Detection of recombinant human EPO and darbepoetin administrated to horses using EPO WGA MAIIA and LC-MS/MS

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ABSTRACT
Doping of horses with recombinant human erythropoietin to enhance their endurance capacity is sometimes occurring in horse-races and has been used and reported on various occasion in Europe, USA, Australia and New Zeeland. Besides unhealthy increased blood viscosity, the horse might start to produce antibodies against the injected human EPO, which can cross-react with the endogenous EPO and lead to severe anaemia and even death.
Two recently developed methods have been compared for their capability to detect administration with epoetin alpha (Eprex) or darbepoetin alpha (Aranesp) in horses in both plasma and urine samples.
The easy-to-use and rapid EPO WGA MAIIA test distinguishes epoetin and darbepoetin from endogenous equine EPO by their different carbohydrate motifs on the protein. LC-MS/MS differentiates human and equine EPO due to the size of some selected peptide structures obtained after trypsin digestion.
Seven horses were s.c. administrated with 40 IU Eprex/kg during six days and one horse obtained a single dose of 0.39 µg Aranesp/kg. It was found that EPO WGA MAIIA could detect (CL 99%) the presence of Eprex four days after last injection for four of the seven tested horses, while LC-MS/MS identified Eprex up to one day after last injection. EPO WGA MAIIA detected (CL 99.9%) the presence of Aranesp up to eight days, while LC-MS/MS could identify Aranesp up to five days after last injection. EPO WGA MAIIA seems to be much more sensitive than LC-MS/MS, while the latter has the mass spectral data required for equine forensic drug testing. Before the advent of more sensitive MS analytical techniques the use of EPO WGA MAIIA might be a valuable means for supressing EPO doping of horses.

Abbreviations used: ESA, erythropoiesis stimulating agents; IEF, isoelectric focusing; N-GlcNAc, N-Acetylglucosamine; PMI, percentage of migrated isoforms; rhEPO, recombinant human EPO; WGA, wheat germ agglutinin

Introduction

Administration in horses of recombinant human erythropoietin (rhEPO) increases the number of red blood cells and the oxygenation improvement of main muscles, and has been proposed to enhance racing performance, even though the fact that horses, unlike humans, have a large red blood cell storage capacity in the spleen. The increase of red blood cells leads to increased blood viscosity, which may result in cerebral thromboembolism, as for humans [1]. Moreover, horses treated with rhEPO have developed immune response, with production of anti-human

EPO antibodies, and obtained fatal pure red cell aplasia. Equine and human EPO have 84% identity [2] of the amino acids building up the protein, but it is likely that also differences in the glycosylation are available, as the posttranslational insertion of carbohydrate structures depends on the specific enzymes in each type of production cells. The requirements of the methodology for detection of the very low concentration of rhEPO required for humans and horses, in the ng/L range, are further obstructed by the short and variable biological half-life for hEPO, 19.4±10.7 h and 6.8±2.7 h, for s.c. and i.v. administration of epoetin alpha, respectively, to humans [3]. Moreover, the varieties of erythropoiesis stimulation agents (ESA) [4,5] based on human EPO or analogues are increasing rapidly, with up to 80 biosimilar epoetin products available in 2009 [6], which makes the doping control even more difficult [7]. The ESA variants on the market are differing in biological activity and structure, e.g. glycosylation, due to cell line expression and details in the manufacturing process, like culture conditions and purification methods.
For detection of recombinant human EPO isoform in humans the methodology combine electrophoretic or chromatographic separation of EPO isoform populations, with sensitive anti-EPO antibody based detection methods. The methods often require an additional purification and/or concentration step before urine and plasma can be analysed [8,9]. Endogenous and recombinant human EPO glycosylation can be distinguished by their charge [10,11], isoelectric point (pI) [12,13,14], molecular mass [15,16,17] or by interaction with the lectin WGA (wheat germ agglutinin) [18,19,20]. The methods differ in how well they distinguish a certain type of glycosylation, and how much EPO is required for analysis.
For detection of exogenous EPO in equine samples, it can be performed by LC-MS/MS preceded with anti-EPO affinity purification of the sample. But as soon recombinant equine EPO is available at the market, methods akin these for humans will be required. In advance to MS the interesting samples are picked out with a screening method for rhEPO using a hEPO immunoassays with low reactivity for equine EPO compared to hEPO, and a detection limit of 2.5 IU/L (20 ng/L) of plasma hEPO [21]. The presently accredited EPO doping method for human athletes based on isoelectric focusing IEF showed that s.c. administration of 36 IU/kg bodyweight of epotin alpha and 0.37 µg/kg of darbepoetin alpha was detectable in equine urine up to one and five days, respectively, after last injection. By LC-MS/MS, with an identification limit of 200 ng/L epoetin alpha, the injection of 8.4 IU/kg and 34 IU/kg of epoetin could be detected up to 24 and 48 h after last i.v. dose [22]. An identification limit of 100 ng/L was obtained for darbepoetin (Aranesp), and an i.v. dose of 0.37 µg/kg could be identified 168 h (7 days) post administration [23]. For the novel EPO WGA MAIIA test it was possible to use urine samples from human athletes with as low as 0.1 ng/L of rhEPO to identify doping [18]. After s.c. injection of 50 IU/kg of epoetin beta the presence of rhEPO could be identified for 63% of the athletes seven days after last injection [19].
In this study the WGA lectin based EPO WGA MAIIA method and LC-MS/MS are compared in their efficiency to detect daily s.c. administration of 40 IU/kg of epoetin alpha during six days and 0.39 µg/kg single administration of darbepoetin alpha to horses.

Material and Methods

Samples from the horse administration series

The details for the administration series performed at LCH (Laboratoire des Courses Hippiques, French horse doping control laboratory) have been recently presented [24]. Briefly, eight thoroughbred horses were administered subcutaneously in the neck. Horses H579 to H585 received 40 IU/kg bodyweight per day of erythropoietin alpha (Eprex) during six days, and horse H626 received a single dose (0.39 µg/kg) of darbepoetin alpha (Aranesp). Heparin plasma samples were collected before, during and after the end of administrations as indicated in Fig.1. Urine samples were also collected. All samples were stored at -20°C until

their analysis. The study was led in agreement with animal welfare rules at the administration and sampling centre of the Fédération Nationale des Courses Françaises (FNCF).

Reference urine and plasma samples for EPO WGA MAIIA

As reference samples 26 plasma samples were used, with16 samples collected from the eight horses before injection, and 10 samples were leftovers from a routine analysis. For the urine testing 6 reference samples was used, 3 samples collected before injection, and 3 samples were leftovers from routine analysis.

Affinity purification of EPO from biological samples for EPO WGA MAIIA analysis

EPO Purification Kit, Art. No. 0250, was obtained from MAIIA Diagnostics (Uppsala, Sweden). EPO from 0.5 mL plasma samples (for 20% of the samples 0.47 mL to 0.06 mL was used) or 20 mL urine samples was purified according to the instructions from the producer, using the recommended addition of detergent and BSA to the reagents. Purified EPO was finally obtained in a volume of 220 µL for urine EPO and for the plasma EPO samples collected one to three days after first injection of Eprex. EPO from the other plasma samples was obtained in a volume of 55 µL. The EPO affinity purification recovery was 75% and 55% for NeoRecormon added to buffer and equine plasma, respectively. The affinity purification recovery of Aranesp added to equine plasma was estimated to 39%. EPO Quantification Kit Art. No 0100 (MAIIA Diagnostics), was used for determination of EPO concentration in the eluate. The affinity purification kit has been evaluated recently [8,9].

EPO WGA MAIIA isoform analysis

EPO WGA MAIIA prototype kit, was obtained from MAIIA Diagnostics and used in accordance with the instructions from the supplier. The MAIIA lateral flow strip contains both a WGA zone and an anti-EPO zone. The procedure has been described recently [18] and takes about 30 min. for processing 56 strips. For each sample four strips were used, one duplicate to obtain retarded migration through the WGA zone using the selected GlcNAc (N-Acetylglucosamine) concentration for elution buffer *low*, while for the other duplicate elution buffer *high* was applied and the high GlcNAc concentration allowed EPO to migrate through the WGA zone without retardation.

The concentration of GlcNAc in elution buffer *low* was optimised to obtain good resolution between equine EPO and rhEPO, with a WGA interaction with epoetin beta at 13 PMI and 31 PMI, respectively, for the plasma and urine runs. In each run two control preparations were included.

Standardisation and concentration determination

A standard curve of epoetin beta, NeoRecormon (Roche GmBH, Mannheim, Germany) was obtained by dilution from the provided stock solution of 10 µg/L (1 U corresponding to 8.3 ng epoetin) to 3-1000 ng/L. The standard series was measured by the EPO WGA MAIIA method using only elution buffer *high.* The concentration values for unknown samples using *high* and *low* elution buffer was calculated by a 4-parameter logistic curve fit program (WorkOut 2, Perkin-Elmer, Turku, Finland) using the signal intensity from the strips for the standard dilution series. Stored signal values were used for runs using the same batch of reagents. The concentration of the EPO analogue Aranesp will be underestimated with 18% as reported earlier for the antibodies used in the sandwich immunoassay part [25] of the test. The EPO concentration of samples containing equine EPO might be underestimated as the used anti-EPO antibodies were obtained by injection with human EPO, and human EPO was also used in the standard curve, as no purified equine EPO preparation was available. The EPO

concentration in the eluates was obtained from their elution buffer *high* values and the concentration in urine or plasma samples was obtained by correcting for the recovery and sample volume used in the affinity purification step.

Calculation of EPO WGA MAIIA results

The EPO WGA MAIIA value, with the unit Percentage of Migrated Isoforms (PMI) was established by calculating the percentage ratio of the apparent concentration values for the strips used with elution buffer *low and high.* Only concentration values between 3 to 500 ng/L, inside the measuring range, were used.

Mass spectrometry analysis, LC-MS/MS

The details for the affinity purification of 4 mL plasma or 10 mL urine with the EPO purification kit, and mass spectrometry analysis developed and performed at LCH, using EPO from samples purified with EPO Purification kit, have been presented recently [24]. Briefly, EPO in the eluate from the affinity purification was denatured and digested with trypsin, and the peptide analysis was carried out on a triple quadrupole MS with heated electrospray ionisation.

Enzyme immunoassay of EPO in plasma samples

An ELISA for EPO, Quantikine EPO, was purchased from R&D Systems (Minneapolis, MN, USA) and used as described by the producer for analysis of plasma samples in advance to the LC-MS/MS analysis.

Statistics

Values are means±SD. Differences between groups were examined by paired *t*-test (SigmaPlot 12, Systat Software, USA) and statistical significance was accepted at $p< 0.05$.

Results

EPO WGA MAIIA results

The PMI values obtained by EPO WGA MAIIA, which indicate the WGA interaction with the carbohydrate motifs of different types of EPO, can be found in Fig.1. The results for plasma samples collected one day after injection showed a highly significant ($p<0.001$) reduction in PMI values (increased WGA interaction) to 22±1.7 PMI and 14 PMI for Eprex and Aranesp respectively, compared to 92±6.7 PMI for the equine EPO present in the 16 samples collected before injection. For the Eprex injection series the mean PMI results for the seven horses collected 2 days ($p < 0.001$) and 4 days ($p=0.003$) after last injection were highly significantly different from the samples collected before injection for each horse. There were no plasma samples collected 3 days after injection. The samples collected at 5 days after last injection showed also a significant difference ($p=0.047$), while EPO in the samples collected 6 days and later after last injection could not be differentiated from equine EPO. For the samples from horse 581, something seems to have happened during the injections at day 4 to 6. The EPO concentrations were considerable lower than for the samples from the other horses, and the PMI value was quite high at day 5, although clearly positive.

The mean value for the 26 plasma reference samples was 90.0±7.3 PMI, and the one-tailed 99% and 99.9% confidence limits (CL) were 73 PMI and 67.3 PMI, respectively. For the Eprex series samples all seven samples showed its presence 2 days after injection using the 99.9% limit. For the samples collected 4 days after injection, one and four samples were positive when using 99.9% and 99% CL, respectively. Five days after injection one sample

was positive when using 99.9% CL. For the Aranesp injection of one horse, the PMI values were positive, below the 99.9% CL, up to 8 days after first injection.
For urine samples, the six reference samples showed a mean PMI value of 77±2.5 PMI, and 99% and 99.9% CL were 71 PMI and 69 PMI, respectively. For the samples collected up to 3 days after injection from the two horses injected with Eprex, the samples were clearly positive using 99.9% CL. For the horse injected with Aranesp the sample collected 8 days after injection was clearly positive using the 99.9% CL. No urine sample was obtained 4 days after last injection with Eprex, or 9 days after Aranesp injection, or later. The urine plasma results at 3 days after collection indicate that positive results would have been obtained also for the plasma samples, if collected.

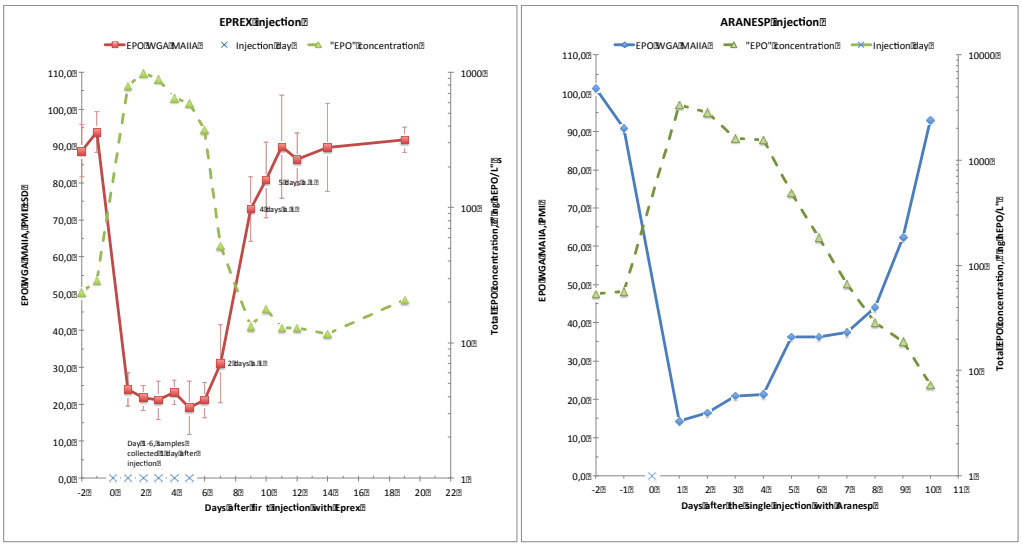

Fig. 1
From the EPO WGA MAIIA results both the total EPO concentration and the PMI value, indicating the strength of WGA interaction with the EPO carbohydrate motifs, can be obtained. Noted in the figure, besides day after first injection on the x-scale, and the days for injection, is also how many days after injection the sample was collected. Equine EPO found in the samples collected before injection showed low WGA interaction (high PMI values), while both Eprex and Aranesp showed low PMI values. For the Eprex injection series the samples collected up to day 5 after injection, and for Aranesp up to day 8 after injection, showed significantly aberrant PMI values compared to equine EPO collected in the samples before injection.

EPO concentration in plasma sample as determined with EPO WGA MAIIA

The estimated EPO concentration in equine plasma, before, during and after s.c. administration, is shown in Fig. 1 for seven horses injected with 0.34 µg Eprex (1 U=8.4 ng epoetin) per kg and one horse injected with 0.39 µg Aranesp per kg bodyweight, respectively. For samples collected one day after injection the EPO concentration was 710 ng/L for Eprex and 3.400 ng/L (4.150 ng Aranesp/L, after correction for underestimation) for Aranesp. The concentration was back to baseline 3-4 days and 7-8 days after injection with Eprex and Aranesp, respectively, but still EPO with aberrant WGA interaction could be found up to day 5 and day 8 after injection. It was also found that the estimated concentration of equine EPO was much lower in the samples collected after injection than before. Equine EPO seemed to be reduced to 52% for samples collected at day 10 to 14 after the initial injection (p=0.026). In Fig. 2 is the difference in the excretion profile for Eprex and the EPO analogue Aranesp plotted, with an estimation of the EPO concentration in plasma at 3 days after injection with Eprex. The concentration of Eprex was reduced to 8% between day 1 and day 2 after s.c. injection, and Aranesp to 84%. Aranesp between day 4 to day 5 was reduced to 33%.

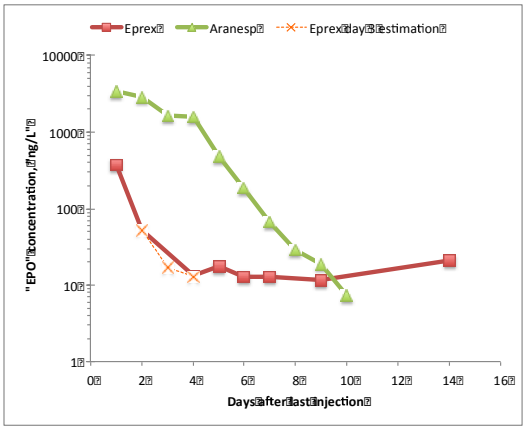

Fig. 2
The concentration of Eprex was rapidly eliminated from blood, while Aranesp had a slower elimination rate. The concentration of Eprex was reduced to 8% between day 1 and day 2 after s.c. injection, and Aranesp to 30% between day 4 to day 5.

Imprecision for EPO WGA MAIIA

The immunoassay measurement of the affinity purified plasma samples included in the injection study tested in *low* and *high* desorption mode showed a median coefficient of variation (CV) of 5.7% (n=163, mean 27 ng/L) and 3.9% (n=163, mean 97 ng/L), respectively, between the duplicates.
The mean inter-assay CV for the PMI values were 10.5% and 15.2% for the controls at 74 PMI (affinity purified equine EPO in plasma) and 13 PMI (Neorecormon in buffer), when measured in duplicate at 11 different test occasions.

Differences in interaction between EPO forms

The interaction with the WGA zone on the EPO WGA MAIIA strip shows different strength depending on the type of carbohydrate structure and the number of interacting forms on the protein. As shown in Fig. 4, Aranesp with two additional carbohydrate structures had the strongest WGA interaction, and thus the lowest PMI value, while equine EPO in plasma showed the weakest WGA interaction. For Aranesp (n=2), NeoRecormon (n=4) and Mircera (n=3), the preparations were added to buffer, and some samples was directly analysed, while some other samples was affinity purified before analysis. The different types of EPO in urine and plasma were affinity purified before tested in EPO WGA MAIIA (n=2 for human EPO in serum, and n=3 for the other EPOs). In that study, epoetin beta was optimised to a value of 8.0 PMI, while in the injection study the interaction with the WGA zone was optimised to be 13 PMI and 30 PMI for epoetin beta in the plasma and urine runs, respectively.

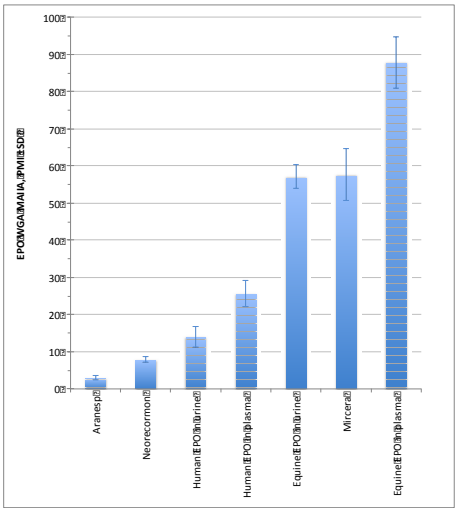

Fig. 4
The interaction with the WGA zone in the EPO WGA MAIIA test was strongest for Aranesp and weakest for equine EPO purified from equine plasma. This means that EPO WGA MAIIA will have higher sensitivity for distinguishing EPO produced in hamster cells from equine samples than from human ones. Surprisingly, the equine EPO in plasma showed even less interaction with WGA than the EPO analogue Mircera containing a 30kDa large PEG group linked to epoetin beta.

EPO concentration measured with ELISA

For screening of plasma samples collected from horses an EPO ELISA, with low reaction with equine EPO, has been used for selecting the most suspicious samples for further analysis with isoelectric focusing or mass spectrometry. Samples with an EPO concentration higher than 20 IU/L (170 ng/L) were selected for the MS determination.

When affinity purified samples were analysed with both with the lateral flow immunoassay (EPO Quantification kit) and the ELISA (Quantikine EPO) it was found that compared to endogenous serum EPO and epoetin beta, the antibody pair in the lateral flow immunoassay reacted 3.0, 2.2 and 1.6 fold-times more with equine EPO, Aranesp and Mircera respectively, than the antibodies used for ELISA did. In the EPO WGA MAIIA kit is the same antibody pair used for the detection of the EPO migration through the WGA zone.

LC-MS/MS, results

A limited number of samples were analysed with MS, one of the samples collected before administration, and the last sample in the series having a plasma concentration above 20 IU/L (170 ng/L). Samples below 170 ng rhEPO/L were expected to be below the detection limit of the MS confirmation.

For six of the horses the plasma sample collected one day after last injection of Eprex were above 20 IU/L (26-42 IU/L), but two days after last injection the concentration was 1.0-11 IU/L. For horse 581 the last sample collected above 20 IU/L was 35 IU/L at day 4, during the period with Eprex injections, while one day after last injection (day 6) the EPO concentration was only 11.5 IU/L. This is in accordance with the results from EPO WGA MAIIA, where the concentration in this sample at day 6 was estimated to be about 30% compared to the values from the other horses.

The six samples collected at day 6, one day after last injection, with an Eprex concentration of 26-42 IU/L, were all positive as determined with MS. For horse 581, the sample collected during the injection period at day 4 after first injection was positive with MS. For the horse obtaining Aranesp the samples collected 4 and 5 days after injection were positive, while the urine sample collected 6 days after injection was negative.

Comparing the results from EPO WGA MAIIA and LC MS/MS

In Tab. 1 are the results compared from the horse that received one dose of the EPO analogue Aranesp. Samples were found to be aberrant for EPO WGA MAIIA up to 8 days (both in urine and in plasma) and with LC-MS/MS were the samples positive up to 5 days after last injection. In Tab. 2 are the results shown for the seven horses that obtained Eprex during six days. EPO WGA MAIIA showed positive results up to four to five days after last injection using CL 99% in plasma samples. When using 99.9% CL, the urine samples were positive up to 3 days after last injection, but as no plasma samples were collected that day the majority of plasma samples were positive only up to 2 days. Horse 579 was positive up to 5 days after last injection, using 99.9% CL, but obtained also a positive sample at nine days after last injection. For LC-MS/MS the samples for five horses were positive up to one day after last injection, while the samples for horse 581 was expected to be negative due to the low EPO concentration.

The EPO WGA MAIIA method seems to be much more sensitive for traces of both Eprex and Aranesp in the samples, with detection up to 3 days later compared to the LC-MS/MS results.

Aranesp	**EPO WGA MAIIA procedure**			**LC-MS/MS procedure**		
	1	**2**		**3**	**4**	**5**
	Plasma	**Urine**		Plasma ELISA	**Plasma**	**Urine**
Days after	EPO WGA MAIIA	EPO WGA MAIIA		EPO, IU/L	MS	MS
last inj	*CL 99.9%*	*CL 99.9%*				
1	POS	NA		118	NT	NA
2	POS	POS		90	NT	NT
3	POS	NA		63	NT	NA
4	POS	NA		35	POS	NA
5	POS	NA		21	POS	NA
6	POS	POS		9	NT	NEG
7	POS	NA		4	NT	NA
8	POS	POS		2	NT	NT
9	NEG	NA		NT	NT	NT
	NA: not available					
	NT: not tested					

Tab.1

The human EPO analogue darbepoetin (Aranesp) was administrated to one horse, and urine and plasma samples were analysed with EPO WGA MAIIA, and with LC-MS/MS after screening with EPO ELISA. EPO WGA MAIIA showed positive results up to 8 days after injection, while LC-MS/MS had positive results up to 5 days after injection.

Eprex								
EPO WGA MAIIA								
	Days after							
	last inj	**Horse 579**	**Horse 580**	**Horse 581**	**Horse 582**	**Horse 583**	**Horse 584**	**Horse 585**
PLASMA		*CL 99.9%*	*CL 99.9%*	*CL 99.9%*	*CL 99.9%*	*CL 99.9%*	*CL 99.9%*	*CL 99.9%*
EPO WGA MAIIA								
99.9% CL	1	POS	POS	POS	POS	POS	POS	POS
	2	POS	POS	POS	POS	POS	POS	POS
	3	NA	NA	NA	NA	NA	NA	NA
	4	POS	NEG	NEG	NEG	NEG	NEG	NEG
	5	POS	NEG	NEG	NEG	NEG	NEG	NEG
PLASMA		*CL 99%*	*CL 99%*	*CL 99%*	*CL 99%*	*CL 99%*	*CL 99%*	*CL 99%*
EPO WGA MAIIA								
99% CL	1	POS	POS	POS	POS	POS	POS	POS
	2	POS	POS	POS	POS	POS	POS	POS
	3	NA	NA	NA	NA	NA	NA	NA
	4	POS	NEG	POS	POS	NEG	POS	NEG
	5	POS	NEG	NEG	NEG	NEG	NEG	NEG
		For EPO WGA MAIIA, 6 to 14 days after last injection the samples were negative						
		but with 99% CL the sample H579, 9 days after injection, was positive						
URINE		*CL 99.9%*	*CL 99.9%*					
EPO WGA MAIIA								
99.9% CL	1	POS	POS					
	2	POS	POS					
	3	POS	POS					
	4	NA	NA					
LC-MS/MS								
	Days after							
	last inj	**Horse 579**	**Horse 580**	**Horse 581**	**Horse 582**	**Horse 583**	**Horse 584**	**Horse 585**
PLASMA								
MS	1	POS	POS	NT	POS	POS	POS	POS
	2	NT	NT	NT	NT	NT	NT	NT
URINE								
MS	1	POS						
	2	NT						
NA: not available								
NT: not tested								

Tab. 2
The horses that received Eprex during six days showed positive results up to four to five days after last injection using CL 99% for EPO WGA MAIIA in plasma samples. When using 99.9% CL, the urine samples were positive up to 3 days after last injection, but as no plasma samples were collected that day it can only be shown that the majority of plasma samples were positive up to 2 days. For LC-MS/MS six of the seven samples were positive up to one day after last injection.

Discussion

Doping confirmation

The confirmation process in doping control is preferably mass spectrometry, which is the best technology for molecular characterisation through specific and characteristic fragmentation pathways. For the determination of recombinant protein hormones, like EPO, the sensitivity for LC-MS/MS compared to e.g. immunoassay seems not to be sufficient so far. The requirement of MS/MS data or "fingerprints" for equine forensic drug testing [23] would limit the potential use of techniques like EPO WGA MAIIA, even though the time window for detection will increase 3-4 times. The IEF based doping test, with double immunoblotting, for humans have been used for doping analysis on equine samples [21]. In this case the image of distribution of the EPO bands with different pI has been used for confirmation. For EPO WGA MAIIA, the migration in the WGA zone is used for distinguishing EPO subpopulations. But instead of images or fingerprints, values that give the percentages of EPO isoforms in the sample that have migrated through the WGA zone are obtained. These values can be used for statistical calculations and compared to the values obtained from thousands of samples from reference populations.

The optimal doping test

Besides high sensitivity and specificity for the doping substance, the potential to set up high-throughput analysis for all steps in the analysis is of high importance. This will reduce the analysis cost and enable more samples to be analysed. More samples analysed with a highly sensitive doping test will increase the risk for identification of doping, and thus reduce the incitement for doping. Further development of both the affinity purification kit and the EPO WGA MAIIA kit seems to be possible for applications on suitable equipment for large-scale analysis.

Conclusion

The EPO WGA MAIIA test seems to have the sensitivity required for doping analysis of epoetin and darbepoetin in plasma and urine samples from horses. Compared to the recently developed LC-MS/MS system, which was able to confirm presence of Eprex up to one day, EPO WGA MAIIA detected it up to 3-4 days after last injection. For Aranesp with longer half-life in the circulation, LC-MS/MS identified administration up to 5 days after last injection compared to 8 days for EPO WGA MAIIA.

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