Detection of EPO injections using a rapid lateral flow isoform test

Lönnberg, M ; Lundby, C

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Detection of EPO injections~~doping~~ using a rapid lateral flow isoform test

M. Lönnberg[a], C. Lundby[b]

[a] Dept. of Chemistry - Biomedical Center, Uppsala University, Uppsala, Sweden

[b] Center for Integrative Human Physiology (ZIHP), Institute of Physiology, University of Zurich, Zürich, Switzerland

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*Corresponding author:

Maria Lönnberg, Dept. of Chemistry - Biomedical Center, Box 599, Uppsala University, SE-751 24 Uppsala, Sweden.

+46 706630481, maria.lonnberg@kemi.uu.se

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Abstract

Misuse of recombinant human erythropoietin (rhEPO) is a major concernproblem in competitive sports and the implementation development of tests allowing for higher detection rates than what current test are capable of is required. In this study a novel lateral flow EPO isoform test kit, EPO WGA MAIIA, is evaluated on plasma and urine samples obtained from eight healthy malesen in connection with a 28-day rhEPO injection period. During the first 14 days rhEPO was injected every second day during the first 14 days of the study and the method proved to be very effective (xx out of xx) in detecting thisdoping infor the concomitantly obtained samples collected prior to injection. Samples obtained 14 and 21 days after the last injection showed no detectable rhEPO. Seven days after the last injection three positive (>99.99% CL) subjects were found with the test kit. When using 99% CL as cut-off limit, six of the eight subjects (75%) were suspectedfound to be suspicious for doping. Samples obtained 14 and 21 days after the last injection showed no detectable trace of rhEPO. A previous study using indirect methods to determinefor detecting EPO doping on the same samples indicated only that two of the subjects hadwere suspicious valuespositive during 7-21 days after the last injection.

We proposesuggest implementing the easy to-use EPO WGA MAIIA rapid lateral flow test as an initial screening procedure in anti-doping work to 1) increase the detection rate of potential rhEPO doping athletes and 2) allow for analysis of a ten to twenty folds higher analytical rate than what is possible todaymore samples than what is possible today.

Keywords: Epoetin beta; erythropoietin; IEF; WGA affinity chromatography.

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Introduction

Recombinant proteins and peptide hormones such as recombinant human erythropoietin (rhEPO) seem to be widely abused in sports to enhance performance [1]. Unfortunately the detection of doping with recombinant proteins is difficult [2] since they only possess minor structural differences as compared to their endogenous forms. Other challenges include that only small concentration of, for example, injected rhEPO is required in order to increase exercise performance [3] and the clearance rate from the body is very high [4]. Endogenous and recombinant EPOs are glycoproteins that can be distinguished by their posttranslational glycosylation pattern, which is specific for the synthesizing cell. The basic approach to analytically distinguish between different EPOs is to combine electrophoretic or chromatographic separations of EPO isoforms with sensitive anti-EPO antibody-based detection methods. Endogenous and recombinant EPO glycosylation can be distinguished by charge [5,6], isoelectric point [7], molecular mass [8,9] or by interaction with specific lectins [10,11] and the methods are described in a recent summary [12]. The methods differ in how well they distinguish certain types of glycosylation (the EPO isoform resolution), and by the minimum required amount of EPO for the analysis (the EPO detection sensitivity). The methods often require an additional purification and/or concentration step before urine and plasma can be analysed [13,14]. The isoform methods rely on that large numbers of samples from a reference population have been tested to select the most suitable cut-off limits for achieving highest possible specificity and sensitivity for detecting a potential doping misuse with rhEPO. With the ~~One of the~~ recently ~~presented~~ developed EPO WGA MAIIA test kit [11,15,16]~~EPO isoform methods show~~ quantitative values for EPO isoforms are obtained~~, the EPO WGA MAIIA test kit [11,15,16],~~ by using percentage of migrated isoforms (PMI) as unit. Quantification allows the use of confidence limits (CL) calculated from the results for the reference population, and makes between method comparison ~~possible~~possible. imprecision studies and comparison with other methods.

~~Hence, t~~The detection of potential rhEPO doping by athletes is hence very challenging [17,18,19,20,21,22] and with the present study we hope to increase the efficacy hereof. The newly developed EPO WGA MAIIA method is suggested to be much more sensitive than the IEF method currently applied~~used~~ by the World Anti Doping Agency (WADA) to fight EPO doping ~~laboratories today~~. In samples collected from a major sport event the method

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determined 6% to be positive whereas the IEF method did not flag one sample to be indicative of EPO doping [11].

This study evaluates the efficiency to detect potential rhEPO abuse~~abuse~~ in plasma and urine samples obtained from volunteers injected with rhEPO. Based on the ~~potential~~hypothesized higher sensitivity, we expected~~hypothesize~~ that the MAIIA method would prove~~s~~ superior as compared to other methods in their ability to determine EPO doping.

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Methods

Samples collected in the rhEPO injection study.

Urine and blood plasma samples were obtained from a recently performed study [17]. Briefly, all samples were obtained from eight healthy male volunteers (23 ± 3 years) before, during and after ten s.c. injections of 5,000 IU (65 IU ± 5/kg bodyweight) of NeoRecormon®. This pharmaceutical glycoprotein belongs to the group epoetin beta. The samples were collected before (-14, -10) and 2 days after injection during day 2-16, and finally 7, 14 and 21 days after injection at day 35, 42 and 49. The injection and collection regime is illustrated in Fig. 1.

Reference population urine and plasma samples.

Urine samples from 25 healthy volunteer donors (men and women between 20 and 60 years of age) were collected after approval by the local ethical committee (No. 2005:307), and combined with 8 pre-samples from the injection series to establish the reference values for EPO WGA MAIIA. The 16 plasma pre-samples from the injections series and 7 additional EDTA plasma samples (leftovers from a routine medical health check-up) were used as reference plasma samples. All specimens were stored in aliquots at -20ºC until analysed.

Affinity purification of EPO prior to MAIIA test.

EPO from 0.4 mL of plasma and 5 mL urine samples was purified according to the instructions from the producer (EPO Purification Kit, MAIIA Diagnostics, Uppsala, Sweden). The EPO affinity purification recovery was 62.5% for the 85 plasma samples and 39% for the 67 urine samples. The EPO concentration in urine, plasma and purified sample was determined using a commercially available kit (EPO Quantification Kit, MAIIA Diagnostics). The affinity purification kit has been evaluated recently [13,14]. Following this procedure it took 20 min to obtain affinity purified and concentrated EPO samples from 5 mL of urine.

The MAIIA isoform analysis.

A MAIIA kit (EPO WGA MAIIA prototype Kit, MAIIA Diagnostics) was used to analyse affinity purified EPO from urine or plasma samples in accordance with the instructions from the supplier. The procedure, standardization and scanner equipment have been described recently [11]. The MAIIA lateral flow strip contains both a WGA (wheat germ agglutinin) lectin zone and downstream from this, an anti-EPO zone. Briefly, after immersing the strip in

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a well containing the sample, all EPO is bound in the first tenth of the 8 mm long WGA zone. Then the strip is moved to a second well for desorption of EPO, from its WGA interaction, by a WGA competing sugar derivate, N-acetylglucosamine (GlcNAc). Two strips were used to obtain total (using high concentration of GlcNAc, 100 mM) desorption and two strips were used for retarded (using low 10 mM of GlcNAc) desorption of EPO for each sample. Desorption from the WGA binding starts the migration of EPO and the most rapidly migrating ones can pass the WGA zone and be captured in the subsequent anti-EPO zone on the strip. Desorption was interrupted after 5 min. by removing the WGA zone by cutting. EPO bound to the anti-EPO zone was reacted with anti-EPO bound to carbon black nano-string, and the obtained grey to black signal intensity was quantified with an image scanner and compared to the signal obtained for an rhEPO standard. The unit PMI (Percentage of Migrated Isoforms) was obtained by calculating [EPO amount released during retarded conditions using 10 mM GlcNAc]/[total amount of EPO released using 100 mM GlcNAc] x 100. It took about 30 min to obtain the results from one test run.

Determination of EPO concentration in urine using lateral flow immunoassay.

The method, combining desalting and quantification using EPO Quantification Kit, has been presented elsewhere [23].

Determination of EPO concentration in plasma using lateral flow immunoassay.

EPO Quantification Kit (MAIIA Diagnostics) was used in accordance with the instructions from the supplier, but some additional steps were required for the plasma measurement. The plasma samples were diluted 8 times in a 20 mM phosphate buffer pH 7.5, supplemented with 6% BSA, 0.1 M NaCl and 0.02% NaN_3. In each well 25 µl of diluted sample was mixed with 25 µl of 20 mM phosphate buffer pH 7.5 supplemented with 1% BSA, 0.1 M NaCl, 0.1% bovine gamma globulin, 0.02% monoclonal mouse IgG, 1% Tween 20 and 0.05% NaN_3. 0.02% monoclonal mouse IgG was added to the working solution of anti-EPO carbon black nano-strings.

Statistics.

Values are presented as means ± SD. Differences in results for the groups before and after administration of drug were examined by paired t-test (SigmaPlot 12, Systat Software, USA)

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after normal distribution control using Shapiro-Wilk test. Statistical significance was accepted at P <0.05 (*), P <0.01 (**) and P <0.001 (***).

Each individual result before and after administration of the drug was also compared to the one-tailed confidence limit (CL) calculated from the results for the reference population. Limits selected at 99.99%, 99.9%, 99.0% and 97.8% allow that 0.1, 1, 10 or 22 normal samples out of 1000 samples are outside the limit, and regarded as aberrant.

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Results

EPO WGA MAIIA results during the boosting, maintenance and wash out period

Eight subjects were each administrated with ten s.c. doses of 5,000 IU epoetin beta (65 IU ± 5/kg) and the injection regime and results are shown in Fig. 1. The EPO WGA MAIIA isoform analysis demonstrated a highly significant (P<0.001) change in values between the pre treatment samples (day -14) and the samples collected 2 days after injection at day 2-16 for both plasma (panel A) and urine samples (panel B). The plasma samples collected on day 35, 7 days after last injection (n=7), showed significantly (P=0.041) decreased values compared to pre treatment values for the isoform test. The results for the urine samples (n=6) collected the same day were highly significantly (P=0.005) decreased compared to samples collected on day -14. The results at14 and 21 days after last injection could not be distinguished from pre treatment sample results.

Plasma and urine EPO concentration during the test period

The plasma and urine EPO concentration was determined by the EPO lateral flow immunoassay and is shown in Fig. 1. For the 16 plasma samples collected before any injection, at days -14 and -10, the EPO concentration was 99.9 ± 43.2 ng/L, while the concentration increased significantly (P<0.001) to 300 ± 90.5 ng/L for the 47 samples collected on days 2 to 16, two days after previous injection. The individual mean plasma EPO concentrations were in the range 240-396 ng/L for the eight subjects during days 2-16. The urine EPO concentration was 20.4 ± 13.1 ng/L for the eight samples collected before injection and 72.9 ± 71 ng/ L at days 2 to 16, collected two days after previous injection. As expected ~~A~~a considerable variability in the urine EPO concentration was present~~, as expected~~.
At day 35 ~~it can be noticed that~~ the total concentration of EPO had returned to normal values, while the EPO WGA MAIIA values still ~~can~~ shows presence of epoetin beta.

Analysis of samples to obtain the reference range

The analysis of the reference samples revealed 73.0 ± 6.5 PMI for the 33 urine specimens, with a range from 61.8 to 87.0 PMI. The 97.8, 99.0, 99.9 and 99.99% CL values were 58.9, 56.1, 49.7 and 44.4 PMI, respectively. For the 23 plasma samples the mean value was 75.7 ± 8.4 PMI, with a range from 62.8 to 92.1 PMI. The 97.8, 99.0, 99.9 and 99.99% CL values were 60, 57.8, 52.8 and 48.7 PMI, respectively.

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The PMI values can be compared to affinity purified recombinant epoetin beta that gave PMI's of 26.6 ± 2.1 and 24.9 ± 2.0 when used as controls for the presented urine (n=13) and plasma (n=8) test runs, respectively.

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In each test run, three control preparations were included containing 1) affinity purified endogenous EPO in urine from one healthy subject 2)~~,~~ affinity purified epoetin beta applied to buffer~~,~~ and 3) a known mixture of 60% endogenous and 40% of epoetin beta. The inter-assay CV was 8.2% when tested on 21 different test occasions for the three controls between 25 to 77 PMI.

The immunoassay measurement for retarded and total desorption mode demonstrated a median coefficient of variation (CV) of 4.7% between the duplicates when 603 samples from the injection series, the reference samples, and several controls were measured.

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Comparing plasma and urine sample analysis

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In order to reveal if the detectability of epoetin beta is different in plasma and urine samples, the results for plasma and urine samples collected on days -14, 16 and 35 are shown in Fig. 2. All samples collected on day 16, 2 days after the last injection, were clearly outside the 99.99% CL. The samples collected on day 35, 7 days after the last injection, were the most interesting ones for sensitivity comparison, as the method seemed close to its doping detecting limit. For the 5 subjects having both urine and plasma results it was found that one plasma sample and four urine samples were outside the 99.0% CL. This indicates that the analysis of urine samples gives a slightly wider detection window than plasma samples.

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The MAIIA results ~~for each subject~~ at day 35, 7 days after last injection

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The individual EPO WGA MAIIA values, haemoglobin and haematocrit values at day 35 are given~~found~~ in Tab.1. In total 7 plasma samples from day 35 were analysed, and one of these was outside 99.99% CL, but unfortunately the corresponding urine sample for that specific sample was not available. There were 6 urine samples available for analysis from that particular day and two of them where outside 99.99% CL.

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By combining the MAIIA results for plasma and urine collected on day 35, results for all eight subjects were obtained. Three of the eight subjects (38%) showed results outside the 99.99% CL while 75% (6/8) was outside the 99.0% CL. The haemoglobin and haematocrit

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values were exceeding the limit [24] of 170 g/L and 50 %, respectively, for only one of the subjects, who would have been excluded from starting competition. This subject had also a haematocrit value above the limit (50.5%) at day 42, 14 days after last injection. Subject 16 had a haematocrit value of 50.4% at day 49, 21 days after last injection, which would have excluded him. In nNone of the other testingsampling days were the limits were exceeded for any of the subjects.

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Discussion

The present study evaluated~~s~~ the detection efficiency of a novel lateral flow isoform test, EPO WGA MAIIA, intended for EPO anti-doping control, on samples collected before, during and after s.c. injections with epoetin beta in eight healthy m~~en~~ales. The main finding is that the EPO WGA MAIIA method can ~~measure~~determine rhEPO injections ~~doping~~ during a 14 day period with a high injection rate ~~the boosting~~ and also up to 7 days after a period with less frequent injections~~maintenance period up to 7 days after last injection, but not at 14 days~~. It is also indicated that urine is a better specimen than plasma for doping analysis.

~~Results were available for all eight subjects on day 35, 7 days after last injection, only by combining results from urine and plasma samples. This revealed that three out of eight subjects were outside the 99.99% CL, and thus 38% of the subjects were regarded as positive for EPO doping. Additional three subjects (38%) exceeded the 99.0% CL, but not 99.9%. Only two subjects were negative for doping (25%).~~

Other tests performed on the samples collected from the present~~ed injection~~ study

The urine samples collected from the same injection series was analysed ~~during 2007~~ by means of IEF in a previois publication [17] and demonstrated 2 out of 7 (33%) tested samples as positive on day 35.

The use of indirect methods [25] to identify EPO doping for the samples from the study presented herein, has also been presented elsewhere [26]. During the entire 49 days sample collection period very few of the eight subjects exceeded the cut-off limit (99.9% CL) for $OFF_{z\ score}$ (two subjects), percentage of reticulocytes (two subjects), $Hb_{z\ score}$ (one subject) and $OFF_{hr\ score}$ (no subjects). The limit for percentage of reticulocytes was exceeded once for two subjects, and only during the injection period where all eight subjects were found to be positive with EPO WGA MAIIA. $OFF_{z\ score}$ showed one subject outside the 99.9% CL on days 35 and 42, while another subject was outside the limit at day 49.

Aerobic exercise capacity was significantly increased on days 35, 42 and 49 [17], with the peak value determined on day 42. During these days, 7-21 days after the last injection, three subjects were positive (99.99%) and another three subjects were suspicious (99.0%) for doping using EPO WGA MAIIA, while only two subjects exceeded the 99.9% limit with $OFF_{z\ score}$. The EPO WGA MAIIA test, which identified rhEPO both during and up to 7 days after injection, and indirect test, which in this study indicated misuse 7 to 21 days after last injection, seems preferable to use in combination.

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Detection of different types of rhEPO

In this study the ability for isoform methods to detect injections specifically with epoetin beta was studied. An optimal EPO doping control method should be able to distinguish all different recombinant EPOs and all available EPO-like molecules [22,27,28] from the at least two to three different endogenous EPO populations. The EPO WGA MAIIA method has previously proven to be successful in the detection of several isoforms of EPO [11] like epoetin alpha, omega, delta, darbepoetin alpha and CERA. The method has also successfully ~~been identifying~~ identified the injection of epoetin- ~~alpha~~ and darbepoetin alpha in horses [15].

EPO WGA MAIIA has also ~~been used for detecting intravenous administration of epoetin beta 72h after last injection.~~

The minimal required amount of rhEPO that can be detected

The EPO detection sensitivity of the isoform method seems sufficient to distinguish isoforms of rhEPO in urine samples containing as little as 0.1 to 1 ng/L (3 to 30 femtomol) of EPO. This is crucial since very low EPO concentrations were found in several urine samples collected during a major competition [11]. The MAIIA method was able to determine that these samples had aberrant EPO forms while the EPO amount was too low for IEF analyses. In the present study ~~the MAIIA method analysed~~ all samples were analysed using only 5 mL urine for affinity purification as compared to the 20 mL required for IEF doping method. It was calculated that even 2.5 mL of urine had been sufficient for analysis of all the samples in the series.

The increased sensitivity may be due to better detection sensitivity as well as to the high EPO recovery of the affinity purification regime required prior to the isoform test procedure.

Conclusion.

The sensitive and easy-to-use MAIIA method with a process time of just 1 hour, and with potential for large-scale testing seems to be a very good candidate for a screening tool against EPO doping. This test demonstrated a higher detection rate than the other direct and indirect EPO doping tests that were tested on the samples from the presented epoetin beta injection series.

The on-going EPO doping in sport needs to be stopped [1].Todays acknowledged methods to determine EPO doping are unfortunately not as efficient as could be whished for. Furthermore

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the analytical procedures are not easy to perform and very expensive. In the present study it is proposed that the inclusion of an affordable and sensitive screening test against rhEPO doping will lead to higher detection rates than what is seen today. Moreover, the use of quantitative results for isoform tests could allow the implementation of different degrees of suspiciousness. Exceeding a certain limit is suggested to lead to an intense out-of-competition sampling program.

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Fig. 1. EPO WGA MAIIA and total EPO concentration results

Subjects were s.c. injected with epoetin beta 10 times (💉) *i.e.* day 0, 2, 4, 6, 8, 10, 12, 14, 21 and 28. Samples for analysis were obtained prior to the rhEPO injections and then two days after each injection. Finally, samples were obtained on day 35, 42 and 49, corresponding to 7, 14, and 21 days after the last rhEPO injection. Directly two days after the first injection the epoetin beta concentration in plasma and urine was increased and the EPO WGA MAIIA values were significantly reduced, indicating doping. Seven days after last injection EPO WGA MAIIA significantly detects rhEPO in the urine and plasma samples while the EPO concentration is back in the normal range. At 14 and 21 days after last injection the EPO WGA MAIIA values are normal.

Significant difference, as compared to the pre-EPO (day -14) values, is indicated with: *=P<0.05, **=P <0.01, ***=P <0.001.

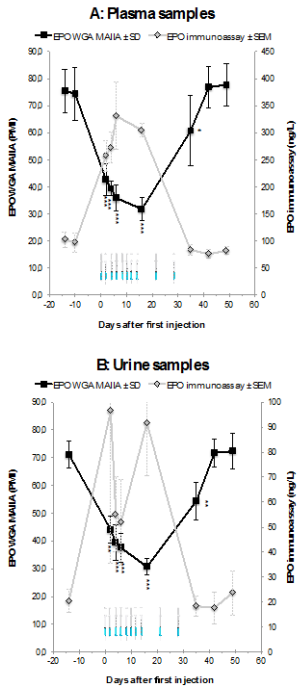
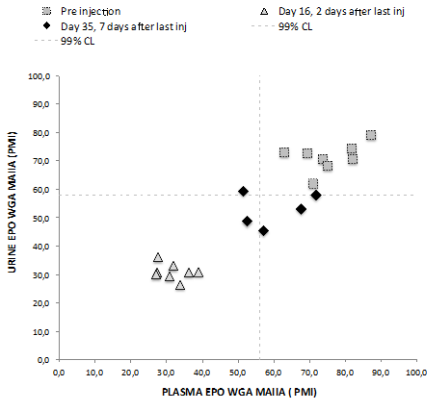


Fig. 2. EPO WGA MAIIA analysed with plasma and urine samples

The samples collected at day 16, 2 days after last injection, are all well outside the 99.99% CL for both urine and plasma. The interesting day for comparing test sensitivity is day 35, 7 days after last injection. For the five subjects having both urine and plasma samples it was found that one plasma samples and four urine samples were outside the 99% CL and regarded as doping suspected samples. This indicates that traceable amounts of epoetin beta remain in urine, while excreted from plasma, at 7 days after last injection.



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Tabel 1

The table shows the individual EPO WGA MAIIA values, and haemoglobin and haematocrit values, for each of the eight subjects on day 35, 7 days after last injection. As not all of the samples were available for testing both the plasma and urine results are used to settle the suspicions for doping. Three of the subjects will be positive for doping (10,11,13) being outside the 99.99% CL. Three of the subjects might be suspected for doping (9, 12,15) being outside the 99% CL, while only two subjects are negative (25%). The haemoglobin and haematocrit values exceed the limits for competition exclusion only for one subject (13).

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Day 35	EPO WGA MAIIA Plasma		EPO WGA MAIIA Urine		Hb	Hct
Subject No	PMI	CL, %	PMI	CL, %	g/L	%
9	71,9	N	57,8	>99	155,3	47,60
10	43,6	>99.99	NA	NA	NA	NA
11	52,7	>99.0	48,5	>99.99	147,0	45,07
12	51,5	>99.0	59,2	>97.8	161,0	49,30
13	57,2	>97.8	45,3	>99.99	178,0	54,33
14	NA	NA	62,9	N	NA	43,84
15	67,8	N	52,9	>99.0	NA	42,25
16	80,5	N	NA	NA	156,3	47,83