti-doping workshop — March 2002

P2000-010: Carbon Isotope Ratio Mass Spectrometry Profiling Study

Detection of endogenous steroid doping using carbon isotope mass spectrometry is a reasonably recent addition to the range of tests used in the IOC accredited laboratories.. Because the test relies on detecting differences in the isotope ratio of steroids present in the body it is necessary to know the normal values found in a large population of elite athletes living in different countries. The reason for this is that isotope ratio is known to vary with diet and subjects of different ethnic origin may have adopted the diet of the country in which they reside.

Summary of Project Purpose and Aims:

This is an ongoing project started in January 2000 with the aim of measuring the isotope ratio of the natural steroids found in the urines of over 1000 elite athletes collected during the EPO2000 study. Its purpose is to produce additional data to support the use of CIR/MS in detecting doping with endogenous steroids.

The project plan developed for year one is set out below:

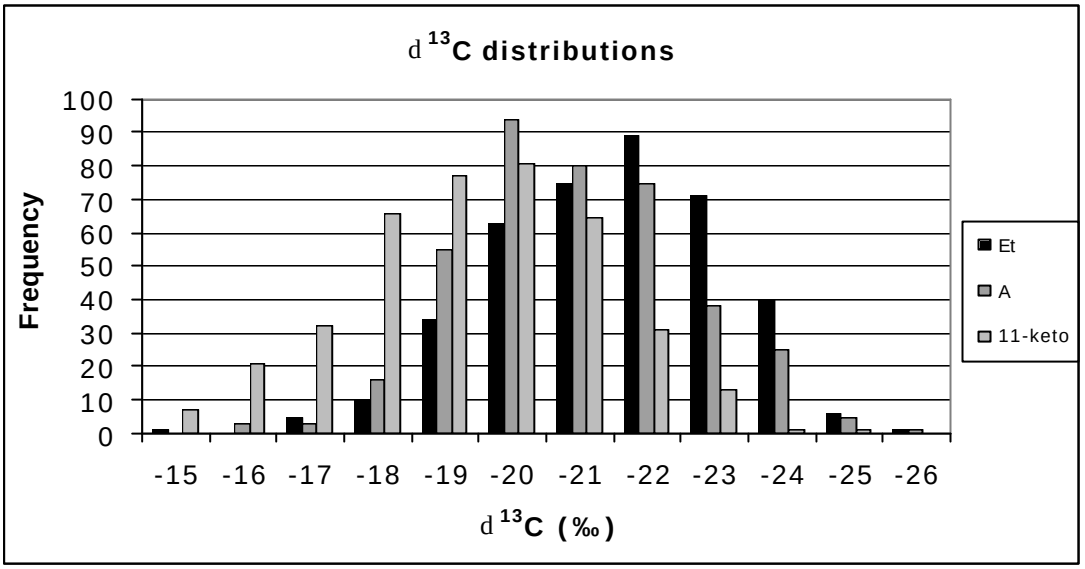
#	*Objective*	*Target*
1	Analysis of 300 samples completed	December 2001
2	Analysis of 600 samples completed	March 2002
3	Analysis of 900 samples completed	June 2002
4	Preliminary results presented at Cologne anti-doping workshop	March 2002
5	Results evaluation completed and paper prepared for publication in peer-reviewed journal	August 2002

Report on Progress

A simplified method to determine the Carbon Isotope Ratio (CIR, $d^{13}C$) of Etiocholanolone (Et), Androsterone (A) and 11-ketoetiocholanolone (11-keto) has been developed at the Australian Sports Drug Testing Laboratory (ASDTL) by Rogerson, Trout and Kazlauskas.. Et and A are inactive isomers that are the end products of the androgen biosynthetic pathway. As such they are usually the most concentrated of the urinary steroids. 11-keto is produced in a separate biosynthetic pathway to that of the androgens such that its CIR can be measured to account for the diet of the individual athlete.
There were 900 urine samples extracted for GC-C-IRMS analysis at June 30th. Of these samples, 395 have been analysed and the data included in this report. There were samples from four (4) countries: China, Kenya, France and Singapore that contributed to this data set. Importantly, no sample in the data set had $d^{13}C$ Et, $d^{13}C$ A

or $d^{13}C$ 11-keto values less than or equal to -27 ‰ (ie. the value at which a metabolite is considered to be a product of endogenous steroid abuse). The minimum $d^{13}C$ values for Et, A and 11-keto were -26 ‰, -26 ‰ and -25 ‰ respectively. The variation in $d^{13}C$ values for Et and A were similar at 7.7 % and 7.8 % respectively. The frequency distribution of $d^{13}C$ Et, $d^{13}C$ A and $d^{13}C$ 11-keto in the data set is shown in Figure 2.

Figure 2: Frequency distribution of $d^{13}C$ values for Et, A and 11-keto obtained from 395 samples



Analysis of the whole data set revealed that $d^{13}C$ Et had an average value of -22 ‰ with a skewed distribution, while $d^{13}C$ A had an average value of -21 ‰ with a more normal distribution. A noticeable difference was observed in $d^{13}C$ values of Et and A less than -22 ‰ (^{13}C depleted, more negative) arising from 52 % and 36 % of samples respectively. This difference is believed to result in the apparent skewed distribution of $d^{13}C$ Et relative to that of $d^{13}C$ A. The other $d^{13}C$ interval frequencies were shown to be similar (Table 1).

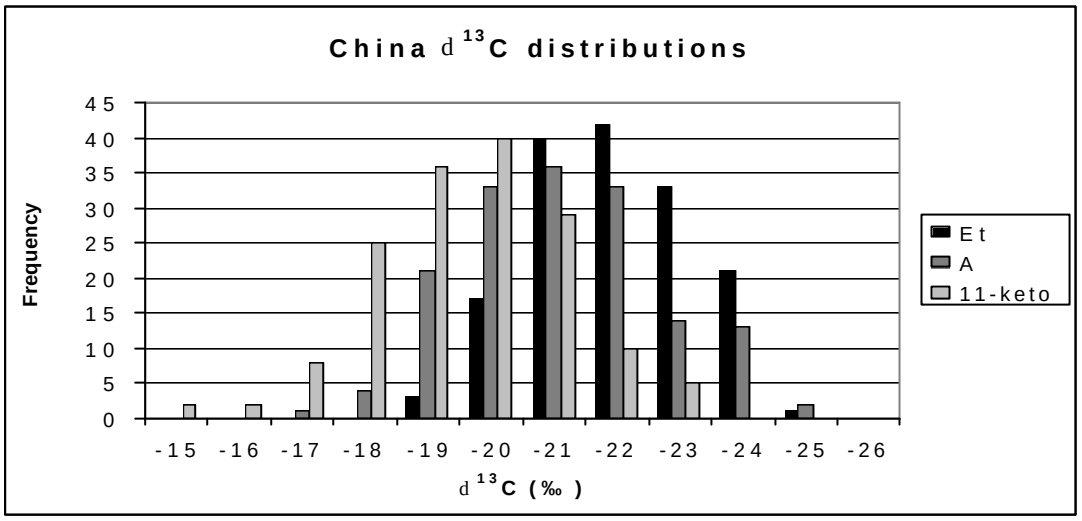
The frequency distribution of $d^{13}C$ 11-keto in Figure 2 shows a normalised pattern around the average value of -20 ‰. In general, $d^{13}C$ 11-keto values were observed to be ^{13}C enriched (less negative) in relation to $d^{13}C$ Et and $d^{13}C$ A values, with 32 % being greater (less negative) than -19 ‰.

Table 1: $d^{13}C$ interval frequencies (as percentage of total 395 samples) for Et, A and 11-keto

	Et (% of total)	A (% of total)	11-keto (% of total)
-19 ‰ to –25 ‰	94	93	68
-20 ‰ to –24 ‰	75	73	48
-21 ‰ to –23 ‰	42	39	24
< -25 ‰	2	2	0.3
> -19 ‰	4	6	32
< -22 ‰	52	36	12

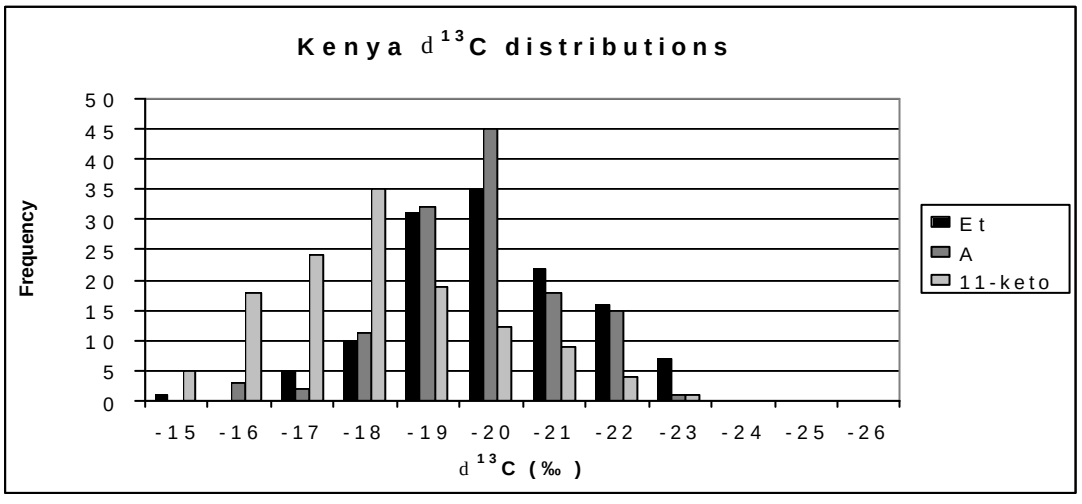
Analysis of the data set per country of origin also revealed some interesting trends. Of the 395 samples contained in the complete data set, only 392 were analysed per country of origin due to inconsistencies in the information supplied by three (3) of the subjects. Figure 3 shows that the China $d^{13}C$ distributions obtained from 157 samples reflect those of the whole data set in Figure 2. Accounting for the fact that this subset represents 40 % of the samples in the total data set, the relative frequencies of the $d^{13}C$ values of Et, A and 11-keto were observed to be similar for each of the delta intervals.

Figure 3: China $d^{13}C$ distributions of Et, A and 11-keto obtained from 157 samples



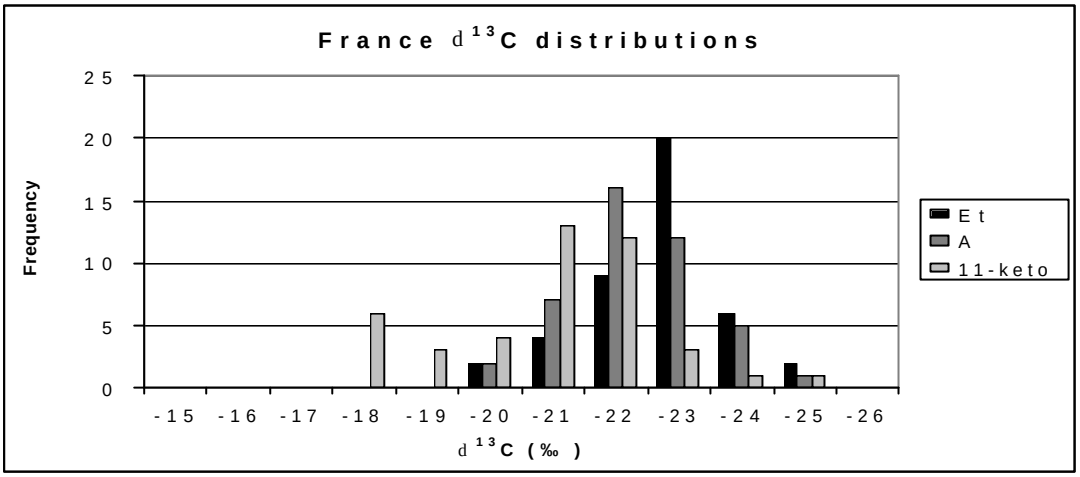
In contrast, a marked difference was observed in the Kenya $d^{13}C$ distributions shown in Figure 4. The 127 samples contained in this subset had $d^{13}C$ values that were generally ^{13}C enriched with respect to those from China. The average $d^{13}C$ of Et, A and 11-keto were -21 ‰, -20 ‰ and -19 ‰ respectively.

Figure 4: Kenya $d^{13}C$ distributions of Et, A and 11-keto (127 samples)



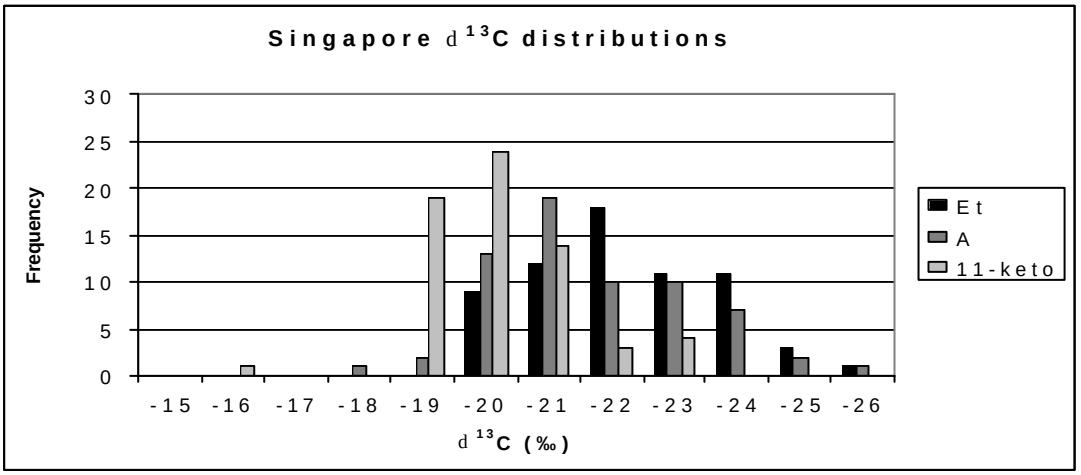
The France $d^{13}C$ distributions obtained from 43 samples are displayed in Figure 5. In this figure a smaller $d^{13}C$ range for Et, A and 11-keto was observed. A larger subset is required to verify if this smaller $d^{13}C$ range is indeed characteristic for France or simply the result of a smaller subset. The average $d^{13}C$ values of Et, A and 11-keto were -23 ‰, -23 ‰ and -21 ‰ respectively, resulting in their distributions being ^{13}C depleted (more negative) in relation to those for China.

Figure 5: France $d^{13}C$ distributions of Et, A and 11-keto obtained from 43 samples



The Singapore $d^{13}C$ distributions obtained from 65 samples shown by Figure 6 are also ^{13}C depleted with respect to the Et and 11-keto of China judging by the average $d^{13}C$ Et (-23 ‰) and $d^{13}C$ 11-keto (-21 ‰). This is interesting since most of the subjects came from the same ethnic group. Like the France $d^{13}C$ distributions, those from Singapore have a relatively small $d^{13}C$ range but this may also be due to the small data subset.

Figure 6: Singapore $d^{13}C$ distributions of Et, A and 11-keto obtained from 65 samples



The data set obtained from GC-C-IRMS analysis of urine samples in the BASA research program provides ASDTL with preliminary CIR reference ranges of Et, A and 11-keto. This is the first stage in establishing CIR reference ranges for these urinary steroid metabolites such that doping control laboratories may gain a more complete understanding of what defines a natural (or completely endogenous) $d^{13}C$ value. This information can be used in more accurate GC-C-IRMS analysis of athlete urine samples containing metabolites that are derived from administration of endogenous steroids. As expected, no sample in this data set had a $d^{13}C$ Et, $d^{13}C$ A or $d^{13}C$ 11-keto value less than (lighter/more ^{13}C depleted) -27 ‰. This result may allow the use of this absolute CIR as a criterion for reporting administration of endogenous steroids. For this to occur would rely on harmonisation of CIR values from different laboratories which is one of the aims of the other CIRMS project P2002-09. The results from different countries illustrate the diverse metabolism and diet of individuals and their effects on measured steroid isotope ratios.

Owing to the late commitment of funds to this project and instrumental breakdowns it has not been possible to complete all the analyses. All 900 samples have been extracted and prepared but only 400 have been analysed. The objectives achieved are:

1	Analysis of 300 samples completed	May 2002
2	Analysis of 400 samples completed	June 2002
3	Preparation of 900 samples completed	June 2002

It is anticipated that the project will continue so that a more statistically significant number of samples from additional countries can be analysed for publication to support the use of CIRMS in doping analysis.