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An Immuno-PCR screen for the detection of CJC-1295 and other Growth Hormone Releasing Hormone analogues in equine plasma.

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Abstract

CJC-1295 is a 30 amino acid peptide-based drug that stimulates the release of growth hormone (GH) from the pituitary gland. It is unique among performance enhancing peptides due to the presence of a reactive maleimidopropionic acid group that covalently links the peptide to free thiols on the surface of plasma proteins. Once conjugated, CJC-1295 remains active in the bloodstream for significantly longer than non-conjugated peptide-based drugs that are rapidly excreted. Conjugation of CJC-1295 to plasma proteins prevents its detection by top-down mass spectrometry based peptide screening protocols as it effectively becomes a macromolecular protein with an undefined molecular weight. Using a pair of monoclonal antibodies raised against the CJC-1295 peptide, we present an Immuno-Polymerase Chain Reaction (I-PCR) assay that is capable of detecting the CJC-1295-protein conjugate at concentrations down to 0.8 pg/mL. Detection of endogenous equine GHRH necessitated a screening threshold for CJC-1295 in equine plasma of 50 pg/mL. The effectiveness of the assay for controlling the illicit use of CJC-1295 was confirmed in equine blood samples after administration in thoroughbred race horses.

Introduction

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CJC-1295 was first described in 2005 as a 30 amino acid peptide analogue of growth hormone releasing factor 1-29 (GRF 1-29, also known as sermorelin) [1,2]. GRF 1-29 is a truncated form of the 44 amino acid growth hormone releasing hormone (GHRH). Compared to the original sequence, CJC-1295 incorporates 4 amino acid substitutions to decrease the rate of degradation by proteases, and an additional lysine residue at the C-terminus (Table 1). This C-terminal lysine has been derivatised with a maleimidopropionic acid group, sometimes called a drug affinity complex (DAC). The DAC allows the peptide to be covalently linked to free thiol groups present on the surface of plasma proteins including serum albumin [3]. The conjugation of CJC-1295 to plasma proteins dramatically increased the biological stability of the drug, extending plasma half-life up to 6 – 8 days in humans [3]. The same study showed that a single dose of CJC-1295 increased plasma levels of GH (2-10 fold) and IGF-1 (1.5-3.0 fold) which persisted for up to 6 days and 14 days respectively.

CJC-1295 was originally developed as an alternative form of growth hormone (GH) therapy. While recombinant growth hormone (rGH) is now commonly used for treating forms of growth hormone deficiency, it has been noted that rGH does not mimic the normal pulsatile expression of endogenous GH [4]. In comparison, CJC-1295 elevates endogenous levels of GH and IGF-1 while maintaining more normal pulsatile levels of GH [5]. Development of CJC-1295 for clinical use ceased in 2006 in response to adverse drug reactions including the death of a patient during a phase II clinical trial for the treatment of HIV related lipodystrophy [6]. Despite never being accredited by the FDA or other regulatory bodies, CJC-1295 is now commonly sold over the internet as a peptide-based drug [7,8], being marketed for its alleged performance-enhancing and anti-aging properties, alongside similar peptides such as modified GRF 1-29 (mGRF 1-29) and CJC-1295-without-DAC (Table 1). In 2011 twelve Australian rugby league players were alleged to have used CJC-1295 and GHRP-6. Despite the lack of analytical evidence, the players pleaded guilty to the use of these performance enhancing drugs [9]. Given the known use of CJC-1295 in professional human sport, it clearly presents a potential threat to the integrity of the horse racing industry.

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The ready availability of CJC-1295 and its potential as a sports doping agent has prompted the publication of several analytical methods that claim to detect CJC-1295, but in all cases these tests have targeted peptides lacking the DAC and are thus chemically distinct from the peptide defined by Teichman et al. [10–13]. The CJC-1295-protein conjugates formed in the blood stream have an undefined molecular mass too large for effective mass spectrometry analysis. They would also be difficult to extract from plasma or serum by SPE due to the abundance of unmodified serum proteins in the sample matrix. Yet the extended plasma half-life of CJC-1295 compared to other peptide-based drugs makes it an attractive analytical target. As the conjugated CJC-1295 has physical properties equivalent to a protein instead of a peptide, a pair of complimentary monoclonal antibodies were generated, allowing the development of a sandwich immunoassay. Here we describe an immuno polymerase chain reaction (I-PCR) assay capable of detecting CJC-1295 protein conjugates as well as other GHRH analogues in equine plasma.

Experimental Methods

Reagents and materials

Nalgene rapid flow sterile disposable filter units, Zeba columns (0.5 mL), sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, 10 x PBS solution, EDTA, sodium chloride, sodium hydrogen phosphate, sodium dihydrogen phosphate, 10x Dulbecco's PBS, MicroAmp Fast 96-well reaction plates, TaqMan gene expression master mix, 20% w/v sodium dodecyl sulphate (SDS), bromophenol blue and SuperSignal West Femto maximum sensitivity substrate were purchased from Thermo Scientific (Waltham, MA, USA). L-cysteine, dithiothreitol, tween 20, glycerol, tris base, carbonate-bicarbonate buffer, Gelatin from porcine skin, N-Laurosarcosine, and Proclin 300 were purchased from Sigma-Aldrich (St Louis, MO, USA). BSA was purchased from Bovogen (Victoria, Australia). RD4 and RD6 monoclonal antibodies to CJC-1295 were generated by the Monash Antibody Technology Facility (Clayton, Australia). GRF 1-29, mGRF 1-29, CJC-1295 and CJC-1295-without-DAC (Table 1) were synthesized by Auspep (Tullamarine, Australia). Donkey $F(ab')^2$ polyclonal secondary antibody to mouse IgG, HRP, preabsorbed (ab98771) was

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purchased from Abcam (Cambridge, MA, USA). Anhydrous disodium hydrogen orthophosphate was from Biolab (Victoria, Australia). Mini Protean TGX stain free Any kD gradient Gels (10-well), 0.2uM PVDF Trans-blot Turbo Transfer Packs, 10x Tris/Glycine/SDS running buffer, Precision Protein StrepTactin-HRP conjugate and Precision plus western c standards were all purchased from Bio-Rad (Hercules, CA, USA). All aqueous buffers were prepared in Milli Q grade water (Millipore, Billerica, MA, USA).

Oligonucleotides

DNA primers, probe and template were synthesized to order by either Integrated DNA Technologies (Coralville, Iowa, USA) or Bioneer Pacific (Kew, Australia). The oligonucleotide label attached to the secondary antibody was 5'-SH-ATGTTTTCCCAGTCACGACGTTGAGGAATGCCCGTTCTGCGAGGCGGTGCTGTGTGAAATTGTTATCCGCTCA-3'. A sulfhydryl linker group was incorporated at the 5' end to allow reactivity with the sulfo-SMCC bifunctional crosslinker. The forward (5'-GTTTTCCCAGTCACGACGTTG) and reverse (5'-TGAGCGGATAACAATTTCACACAG-3') primers were complimentary to the 5' and 3' ends of the oligonucleotide label respectively, allowing PCR amplification while the TaqMan probe (5'-CCTTACGGGCAAGACGCTCC-3') labelled with a FAM (Carboxyfluorescein) fluorophore and a TAMRA (Tetramethylrhodamine) quencher is complimentary to the central region of the oligonucleotide label and is degraded by the TAQ polymerase. The relationship between the four different oligonucleotides is detailed in Figure 1.

Plasma samples

Equine blood samples were obtained from Racing Victoria Limited (Flemington, Australia), and supplied as part of a routine testing program. Whole blood was collected into heparinised tubes. Plasma was prepared from whole blood samples by spinning vials at 3000 x g for 15 minutes and decanting the clear liquid above the cell pellet.

Surface plasmon resonance (SPR) analysis

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Surface plasmon resonance studies were conducted on a ProteOn XPR36 SPRi biosensor equipped with GLH 6x6 array chips (BioRad, Hercules, CA, USA). The chips were conditioned with 0.5% w/v SDS, 50 mM NaOH and 100 mM HCl. Five channels of the GLH chip were immobilised with different monoclonal antibody clones, and the fifth channel was treated as a blank. All immobilisations were done vertically with an antibody concentration of 50 µg/mL in sodium acetate buffer (pH 4.5). Following immobilisation, all six channels were deactivated using ethanolamine. CJC-1295-without-DAC peptide (500 nM) was then pumped across all six channels to be captured on the immobilised antibodies. The chip was rotated horizontally and monoclonal antibody clones pumped across individual channels to identify those antibodies that bound to the peptides already captured by the immobilised antibodies.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

SDS-PAGE and western blotting were performed as described in Timms *et al.* [14]. RD4 and RD6 monoclonal antibodies were diluted 1 in 1000 for use. Donkey $F(ab')^2$ polyclonal secondary antibody to mouse IgG (HRP conjugated) was used at 1 in 20000. StrepTactin-HRP conjugate was used at 1 in 50000.

Standard solutions and buffers

GRF 1-29, mGRF 1-29 and CJC-1295-without-DAC were each prepared as 1 mg/mL stock solutions in Milli Q water, before being stored as 100 µL aliquots at -70°C. Working solutions of CJC-1295-without-DAC were prepared by diluting stock solutions with 1% BSA-PBS-T (blocking solution). The blocking solution was prepared by adding 10 g BSA to 1 mL ProClin 300 and 800 mL of 1 x PBS and 500 µL of Tween 20. The solution was adjusted to pH 7.4 and made up to 1 litre with 1 x PBS. It was then filter sterilised by passage through a 0.2 µm rapid flow sterile disposable filter unit. The solution was stored at 2-8°C for up to 6 months.

Preparation of Quantitative Analytical Standards

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The standard curve was prepared by diluting calibrant peptide (CJC-1295-without-DAC) in blocking solution. Calibrant concentrations were 0, 0.8, 4, 20, 100, 500 and 2500 pg/mL. Blocking solution was used as a negative control.

Preparation of Bovine and Equine Serum Albumin-CJC-1295 conjugates

A single 0.5 mg vial of CJC-1295 was dissolved in 300 µL of 10mM EDTA, 100mM sodium phosphate (pH 7.0), and 200 µL of 25 mg/mL BSA, prepared in the same solution, added to give a final volume of 500 µL. The vial was incubated for 4 hours at 25°C after which the sample was transferred to a 1.5 mL microfuge tube and centrifuged for 10 minutes at 17000 x g. The sample was then divided into 4 equal aliquots and desalted with 0.5 mL zeba desalting columns equilibrated in 1 x PBS according to the manufacturer's instructions. ESA CJC-1295 was prepared in the same manner except 110 µL of equine plasma mixed with 90 µL of 10mM EDTA, 100mM sodium phosphate, pH 7.0 was used in place of BSA.

Preparation of DNA labelled RD6 antibody:

A 10 mM solution of Sulfo-SMCC was prepared in 100 mM sodium phosphate (pH 7.4), 1 mM EDTA and 4 µL added to 100 µL of the RD6 detection antibody diluted to 800 µg/mL in 100 mM sodium phosphate (pH 7.4), 1 mM EDTA. The reaction was allowed to proceed for 2 hours at 5°C or 30 minutes at room temperature. Free SMCC was removed from the RD6-SMCC antibody using a 0.5 mL Zeba desalting column according to the manufacturer's instructions. The column was equilibrated with 100 mM sodium phosphate (pH 7.4), 1 mM EDTA. Taqman Label 2 Thiol oligonucleotide was resuspended in Milli Q water to a stock concentration of 2 µg/µL. To activate the oligonucleotide a 50 µL aliquot of the stock was diluted with an equal volume of 100 mM sodium phosphate (pH 8.4), 1 mM EDTA before adding 25 µL of 500 mM DTT prepared in the same buffer. Activation was allowed to proceed for 2 hours at room temperature. The activated oligonucleotide label was desalted using 0.5 mL Zeba column equilibrated with 100 mM sodium phosphate (pH 7.4), 1 mM EDTA. The recovered oligonucleotide was mixed with the RD6-SMCC detection antibody and the reaction allowed to proceed overnight at 4°C. The following day the reaction was

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quenched by addition of 1 µL of 500 mM cysteine in 1 x PBS. The RD6-SMCC-DNA detection antibody was stored at 4°C.

Preparation of CJC-1295 immunoassay plates

The RD4 capture antibody was diluted to 1600 ng/mL in 50 mM carbonate-bicarbonate buffer (pH 9.6), and 25 µL added to each well of a MicroAmp Fast 96-well reaction plate. Plates were sealed and left at 2-8°C for a minimum of 16 hours after which the contents were discarded and 195 µL of 1% (w/v) BSA-PBS-T blocking solution added. Plates could be stored in 1% (w/v) BSA-PBS-T blocking solution at 2-8°C for up to 14 days before use.

Immuno PCR Assay

The blocking solution was discarded from a RD4 coated reaction plate and 50 µL of standards, controls and plasma for testing were transferred to individual wells. After 2 hours at room temperature, the contents of the plate were discarded and the plate washed 4 times with 195 µL/well of PBS-T. Plates were blotted onto absorbent paper between each wash. Following the last wash, 50 µL of 50 ng/mL RD6-oligonucleotide conjugate was added to each well. After 2 hours at room temperature, the contents of the plate were discarded and the plate washed 8 times with 195 µL /well of wash buffer (0.8% (v/v) sarcosyl, 2 M NaCl, 5 mM EDTA and 50 mM Tris-HCl, pH 7.4), followed by 8 times with Milli Q water. Plates were blotted onto absorbent paper between washes. 25 µL of Taq gene expression mix (1x Taq gene expression master mix, 1 µM forward primer, 1 µM reverse primer and 240 pM TaqMan probe) was added to each sample well. Real time PCR was performed using a StepOnePlus thermal cycler (Applied Biosystems). The reaction was activated for 10 minutes at 95°C, after which 35 cycles were performed using a 15 second denaturation step at 95°C and a 55°C annealing/extension step for 1 min. Data analysis was performed using the StepOne software v2.3. Total analysis time is 55 minutes.

Validation

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The method was validated with respect to linearity, limit of detection, specificity, precision and accuracy. Linearity was determined over a range form 0.8-2500 pg/mL using six samples at the following concentrations 0.8, 4, 20 100, 500, 2500 pg/mL. The limit of detection was defined as the threshold cycle (Ct) of the 0 pg/mL negative control minus 3 standard deviations and was assessed over a range of concentrations from 0.8-500 pg/mL. Specificity was established using 974 equine plasma samples. Precision was assessed by analysis of administration samples with three separate experiments performed by two operators on three different days, using eight samples tested in triplicate. Accuracy was determined by spiking mGRF 1-29 into equine plasma at 500 and 1000 pg/mL and analysing samples in triplicate. To limit protease activity, EDTA was added to equine plasma to a final concentration of 20mM before the addition of mGRF 1-29.

Administration Study

Equine administration studies were performed using four adult Thoroughbred geldings. All were in race fit condition but no longer being entered for racing competition. A 10 mg injection of CJC-1295 was delivered by intramuscular injection. Blood samples were collected on days 1, 2, 3, 4, 5, 8, 12 and 15. The same 4 horses were also used for administration of mGRF 1-29 with a washout period of more than 6 months between administrations of the two peptides. Modified GRF 1-29 was administered at a dose of 500 μg/animal, also by intramuscular injection, on three successive days. After the final administration, blood samples were collected at 0.25, 0.5, 1, 2, 3, 4, 5 and 6 hrs. All negative control samples were collected prior to administration. Whole blood was collected into heparinised vacuum tubes, immediately centrifuged and the plasma transferred to fresh polypropylene tubes before being immediately frozen. All samples were stored frozen at -70°C until used for analysis. Dosage rates for both CJC-1295 and mGRF 1-29 were based on internet research of dosages recommended for human clients. These experiments were performed with the approval of the University of Melbourne Animal Ethics Committee (Ethics approval number 1413071.1)

Results

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Validation of the anti-CJC-1295 antibodies.

To generate antibodies specific for conjugated CJC-1295, mice were immunised with CJC-1295 conjugated to bovine serum albumin. Hybridomas were prepared and clones selected for their ability to bind to the peptide CJC-1295-without-DAC (data not shown). Two clones (RD4 and RD6) were ultimately selected based on their high affinity for CJC-1295, both as a free peptide and as a protein conjugate.

SPR was used to confirm the non-competitive binding of these antibodies to CJC-1295, ensuring the antibodies were compatible for their use in a sandwich type immunoassay (Figure 2). In the absence of any immobilised primary antibodies, no CJC-1295-without-DAC peptide would be retained on the chip and as a result no secondary antibody binding was detected (Figure 2A and C). When the RD4 antibody was immobilised on the surface of the chip and incubated with CJC-1295-without-DAC peptide, the RD6 antibody showed high affinity binding to the peptide bound to RD4 (Figure 2B). In the reverse experiment, RD6 antibody was immobilised on the chip and bound to CJC-1295-without-DAC peptide before being exposed to different antibody clones. In this experiment the RD4 antibody showed high affinity binding to the captured peptide (figure 2D). In comparison, the RE1, RE3 and RG5 antibodies did not show any peptide binding activity to the RD6-peptide complex, despite being able to bind to CJC-1295 peptide alone (data not shown). SPR was also used to measure the binding affinity of the RD6 antibody to either RD4 captured CJC-1295-without-DAC or to RD4 captured BSA-CJC-1295 conjugate. Both CJC-1295-without-DAC and the BSA-CJC-1295 conjugate bound to RD6 with high affinity, with Kd of 1.03 nM and 2.3 nM respectively.

The specificity of these antibodies was confirmed by western blotting. RD6 identified several protein bands in the equine plasma sample treated with CJC-1295 (Figure 3, Lane 5). The molecular weights of these bands matched those of plasma proteins known to be associated with CJC-1295, ie albumin at 67 kDa, the IgG light chain at 27 kDa, the IgG heavy chain at 50 kDa and fibrinogen monomers at 47 and 56 kDa [3]. No bands were detected in untreated equine plasma (Lane 4). Likewise, the BSA-CJC-1295 conjugate (Lane 3) but not

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unmodified BSA (Lane 2) was detected by the RD6 antibody. Similar results were obtained from western blotting with the RD4 antibody (data not shown). These western blotting results indicate that the RD6 and RD4 antibodies are capable of identifying CJC-1295 conjugated to a number of different proteins present in equine plasma.

Development of the immuno-PCR assay

Having validated the RD4 and RD6 antibodies against target antigen, an I-PCR assay was developed. The first monoclonal antibody (RD4) is immobilised on the surface of a 96-well polypropylene PCR plate and captures the CJC-1295-protein conjugate in solution. The second antibody (RD6) serves as the detection antibody and carries the oligonucleotide label instead of the more typical horse radish peroxidase enzyme. The presence of the secondary antibody is detected by a quantitative PCR (qPCR) reaction using a TaqMan fluorescent probe. The oligonucleotide label was designed to be as compact as possible with a DNA sequence optimised for efficient PCR amplification. The compact size also allowed the chemical synthesis of the oligonucleotide label including the sulfhydryl tag at the 5' terminus (Figure 1).

Amplification curves for CJC-1295-without-DAC peptide standards and negative controls are shown in Figure 4 with standards ranging from 0.8 to 2500 pg/mL. The CJC-1295-without-DAC peptide was used as the analytical standard to ensure accurate quantitation. The assay was also capable of detecting the mGRF 1-29 and GRF 1-29 peptides (table 1. Data not shown). Technical difficulties prevented the preparation of a reproducible CJC-1295-protein conjugate with a defined concentration. Standards were prepared in 1% BSA-PBS-T following the convention of most commercial ELISA kits.

The amplification threshold cycle (Ct) is defined as the PCR cycle number at which the fluorescence intersects the threshold in the amplification plot and was determined automatically by the StepOnePlus software. In Figure 4 the threshold is shown as the horizontal line drawn across the graph from a log value of 0.398789 on the y axis. Both the no antigen control (Figure 4G) and the no template control (Figure 4H) show amplification

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below that of the 0.8 pg/mL standard (Figure 4F). The limit of detection for the CJC-1295 I-PCR assay was investigated using 8 replicates across a range from 0.8-500 pg/mL (Table 2). The LOD was defined as the average threshold cycle value of the no antigen control minus 3 standard deviations. Given an average threshold cycle value of 26.18 for the no antigen control and a standard deviation of 0.72, the LOD was set at 0.8 pg/mL.

The high background observed in the no antigen control is typical for I-PCR assays which tend to suffer from background issues associated with low level detection of analytes [15]. Background can be associated with low level hydrolysis of the TaqMan probe, incomplete removal of the oligo-labelled detection antibody, inadequate blocking of the assay wells or any combination of these factors. In order to reduce background signal levels, a number of different blocking agents were trialled including ovalbumin, BSA and milk powder, with and without tween 20, herring sperm DNA or tRNA. Best results were obtained with plates blocked with 1% BSA PBS-T overnight. Background levels were also minimised by extensive washing after incubation with the oligonucleotide labelled secondary antibody.

A summary of the validation experiments is presented in Table 3. There was a high level of linearity over the concentration range with correlation coefficients of $R^2 > 0.99$ obtained with PCR efficiencies of 92%. The amplification efficiency is calculated by the StepOnePlus software using the slope of the regression line in the standard curve. A slope close to -3.32 indicates optimal, 100% PCR amplification efficiency. Assay accuracy was established by spiking the GHRH analogue, mGRF1-29 into EDTA treated equine plasma at 500 and 1000 pg/mL with an average accuracy of 16%.

Precision was assessed in terms of both threshold cycle and recorded drug concentration. On a semi log plot such as that in Figure 4, a small change in the cycle threshold can lead to a large variation in the experimentally determined concentration, leading to large errors. Thus the coefficient of variation for experimentally determined drug concentration was always much greater than that for cycle threshold. Accurate pipetting was essential for reproducibility, as was complete removal of residual liquid from wells. In a 25 µL reaction an additional 2.5 µL in residual volume represents a 10% error. Thus PCR plates were

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vigorously tapped onto blotting paper to remove as much residual liquid as possible. This led to an average percentage CV for precision of 1.26 % for threshold cycle and 16.79% for concentration when measured in three experiments performed by 2 operators using 8 samples, each with three replicates (Table 3). The latter value is similar to those of typical ELISA assays, upon which the I-PCR assay is based. In comparison, the percentage CV for cycle threshold was always substantially lower.

Endogenous levels of GHRH in equine plasma.

A high degree of sequence homology exists between equine and human GHRH (up to 86% identity across amino acids 1-29 as shown in table 1). Thus it was not surprising that the RD4 and RD6 monoclonal antibodies recognised and bound to equine GHRH. In order to establish the endogenous level of GHRH detected in equine plasma, 974 samples were analysed. The observed concentration in these samples ranged from 0.12 pg/mL to 82 pg/mL with a median of 4.31 pg/mL and an average concentration across all samples of 5.75 pg/mL. These values are similar to those observed in humans where the mean fasting GHRH level observed in normal adults was 10.3 pg/mL [16]. Levels of GHRH fluctuate during the course of a day to mirror those of GH, whose release it governs. Growth hormone levels have been shown to increase in response to food, exercise and sleep, so it is expected that these same activities will regulate levels of GHRH in the blood. The detection of endogenous GHRH by this assay necessitates the establishment of a screening threshold that allows for such fluctuations. If a screening limit of 50 pg/mL is adopted, the assay is expected to have a false positive rate of only 0.3% (figure 5). Due to the cross-reactivity of the RD4 and RD6 antibodies with equine GHRH, mass spectrometry analysis of the CJC-1295 peptide will be required to confirm the presence of CJC-1295 in a sample. This will be achieved by antibody capture and tryptic digestion to produce proteotypic CJC-1295 peptides.

Equine administration studies

In order to determine that CJC-1295 could be detected over and above background levels of equine GHRH, an equine administration study was performed in four thoroughbred geldings.

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CJC-1295 was administered to each horse by intramuscular injection at a dose of approximately 20 μg/kg, which is in the low range compared to other administration studies (30 μg/kg to 250 μg/kg) [3,17]. Blood was collected for a period of 15 days after administration, with CJC-1295 showing a biphasic loss with time (Figure 6A), similar to that observed in human administration studies [3]. CJC-1295 concentrations peaked on the day of administration at 1200 pg/mL and rapidly dropped over the next 48 hrs, before showing a slower decline over the next 12 days. Using the 50 pg/mL screening limit based on the equine population study, CJC-1295 can be detected for up to eight days in three of the horses while horse 2 displayed a more rapid excretion profile and was detected for only three days. The data from the equine plasma population study and from the CJC-1295 administration were used to generate a ROC curve, showing that the test provides a high level of discrimination between normal samples and samples from treated animals (Figure 6B).

As a comparison to CJC-1295, the modified GRF 1-29 peptide (mGRF 1-29) was also administered to horses. As mGRF 1-29 is known to have a short half-life in the body, the drug is usually administered by human users up to three times daily according to information posted on body building internet sites. In this experiment, 500 μg of mGRF 1-29 was administered once daily on three successive days and a time course of plasma samples collected after the final administration. Approximately 15 minutes after administration mGRF 1-29 was detected substantially above background levels with concentration peaking at over 400 pg/mL in horse 4 (Figure 7). The rate of clearance from the blood was rapid with mGRF 1-29 detected above 100 pg/mL for only 30 minutes in three horses and horse 1 never exceeding 100 pg/mL but displaying a slower excretion profile. The rapid clearance of mGRF 1-29 from plasma is similar to that observed in other peptide-based drugs administered to horses [18,19].

Discussion

CJC-1295 was developed as a GHRH analogue with a long biological half-life, stimulating elevated GH levels in humans for up to a week after administration. This provides a significant advantage over other GHRH and GHRP peptides which require daily injections or

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constant infusions for effective pharmacological responses. As a result, CJC-1295 is one of the common GH stimulating peptides sold to athletes via grey-market internet websites [8]. The ready availability of this peptide means that CJC-1295 may also be used as a doping agent in racehorses and greyhounds.

Direct detection of CJC-1295 by mass spectrometry is confounded by the maleimidopropionic acid group (DAC) which covalently links this peptide to any free cysteine present on the surface of a protein, including common plasma proteins such as serum albumin, immunoglobulins and fibrinogen. This prevents direct detection of CJC-1295 by mass spectrometry as the peptide-protein conjugates are difficult to resolve from native proteins and have undefined chemical compositions. Unlike mass spectrometry based approaches, antibodies allow the specific detection of CJC-1295 regardless of the protein that is conjugated to the C-terminus of the peptide. Antibodies also allow the efficient extraction of the CJC-1295-protein conjugates from the abundant and highly similar plasma proteins present in the sample. By using two CJC-1295 specific antibodies with non-competitive binding sites to independently extract and identify the drug, a highly specific assay has been developed for this peptide-based drug.

Enzyme linked immunosorbent-assays have proven to be an effective method for the detection of analytes in complex matrices. These assays typically rely on a colorimetric change brought about by conversion of a chromogenic substrate by an enzyme covalently linked to an antibody. This system, while robust, can suffer from a lack of sensitivity for low abundance analytes as well as a limited dynamic range. By changing from a colorimetric assay to an assay based on the exponential amplification of DNA by PCR, it is possible to extend the dynamic range of the assay as well as increase its sensitivity [20]. A typical commercial ELISA assay for GHRH has a linear range of 12.35-1000 pg/mL and requires 50-100 μL of sample [21]. By comparison the I-PCR assay described here has a linear range of 0.8-2500 pg/mL using only 25 μL of sample. Thus detection of GHRH by I-PCR is approximately 15 times more sensitive than an equivalent ELISA assay, with a 37 fold increase in dynamic range.

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The high degree of sequence homology between equine GHRH and CJC-1295 means this assay will also detect endogenous equine GHRH. The ability to detect equine GHRH necessitates establishing a threshold level of GHRH above which a sample may be screened as positive and submitted for further analysis. Analysis of 974 equine plasma samples indicated that a threshold of 50 pg/ml would produce an expected false positive rate of only 0.3%. After a single 10 mg dose of CJC-1295, a 50 pg/mL screening limit allowed the detection of CJC-1295 for up to 8 days post administration. The highest recorded value from the equine population study was 82 pg/mL, although higher concentrations may be possible given that GH levels are known to increase with physical exercise [22,23]. Due to the possibility of endogenous GHRH producing an elevated result on this screen, confirmation by mass spectrometry will be required to ensure the specificity of the test. This confirmation will be performed by antibody capture, tryptic digestion and the detection of proteotypic peptides in a process similar to the published methods for confirming rhEPO in an equine plasma samples [24,25].

The I-PCR assay based on the RD4 and RD6 antibodies is also capable of identifying other GHRH-based drugs that lack the DAC group. Binding affinity studies showed that the antibodies had a high affinity for both CJC-1295-without-DAC and for a protein bound CJC-1295 conjugate, indicating that the test was equally effective for both kinds of targets. However, the ability to police the use of these non-conjugated peptides is limited by their rapid clearance from the blood stream after administration. An administration of 500 µg of mGRF 1-29 produced peak plasma concentrations of approximately 400 pg/mL, and these levels persisted above the 50 pg/mL threshold for no more than one hour after administration. These results highlight not only the challenge of policing the use of these non-conjugated drugs through blood testing, but also the poor stability of most peptide-based drugs that limits their pharmacological effects. Despite the short detection window, the effective use of mGRF 1-29 requires regular high doses of the peptide, making detection of the drug more likely [26].

With the advent of strategies such as micro-dosing that are designed to thwart screening tests, there is a need to develop ever more sensitive drug detection assays. The sensitivity and

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specificity offered by I-PCR makes the technique ideal for the low level detection of protein and peptide based doping agents. To our knowledge, this is the first published example of I-PCR used for this application. Another advantage for I-PCR over ELISA screening is the ability to multiplex reactions in a single assay well. Real-time PCR instruments are capable of monitoring up to 6 different fluorescent channels simultaneously. Combined with enhanced sensitivity, it should be possible to immobilise multiple different capture antibodies in a 96-well plate and detect the captured analytes with specific secondary antibodies, each carrying a unique oligonucleotide label recognised by different TaqMan probes. The almost limitless ability of PCR to amplify specific DNA sequences in multiplexed reactions, allows the effective discrimination between large numbers of different secondary antibodies, making I-PCR assays a useful tool for forensic drug screening.

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Tables and Figures

Table 1. GHRH and GHRH analogues

Peptide Hormone	Sequence	MH+	Half Life
Human GHRH	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARAR	4925.5664	7 min
Equine GHRH	YADAIFTNNYRKVLGQLSARKILQDIMSRQHGERNQEQGAKTRF	5151.6770	N/A
GRF 1-29 (Sermorelin)	YADAIFTNSYRKVLGQLSARKLLQDIMSR-NH2	3356.8260	<10 min
Modified GRF 1-29	Y[D-Ala]DAIFTQSYRKVLAQLSARKLLQDILSR-NH2	3366.9009	30 min
CJC-1295-without-DAC	Y[D-Ala]DAIFTQSYRKVLAQLSARKLLQDILSRK-NH2	3494.9958	<30 min
CJC-1295	Y[D-Ala]DAIFTQSYRKVLAQLSARKLLQDILSRK[Maleimido]-NH2	3646.0228	6-8 days
Tesamorelin	N-(trans-3-hexenoyl)-YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL-NH2	5133.7239	26 min

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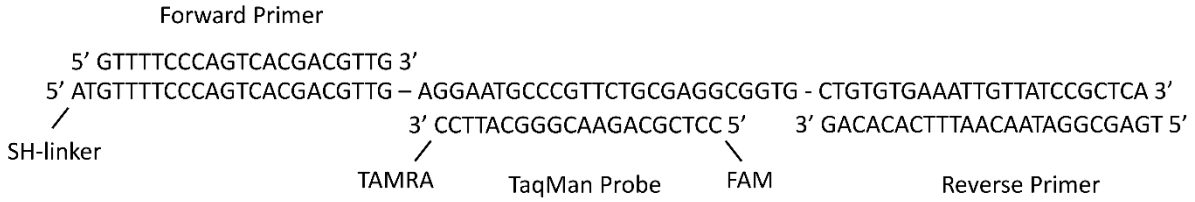

Figure 1. Schematic showing the design of the oligonucleotide label and the three probes and primers used for TaqMan PCR analysis.

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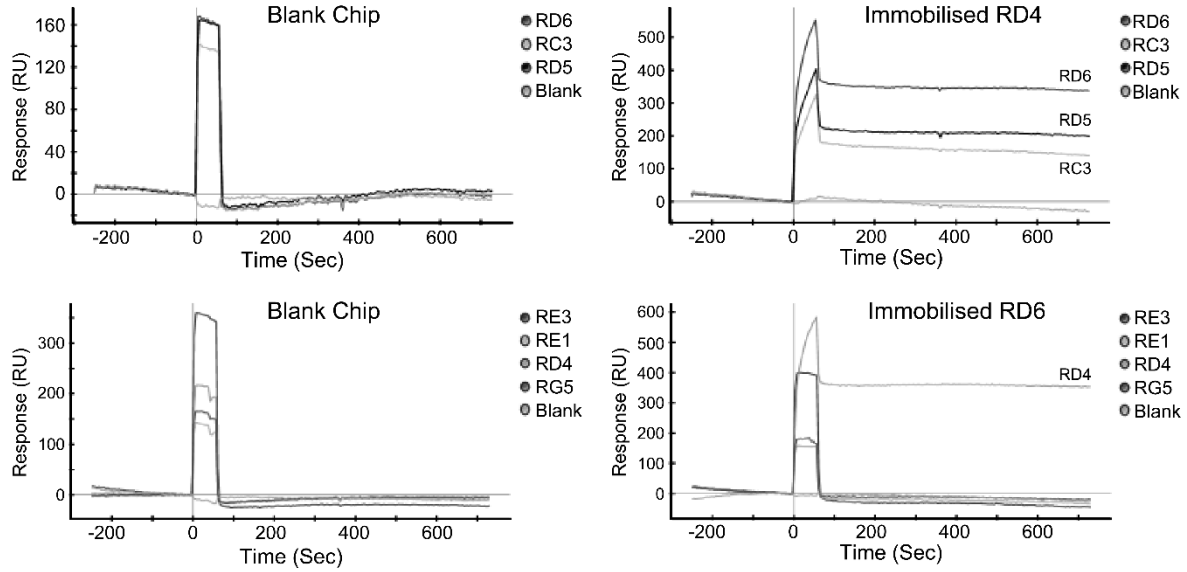

Figure 2. Surface Plasmon Resonance (SPR) analysis confirming the non-competitive binding of RD4 and RD6 antibodies to CJC-1295.

The SPR chip was prepared by coating with either RD4 antibody (B), RD6 antibody (D) or no antibody (A & C). The chip was then incubated with CJC-1295-without-DAC peptide before the binding of different secondary antibodies was analysed by SPR.

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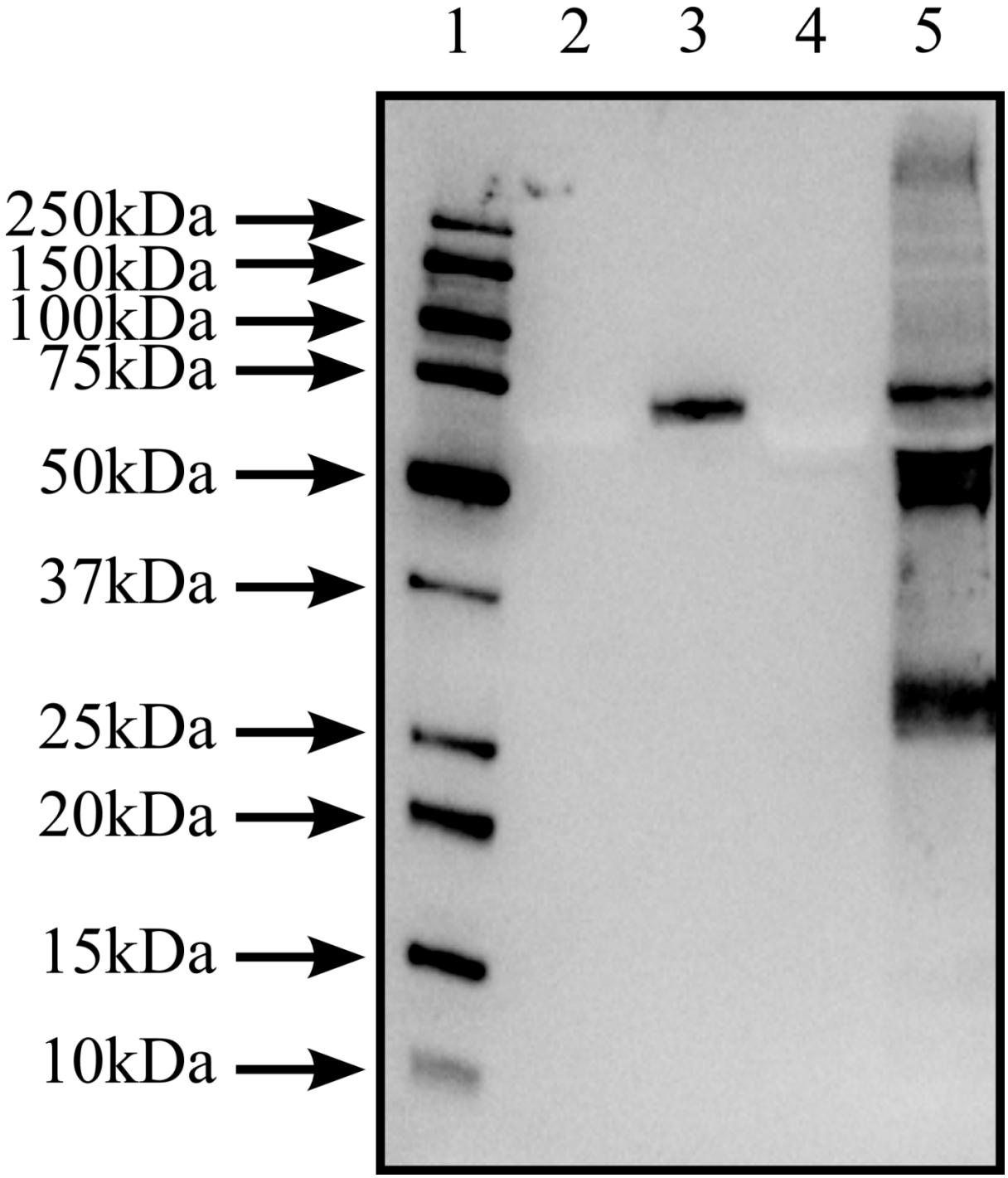

Figure 3. Detection of CJC-1295 conjugated to equine plasma proteins by western blot

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The monoclonal antibody RD6 was used to detect CJC-1295 bound to plasma proteins. Lane 1, Biorad western c markers. Lane 2, BSA. Lane 3, BSA spiked with CJC-1295. Lane 4, equine plasma. Lane 5, equine plasma spiked with CJC-1295. Similar results were obtained with the RD4 antibody (data not shown).

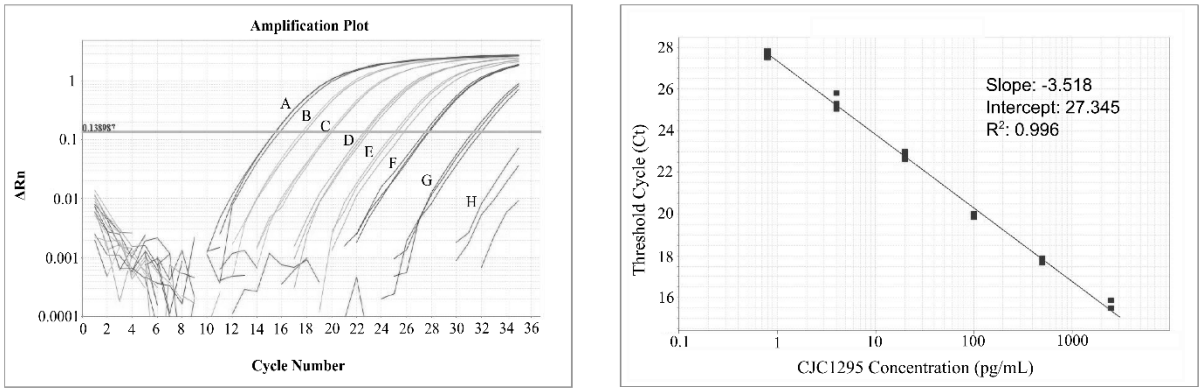

Figure 4. Standard curve and amplification plots for CJC-1295 standards.

Log change in fluorescence is monitored against time for various concentrations of CJC-1295-without-DAC. A, 2500 pg/mL. B, 500 pg/mL. C, 100 pg/mL. D, 20 pg/mL. E, 4 pg/mL. F, 0.8pg/mL. G, 0 pg/mL (no antigen control). H, No RD6 antibody (No template control). The threshold cycle (Ct) of standards was plotted against the log concentration of CJC-1295 to obtain a linear correlation coefficient of 0.996.

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Table 2. Limit of detection study

Sample	Threshold Cycle (Ct)								Avg	SD	%CV
500 pg/mL CJC1295	13.68	14.38	13.85	13.73	13.73	13.77	13.86	13.5	13.81	0.26	1.85
100 pg/mL CJC1295	16.38	16.64	16.91	16.59	16.57	16.78	16.82	16.56	16.66	0.17	1.03
20 pg/mL CJC1295	18.68	18.78	18.88	18.89	18.97	19.13	19.19	18.92	18.93	0.18	0.96
4 pg/mL CJC1295	21.62	21.42	21.57	21.58	21.28	21.61	21.64	21.59	21.54	0.12	0.58
0.8 pg/mL CJC1295	23.54	23.3	23.84	23.89	23.83	23.45	23.86	23.94	23.71	0.24	1.01
0 pg/mL CJC1295	25.62	25.61	26.09	25.88	26.94	25.26	27.23	26.79	26.18	0.72	2.75

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Table 3. Validation summary

LOD	Accuracy	Linearity			PCR Eff	Precision (Ct)	Precision (pg/mL)
pg/mL	%CV	Intercept	Slope	R^2	%	%CV	%CV
0.8	16.31	27.345	-3.518	0.996	92.43	1.26	16.79

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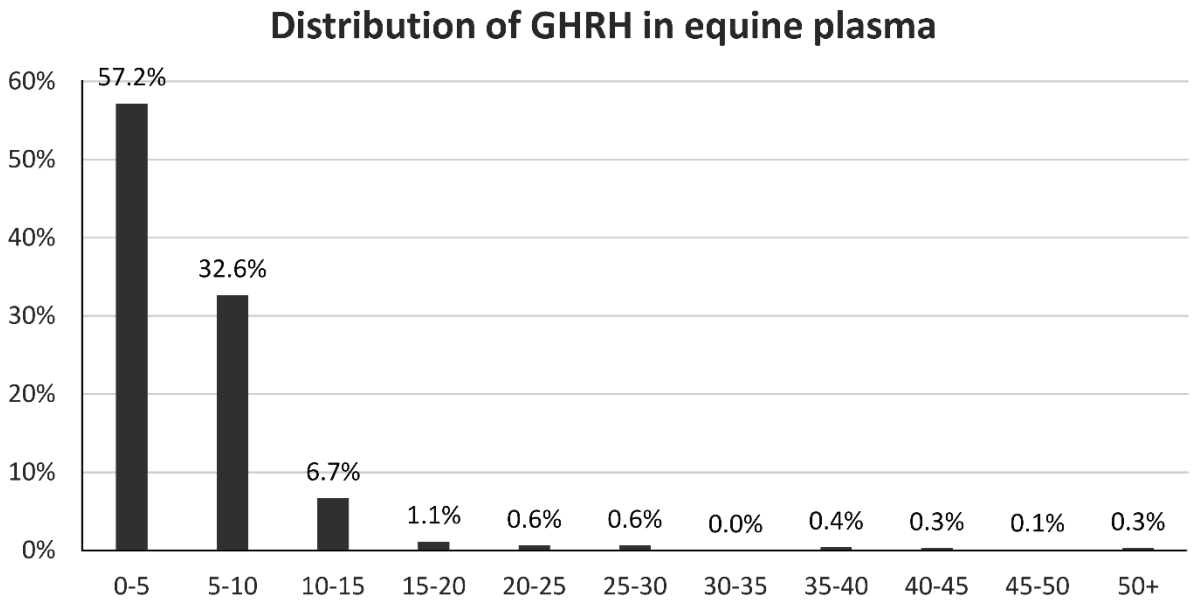

Figure 5. Background levels of CJC-1295 screening in equine plasma

974 equine plasma samples were tested in the I-PCR CJC-1295 assay in order to determine the range for background signals in plasma. The peak concentration observed was 82 pg/mL.

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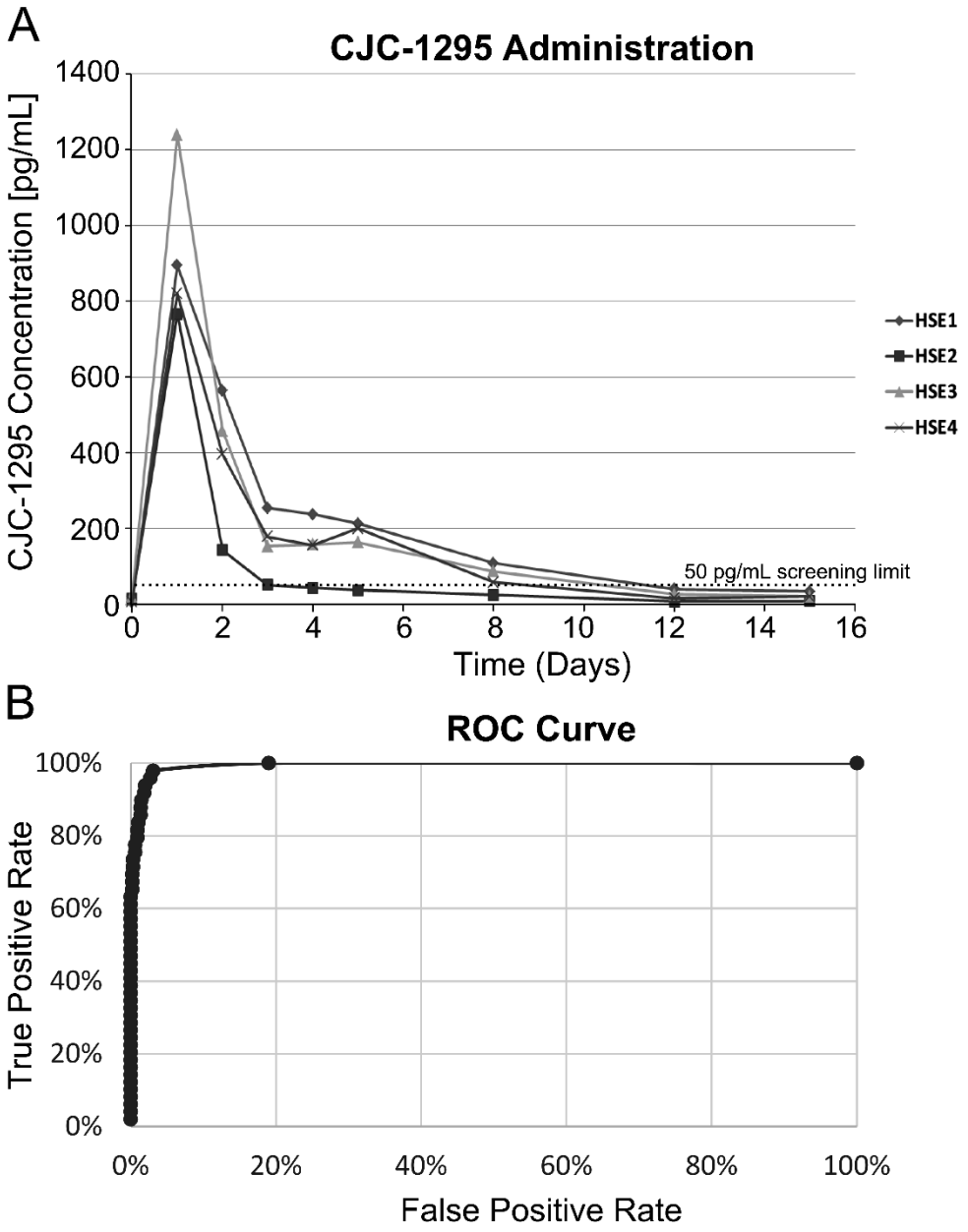

Figure 6. Detection of CJC-1295 in equine plasma following drug administration

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A: The concentration profile of CJC-1295 in equine plasma was monitored over 15 days using the I-PCR assay after a single 10 mg intramuscular injection of CJC-1295 administered on day 0. Negative control plasma was collected prior to drug administration.

B: The ROC curve plotted from the equine population and administration data.

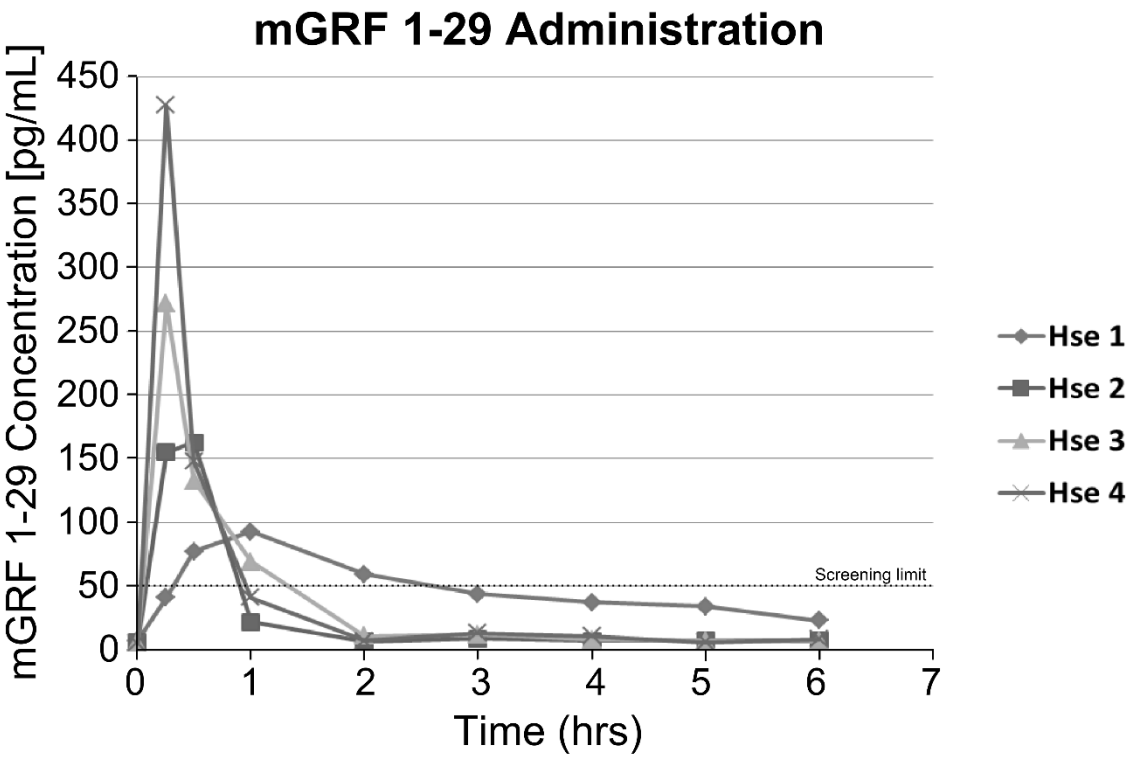

Figure 7. Detection of mGRF 1-29 in equine plasma following drug administration

The concentration profile of mGRF 1-29 in equine plasma was monitored over 6 hours using the I-PCR assay after the third of three daily administrations of 500 μg of mGRF 1-29 by intramuscular injection. Negative control plasma was collected prior to drug administration.

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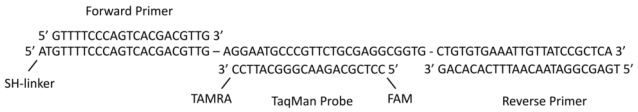

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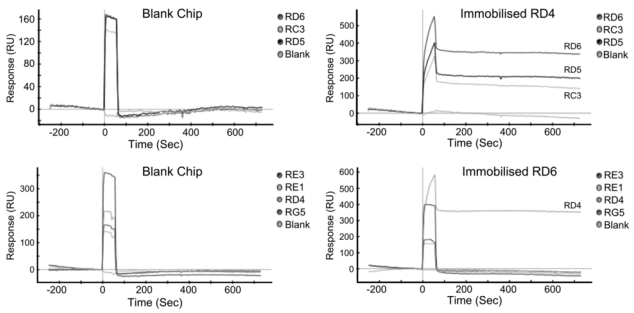

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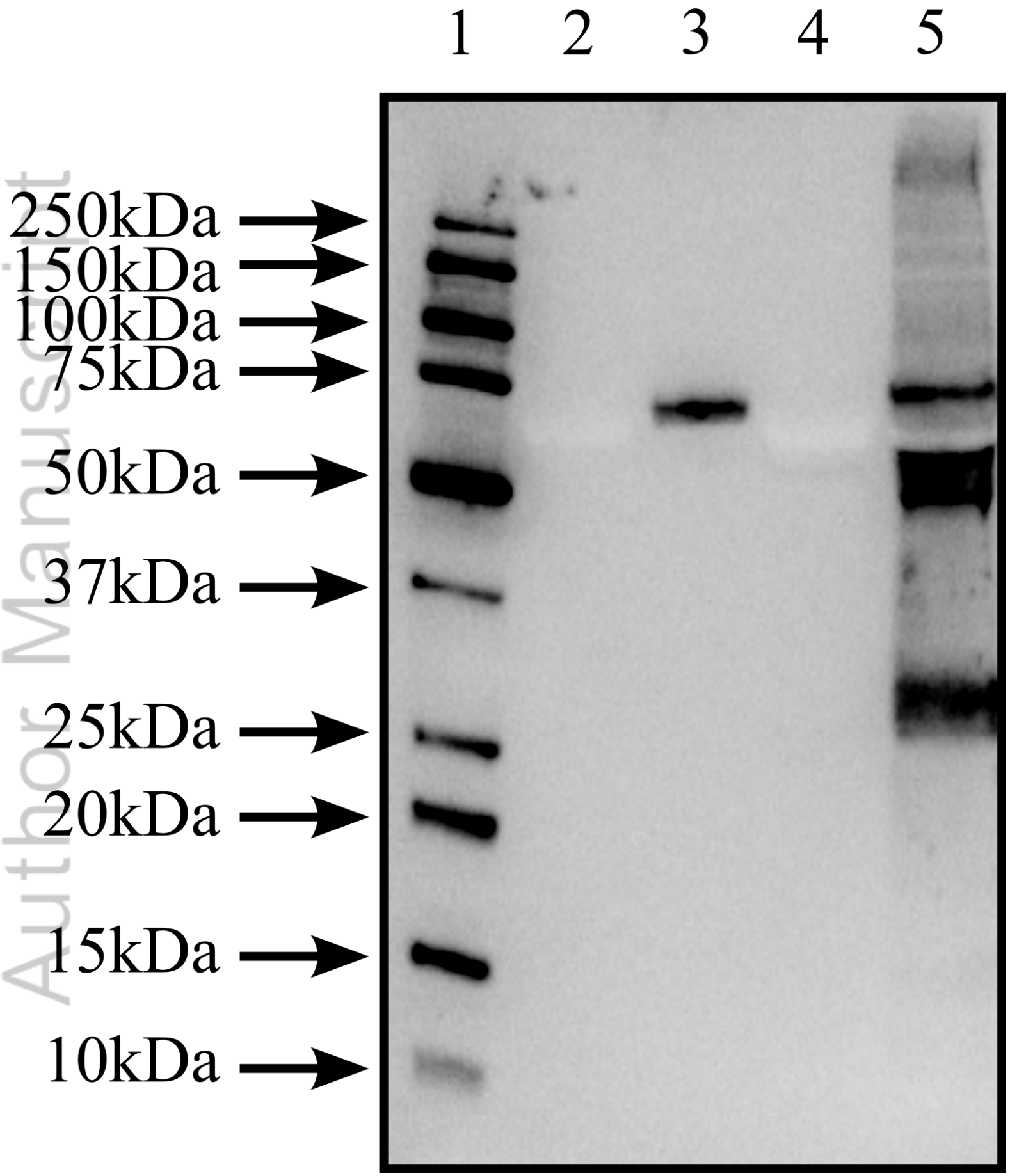

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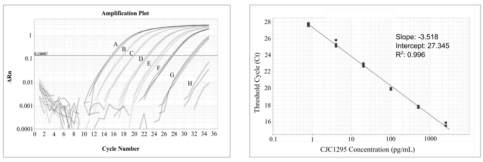

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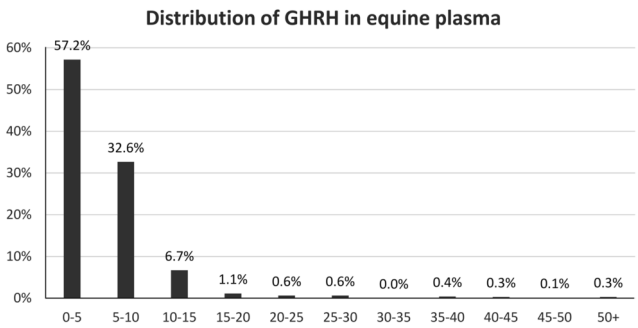

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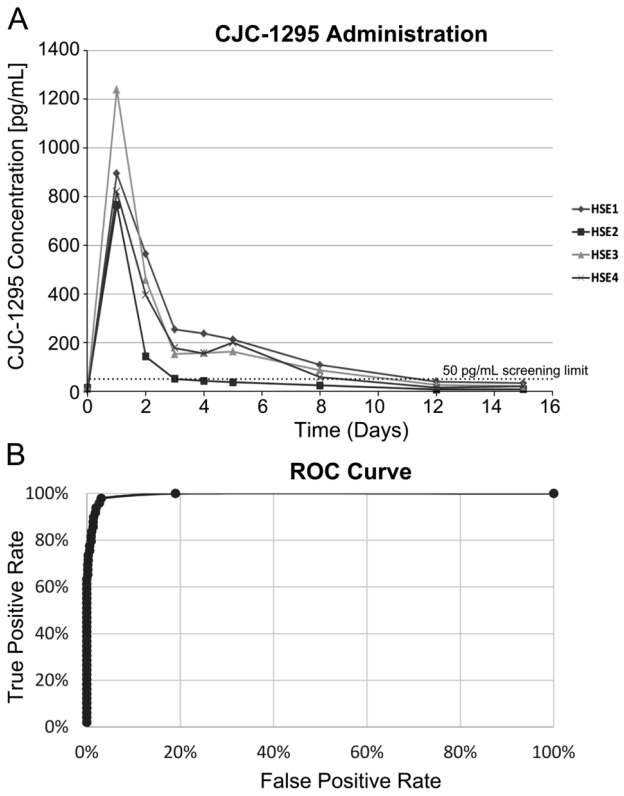

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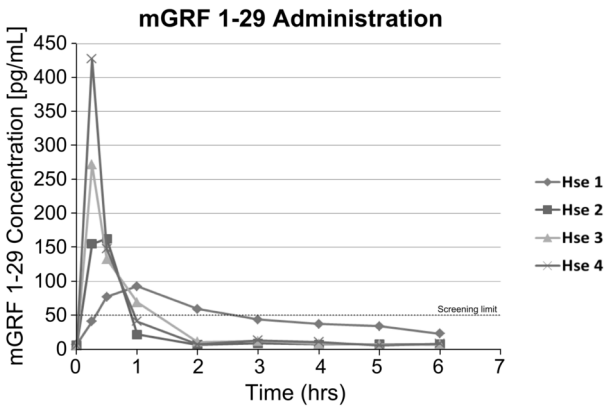

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