Detection of secondary biomarker of met-eGH as a strategy to screen for somatotropin misuse in horseracing

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Since the Australian commercialisation of the recombinant equine growth hormone (reGH) in 1998 (EquiGen-5®, Bresagen), this reGH, which differs only from eGH by an additional methionine at the N-terminal end (met-eGH), is worldwide suspected to be administered to racehorses as a doping agent. Indeed, the use of this biological drug is considered as a threat to horseracing since it acts both on growth, development or reproductive functions, and on the improvement of performances. In this work, we describe two reliable techniques based on surface plasmon resonance biosensor immunoassay (SPR-BIA) and solid-phase enzyme-linked immunosorbent assay (ELISA) as new, rapid and efficient long-term screening methods applicable to horseracing antidoping analysis. The ELISA and SPR-BIA tests were applied to octanoic acid purified IgGs from serum/plasma samples collected on two thoroughbreds treated with recombinant equine growth hormone for a period of two weeks. The first kinetic study of serum/plasma antibodies raised as a consequence of recombinant equine growth hormone administrations, which allows the detection from eight days up to 200 days after the beginning of the treatment, was performed. In order to trace the occurrence of anti-reGH antibodies in routine analysis and to monitor the animal level exposure to this forbidden molecule, a random population study was conducted on 233 post-race horses.

1 Introduction

Equine growth hormone (eGH), also known as equine somatotropin (eST) is a 190 amino acid peptidic hormone produced by the anterior pituitary gland.[1] Its biological effects are numerous and are associated with growth, development and reproductive functions.[2,3] These properties are thought to be useful to improve horse athletic efficiency and to speed up recovery time after wound.[4] Recombinant DNA techniques allow for the production of large quantities of recombinant equine growth hormone (reGH),[5,6] which has been commercialised in Australia (EquiGen-5®, Bresagen) since 1998, to improve the nitrogen balance in horses aged over 15 years.[7] The significant difference between reGH and eGH is its additional methionine at the N-terminal end (met-eGH). The hypothesis of its worldwide illegal use to improve horse athletic efficiency cannot be ignored in spite of the racing rules ban (International Federation of Horseracing Authorities 2007, IFHA). No direct detection method is currently sensitive and specific enough to achieve reGH detection in plasma at the required level (1–5 μg L^{-1}).[2,8,9] Various methods have been developed and are used routinely in official racing laboratories to screen for GH abuse, such as quantification of secondary markers of GH administration: insulin-like growth factor 1 (IGF-1)[10–12] or IGF binding protein 3 (IGFBP-3).[13] The effects of exercise, training, circadian rhythm, age and sex on IGF-1 in the horse are now well documented[11,12] and enable IGF-1 detection above a threshold currently proposed at 860 ng mL^{-1}.

In this paper, the monitoring of another secondary marker, which might allow screening over a longer period than IGF-1, has been studied. The appearance of specific antibodies after recombinant GH administration has been documented since the 1960s. In humans, it is well known that a treatment with human growth hormone (hGH) can induce the production of specific antibodies.[14–16] Antibodies raised against animal growth hormones have also been reported in patients treated with human growth hormone[17,18] and slight modifications on growth rate have been shown.[19,20] In this context, three tests have been described in the literature to detect the presence of antibodies in patients treated with hGH: one method widely used for 25 years is based on chromatoelectrophoresis using the GH-I^{131} tracer,[15] while enzyme-linked immunosorbent assay (ELISA)[21,22] and a High-Performance protein G Affinity Chromatography (HPAC) method using fluorescence detection[23] have also been used. In breeding animals, production of growth hormone antibodies upon recombinant GH administration is well documented in cows[24,25] and is detected by Western Blot analysis 28 days after recombinant bovine growth hormone (rbGH) administration.[26] The production of antibodies raised against biosynthetic human growth

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hormone in dogs has also been reported.[27] In horses, before the commercial introduction of the recombinant equine growth hormone, the detection of antibodies consecutive to human and bovine growth hormone treatment can be achieved by radioimmunoassay (RIA) after 40 days[28] with higher levels observed with the human than with the bovine form.

This paper describes the development of two methods applied to horseracing antidoping purposes for the detection of horse antibodies in serum or plasma produced after EquiGen-5® treatment. The method involved the purification of antibodies by caprylic acid (octanoic acid) precipitation. Two methods for anti-reGH antibody screening with high-throughput performances are described, the first one based on SPR-BIA (Surface Plasmon Resonance Biosensor ImmunoAssay) allowing antibody detection beyond 80 days, and the second based on ELISA (Enzyme-Linked ImmunoSorbent Assay) allowing antibody detection up to 200 days after the first injection of reGH.

2. Materials and methods

2.1 Chemicals and biochemicals

All buffers and solutions were prepared exclusively with high purity water, conductivity checked above 18 MΩ cm, and filtered online through a 0.22 µm membrane; purified plasma immunoglobulin (IgG) clarification was performed with a syringe-driven filter 0.22 µm, 13 mm, PVDF (Millipore, Bedford, MA, USA). Hydrochloric acid, Tris, glycine, phosphate buffered saline (powder, pH 7.4), hydrogen peroxide, citric acid, di-sodium hydrogenphosphate, sodium carbonate, sodium hydrogencarbonate, Tween® 20, caprylic acid and pig pancreatic insulin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). F16 MaxiSorp™ flat-bottomed 96-well microplates and sealing tapes were from Nunc A/S (Roskilde, Denmark). ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt] was from Roche Diagnostics (Meylan, France). SeaBlock™ used to prevent non-specific binding was from Interchim (Montluçon, France). Recombinant equine growth hormone, reGH (EquiGen-5®), was purchased from Bresagen Limited (Thebarton, Australia). Alkaline phosphatase-conjugated polyclonal goat anti-horse IgG chains and horseradish peroxidase (POX)-conjugated polyclonal goat anti-horse IgG chains were from Jackson ImmunoResearch (Newmarket, England). Most of the chemicals used for protein quantification, electrophoretic and Western Blot purposes were from BioRad (Marnes la Coquette, France). Chemicals for SPR were purchased from Biacore AB (Uppsala, Sweden). Serum was collected with 10 mL VT 100SP plain silicone-coated tubes (Terumo Europe, Leuven, Belgium) and plasma with 9 mL lithium heparinate tubes (Greiner Bio-One SAS, Courtaboeuf, France).

2.2 Animals and treatment

The experimental procedure was performed in agreement with animal welfare rules at the administration and sampling center of the Fédération Nationale des Courses Françaises (FNCF). Two five-year-old thoroughbreds identified as H497 (gelding) and H498 (stallion) were injected daily subcutaneously with 18 µg kg^{-1} of recombinant equine somatotropin (EquiGen-5®) for the first week of the experiment and with 25 µg kg^{-1} for the second week as described in the protocol supplied by the manufacturer. Two additional injections were given at 30 µg kg^{-1}. 50 mL of blood were collected daily in silicone-coated tubes to obtain 25 mL of serum, and in lithium heparinate tubes to obtain 25 mL of plasma. Samples were collected everyday from D_{-7} to D_{+20} and then every two days from D_{+22} to D_{+30}. Serum samples were further collected every two weeks for a period of six months (D_{+45}–D_{+210}). All samples were stored at −80 °C until their analysis. Post-race samples from a random, anonymous selection of racing horses (n = 233) were obtained from the Laboratoire des Courses Hippiques (Verrières-le-Buisson, France) and then treated under the same conditions as H497 and H498.

2.3 IgG purification and specificity against reGH

According to the modified protocol of Rojas *et al.*,[29] 870 µL of horse plasma or serum were adjusted to pH 5–6 by adding 80 µL of 0.5 M HCl and gently vortexing. Then, to each sample, 50 µL of caprylic acid were added, dropwise. The mixture was vigorously stirred every 15 min for 1 h at room temperature. After centrifugation (9000 g, 10 min), the supernatant was clarified and sterilized by filtration through a 0.22 µm membrane and stored at −20 °C up to their analysis. IgGs contained in the supernatants were quantified by a colorimetric method and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6% of acrylamide). Qualitative caprylic acid purification efficiency, in serum samples D_{-7}, D_{+15}, D_{+180}, was assessed by comparison of 14 µg of both crude serum and their corresponding purified extracts to observe the most abundant proteins present in each case. Electrophoresis separations were performed in triplicate under non-reducing conditions[30] with molecular mass standards and bovine IgG as reference. Proteins were stained with Coomassie Brilliant Blue R-250 and their concentrations were calculated using bovine gamma globulin as the standard.[31]

2.4 Western Blot

To check for any analytical pitfall resulting from a potential production of antibodies against any excipient or impurity contained in the EquiGen-5® preparation, coloured molecular mass standards and 1.5 µg of recombinant equine somatotropin separated by SDS-PAGE (4–12% acrylamide) were electrophoretically transferred to a nitrocellulose membrane (0.45 µm) in transfer buffer (Tris-glycine-SDS) at 3 mA cm^{-2} for 30 min. After a blocking step, strips of nitrocellulose were exposed for 2 h to H497 purified antibodies (D_{-2}, D_{-1}, D_{+20}, D_{+22}, D_{+135}) diluted 200 times in TBST (Tris buffered saline Tween® 20). Anti-reGH horse antibodies were detected with goat alkaline phosphatase-conjugated anti-horse IgGs. NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) allowed revelation.

2.5 Biosensor immunoassay (BIA)

Bresagen reGH was immobilized on the carboxymethylated dextran layer sensor surface of a CM5 chip using the amine

coupling kit in combination with the surface preparation wizard available from the Biacore® Q control software (Version 3.0.1, Uppsala, Sweden). The reGH was diluted to 50 μg mL^{-1} in a coupling buffer (10 mM sodium acetate, pH 4.5) and injected in flow channel 1 (Fc1) giving a total immobilization level of 5985 response units (RU). A free reference flow channel (Fc2) was used to determine the non-specific interaction between the sensor surface and the sample. The reference and detection Fcs were not serially connected and the response of the reference Fc2 was manually subtracted from the Fc1 signal. For all tests, 30 μL of purified IgGs, diluted ten times in a running buffer, were applied at a flow rate of 20 μL min^{-1} with HBS-EP as the running buffer. Regeneration was performed with 30 μL of a solution containing 5 mM NaOH and 200 mM NaCl at a flow rate of 20 μL min^{-1}. Analyses were performed at 25 °C.

2.6 Enzyme-linked immunosorbent assay (ELISA)

Flat-bottomed 96-well microplates were incubated overnight at 4 °C with 100 μL of a daily-prepared reGH solution (5 μg mL^{-1}) in 50 mM sodium carbonate/bicarbonate buffer pH 9.6 and covered with sealing tapes to prevent evaporation, edge effect and well-to-well cross-contamination. Microplates were stored for 20 min at room temperature, then the coating buffer was removed by inverting the microplates. The wells were washed three times in PBS Tween® 20 0.2%, pH 7.4 (PBST), then blocked with 100 μL of SeaBlock™ solution diluted 50 times in PBST for 1 h at 37 °C followed by three washings with 300 μL PBST. Then, samples were diluted 100 times in PBST, 2% SeaBlock™ pH 7.4. 100 μL of the samples were incubated in duplicate for 1 h 30 min at 37 °C then washed three times with 300 μL PBST. After the addition of 100 μL POX-labelled polyclonal goat anti-horse IgG chain (1 : 8000 diluted daily in PBS) to the wells, the microplates were stored for 1 h at 37 °C and washed three times with 300 μL PBST. Finally, 100 μL of the substrate solution containing 5 mg mL^{-1} ABTS and 0.03% hydrogen peroxide in 150 mM citrate/phosphate buffer pH 5 were added to each well. The conversion of substrate was allowed for over 80 min (with shaking steps every 20 min at 37 °C). The optical density was read by a microplate reader (Opsys MR Reader, Thermo Labsystems) at a wavelength of 405 nm, optical range between 0.00 and 3.50, linearity ± 1.5% above 2.50 OD. The ELISA test was applied to pre- and post-administration serum and plasma samples analysed in duplicate on three different days. Negative and positive controls were used in each test and were respectively serum samples collected before and after (D_{+15}) injection of reGH. Non-specific binding was prevented by coating the wells with porcine insulin, which differs from equine insulin by one amino acid, to a final amount of 500 ng per well. H497 and H498 serum samples were applied once in duplicate to this insulin-coated ELISA.

3 Results and discussion

3.1 Caprylic acid purification

Comparison of SDS-PAGE (Fig. 1) loaded by 14 μg of crude and purified serum after caprylic acid precipitation from horse

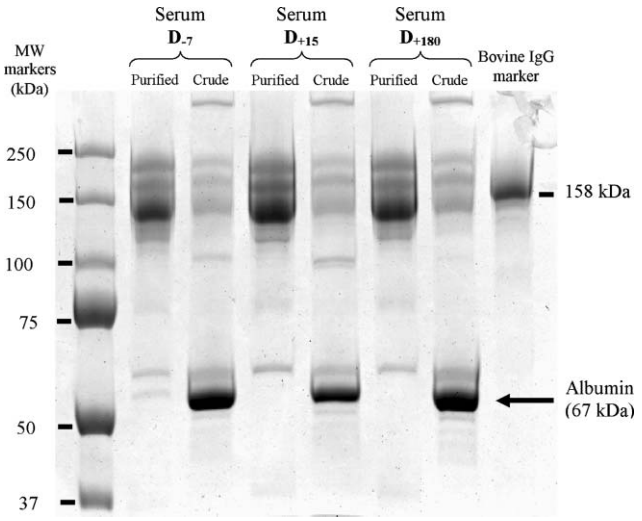

Fig. 1 SDS-PAGE of IgGs from purified serum and crude serum (horse H498). Aliquots of each sample (D_{-7}, D_{+15}, D_{+180}) previously diluted 1 : 2 in buffer and containing 14 μg proteins were separated by electrophoresis in 6% acrylamide gels under non-reducing conditions. Proteins were stained with Coomassie Brilliant Blue R-250.

H498 (D_{-7}, D_{+15} and D_{+180}) demonstrates the elimination of the main proteins. In all crude serum, albumin is present to its expected molecular weight (67 kDa) but is removed from all purified samples. Elimination of one of the most abundant proteins in serum is thus ensured by this precipitation technique. Electrophoretic analysis of the three purified extracts showed a broad predominant band between 130 and 150 kDa which is in accordance with previous data indicating that horse IgGs exhibit a molecular mass around 150 kDa.[32,33] The same location in the gel of the 158 kDa bovine IgG marker provides complementary information which enables confirmation of the presence of horse IgGs at 150 kDa. In this work, the abundance of each antibody isotype was not assessed. A complementary study was carried out to analyze protein concentrations by the method of Lowry *et al.*[31] in plasma and serum samples collected on both horses (H497, H498) at D_{-7}, D_{+15} and D_{+180} (serum only) (Table 1). Owing to the presence of fibrin, the plasma protein content was higher than for the serum. The comparison of the total protein concentration obtained before and after caprylic acid precipitation shows a huge and expected depletion of protein. In all the analysed samples, an average of 70% of proteins were removed by caprylic acid precipitation consisting chiefly of albumins in accordance with the previous result observed by electrophoresis.

The use of caprylic acid in the small-scale purification of IgGs from various mammalian sera has already been described.[34,35] Precipitation conditions with caprylic acid were described in the field of antivenom production from hyper-immune horse plasma.[29,32,33] According to the modified protocol of Rojas *et al.*,[29] the efficiency of caprylic acid precipitation was assessed in this work through electrophoresis and quantification of total proteins and has proved to be a powerful precipitation technique to obtain purified IgGs from serum or plasma.

Table 1 Protein concentration in serum and plasma samples from horses H497 and H498 before and after caprylic acid precipitation with the Folin–Lowry method. Each sample was quantified four times (mean ± SD)

Horse	Matrix	Sample	Protein concentration/g L^{-1}		Elimination yield (%)
			Before precipitation	After precipitation	
H497	Serum	D_{-7}	74.9 ± 0.5	19.9 ± 0.2	73
		D_{+15}	79.1 ± 2.4	23.5 ± 0.6	70
		D_{+180}	78.4 ± 2.0	22.4 ± 0.1	71
	Plasma	D_{-7}	80.5 ± 1.7	20.2 ± 0.1	75
		D_{+15}	88.3 ± 1.5	21.9 ± 0.3	75
H498	Serum	D_{-7}	81.6 ± 3.9	24.6 ± 0.5	70
		D_{+15}	93.6 ± 0.7	27.8 ± 0.5	70
		D_{+180}	82.3 ± 0.7	25.5 ± 0.7	69
	Plasma	D_{-7}	85.8 ± 0.6	25.8 ± 0.6	70
		D_{+15}	95.6 ± 1.5	29.9 ± 0.5	69

3.2 Western Blot characterization of antibodies

Antibody production as a consequence of reGH administration was investigated using the Western Blot technique. Purified IgGs from serum samples collected on animal H497, before (D_{-2}, D_{-1}) and after treatment (D_{+20}, D_{+22}, D_{+135}), were analysed as described previously.[26] Recombinant equine growth hormone from EquiGen-5® and its coloured molecular mass marker, separated on an acrylamide gel and transferred onto nitrocellulose membrane, revealed the presence of antibodies in horse serum collected 20 and 22 days after reGH administration (Fig. 2).

This result demonstrates the production of antibodies raised against reGH following its administration and confirms that antibodies are not raised against any excipient or impurity potentially contained in the EquiGen-5® preparation. This is in good accordance with previous results obtained in cows where antibodies raised against rbGH could be detected with this technique 28 days after rbGH administration.[26] However, no antibody was detected in non-treated horses (H497 D_{-2}, D_{-1} and H498 D_{-2}, D_{-1}) as observed in cows.[26] Antibodies could be detected 20 days after met-eGH administration to horses compared to 40 days with the RIA technique when hGH and bGH were administered to horses.[28] According to this preliminary study, Western Blot appears to be an appropriate technique to detect antibodies to reGH but is incompatible with routine analysis requirements. High-throughput techniques such as SPR-BIA and ELISA were therefore investigated.

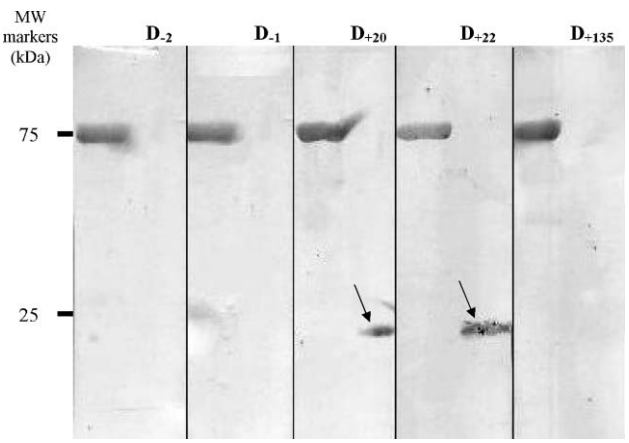

Fig. 2 Western Blot analysis to detect the presence of anti-reGH antibodies from serum collected at D_{-2}, D_{-1} and D_{+20}, D_{+22}, D_{+135} (horse H497). Specific interaction between the reGH and IgGs produced by the treated horse is shown with arrows.

3.3 SPR-biosensor immunoassay (SPR-BIA)

Serum samples collected from horse H497 from D_{-7} to D_{+210} and purified with a caprylic acid precipitation were diluted ten times in HBS-EP running buffer and injected on the Biacore® Q sensor chip coated with reGH. The SPR-BIA technology allowed measurement of the binding of antibodies on the coated chip by an increased response on the sensorgram. The kinetics of antibody production were established from the different sensorgrams obtained from the analysis of horse H497 serum samples (Fig. 3). Specific antibodies were detected as early as ten days after the first reGH injection. The maximum concentration of antibodies was observed after 20 days which was followed by a fast decrease up to 45 days. Then, the decrease slowed down to reach the baseline after 150 days. These results were in good accordance with those obtained with the Western Blot (Fig. 2) where antibodies

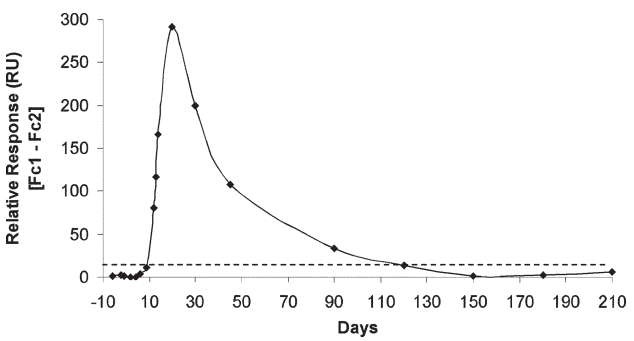

Fig. 3 Kinetics of antibody production from serum (horse H497) measured by SPR-BIA and applied to the Fc1 reGH channel and Fc2 control channel. The figure shows Fc1 − Fc2 obtained from samples collected from D_{-7} to D_{+210}. The threshold value is represented by the dashed line (15 RU).

raised against met-eGH in serum samples collected at D_{+20} and D_{+22} could be detected whereas they could not in the sample collected at D_{+135}. In order to eliminate the noise resulting from the measurement by SPR-BIA and to consider only the signal arising from the binding of antibodies on the coated chip, a threshold value was defined as three times the average baseline value, corresponding to 15 RU. In this context, the SPR-BIA test allowed detection of anti-reGH horse antibodies from ten days up to 90 days after reGH administration, leading to a detection window of 80 days. The SPR-BIA technology has proved to be a powerful device in the field of antibody detection with good sensitivity, specificity and high throughput thanks to its automatic functions and low sample requirement. Recent studies in the field of growth hormone abuse detection have also investigated this SPR technology for the screening of recombinant bovine GH in commercial injection preparations.[36]

3.4 Enzyme-linked immunosorbent assay (ELISA)

Another screening test, dedicated to the detection of antibodies raised against reGH and based on ELISA, was also set up in this study. Serum and plasma samples collected on horses H497 and H498 were purified by a caprylic acid precipitation as described above and analysed by an optimized reGH-coated ELISA test. Even if antibody studies are usually carried out in serum, it was decided to check out both matrices' behaviour for the detection of anti-reGH specific antibodies because plasma is the medium most commonly used in horse racing for drug testing. After caprylic acid purification, samples were diluted 100 times in dilution buffer (PBST, 2% SeaBlock™). To evaluate the repeatability of the measurement, analyses were performed on three different days in duplicate. Samples were also analysed once in duplicate on insulin-coated ELISA to assess specificity. Fig. 4(a) and 4(b) respectively present H497 and H498 antibody production kinetics as obtained by ELISA. For both matrices, $OD_{405\ nm}$ kinetics were very well superimposed, probably due to the similar huge depletion of non-IgG proteins through caprylic acid precipitation. No $OD_{405\ nm}$ evolution was observed when samples were studied on the insulin-coated ELISA, leading to the conclusion that there is no interaction between IgG samples and insulin. A specific interaction between reGH-coated ELISA and samples was observed starting from D_{+7} to D_{+30} with a maximum at D_{+15} with a very low standard deviation leading to a good repeatability of the method. The superimposition of H497 and H498 sera samples collected over the period of six months and applied to reGH- and insulin-coated ELISA is presented in Fig. 5. The large range of collecting days shows an immunisation profile in three steps. A first one arising from samples D_{-7} to D_{+7} is defined as the non-treated animal and latency phase where no antibodies could be detected. The second step is consistent with an exponential antibody production from D_{+8} to D_{+16}. The third is thought to be the step where antibodies are catabolised or removed in immune complexes. These results are consistent with those obtained with the Western Blot (Fig. 2) and SPR-BIA (Fig. 3) techniques. As described in the SPR-BIA part, a threshold value was defined as three times the average baseline value, corresponding to $OD_{405\ nm} = 1.2$.

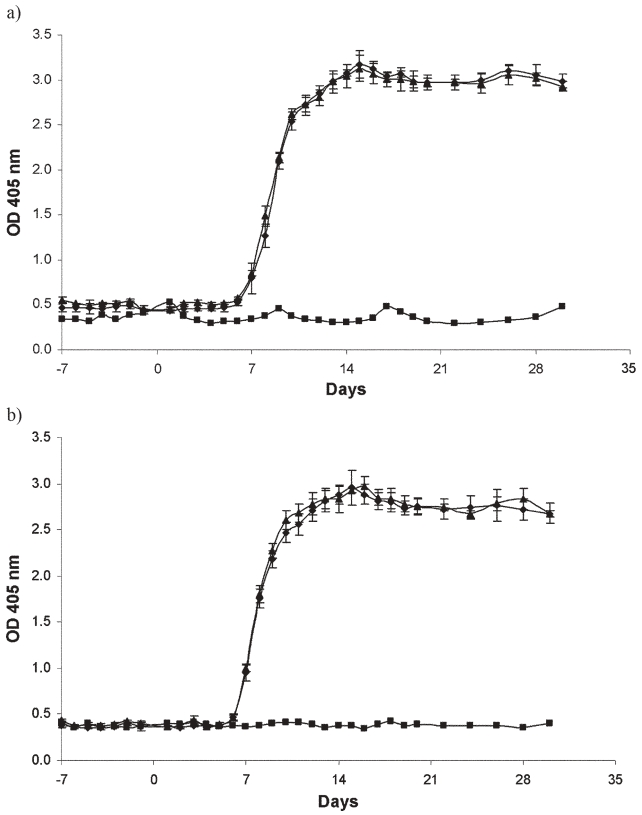

Fig. 4 Kinetics of antibody production from serum extracts applied to reGH-coated wells (◆) and insulin-coated wells (■) [horse H497 (a) and H498 (b)]. Comparison with antibody production from plasma extracts (▲) applied to reGH-coated wells. Samples collected from D_{-7} to D_{+30}.

In this case, the ELISA test allowed the detection of reGH antibodies from eight days to more than 210 days after the first injection of reGH, giving a detection window of 200 days (between six and seven months).

The differences observed between SPR-BIA (Fig. 3) and ELISA (Fig. 4) kinetic shapes could be explained as a result of the strong elution conditions necessary to change specific protein folding to remove all protein–protein interactions between reGH and IgGs on the sensor chip in order to regenerate it. Different elution conditions have been attempted to reach the best regeneration response without decreasing the reGH level linked on the chip. Conditions for horse IgG elution (200 mM NaCl associated with 5 mM NaOH) were optimized with huge ionic strength and basic pH. Recently, similar basic conditions have been reported to remove all interactions on a sensor chip between the recombinant bovine growth hormone (rbGH) and its specific monoclonal and polyclonal antibodies obtained from mouse and rabbit immunisation.[36] The reGH-coated ELISA test was used as a powerful, high-throughput screening method to investigate a post-race random population study on the potential misuse of recombinant equine growth hormone (Table 2). A total of 233 plasma samples arising from 79 mares, 121 geldings and 33 stallions were analyzed in duplicate and their $OD_{405\ nm}$ values were monitored. No differences between mares, geldings, stallions could be noted. The measured $OD_{405\ nm}$

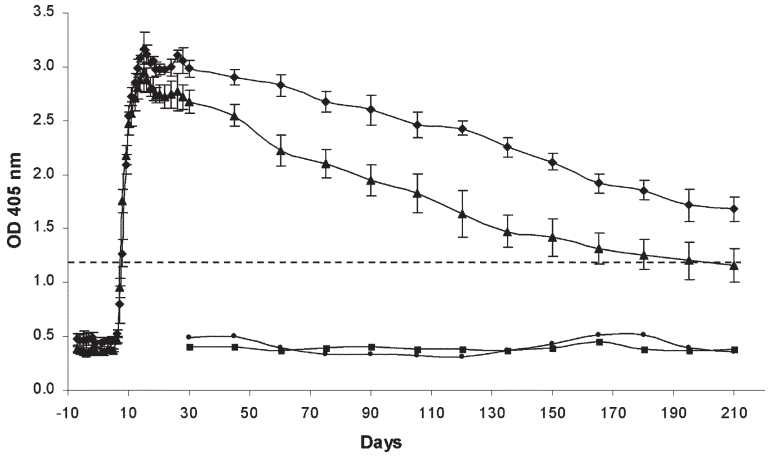

Fig. 5 Superimposition of H497 (◆) and H498 (▲) kinetics of antibody production from serum extracts applied to reGH-coated wells, and H497 (■) and H498 (●) to insulin-coated wells. Samples collected from D_{-7} to D_{+210}. The threshold value is represented by the dashed line ($OD_{405\ nm}$ = 1.2).

Table 2 Random population study of post-race plasma samples and comparison with data obtained from plasma samples collected from horses H497 and H498 for seven days before treatment (mean ± SD)

Horses	Effective	$OD_{405\ nm}$
Mares	79	0.36 ± 0.05
Geldings	121	0.38 ± 0.08
Stallions	33	0.36 ± 0.05
H497 (gelding)	7 blanks	0.51 ± 0.03
H498 (stallion)	7 blanks	0.40 ± 0.02

signals were furthermore consistent with the measurements of plasma samples obtained from horses H497 and H498 before reGH administration.

The characterization of anti-reGH horse antibody production following treatment with recombinant equine growth hormone can be explained by the supernumerary methionine and immunoreactive sequences already reported for the equine growth hormone[37] associated with a probable low inflammation reaction at the injection point. Indeed, low inflammation reactions at the injection point have been observed for both horses. Besides the additional methionine of this reGH, it was proposed that human, ovine, bovine and equine GHs share a common antigenic area corresponding to the amino acid sequence 73–128. This suggests that SPR-BIA or ELISA tests coated with GHs containing this sequence would be appropriate for the detection of anti-GH antibodies from other species.

It must nevertheless be kept in mind that the immunological response obtained after treatment with EquiGen-5® might be different from one horse to another, resulting in inter-individual variability of antibody production. Indeed, some horses might have no production of anti-reGH antibodies or have antibodies lower than the decision limit; consequently, a further population study with 1000 post-race horses and some animals treated with reGH different from thoroughbreds is being performed. Furthermore, an evaluation to give more information on the animals' exposure levels to this forbidden molecule in order to adjust the background noise and to assess the ELISA threshold according to statistical analysis is in progress.

The detection of such antibodies in the field of human or animal protein treatment is not only reported in the case of GH but also when insulin,[38] prolactin[39] or EPO[40] treatments are performed. In this context, the monitoring of antibodies produced as a consequence of the exogenous administration of a xenobiotic protein could be used as a screening method in horse antidoping analysis.

4 Conclusion

Two high-throughput screening methods to detect the production of anti-reGH horse antibodies as a consequence of EquiGen-5® treatment were set up in serum or plasma samples. Caprylic acid precipitation is a key step in the results obtained since it allows for an efficient IgG purification, leading to a huge improvement in the sensitivity of detection. Results obtained from both matrices are similar, leading to the conclusion that the antibody screening can be performed with blood collecting tubes already used in racecourses. The ELISA and SPR-BIA technologies described in the present article allow the detection of anti-reGH horse antibodies for a period of 80 days with SPR-BIA or 200 days with ELISA after reGH administration, with efficient repeatability and specificity of the methods. An experiment with eight other horses (standardbreds) is currently being performed to follow the production of anti-reGH horse antibodies and get more information concerning this detection of antibodies in order to cross the results with the IGF-1 level. Obviously, this detection window will never be reached with any direct detection method of met-eGH and seems to be a powerful tool as a screening method in horseracing antidoping control. Indeed, it could be used as a complementary analysis when the IGF-1 level is slightly below or above its proposed threshold. In the field of horse antidoping, this could also provide an inexpensive and important tool for the indirect detection of insulin or rhEPO misuse through their anti-insulin or anti-rhEPO horse antibodies, supposing that it can be adapted.

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