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A Method for Confirming CJC-1295 abuse in equine plasma samples by LC-MS/MS

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Abstract

CJC-1295 is a peptide-based drug that stimulates the production of growth hormone (GH) from the pituitary gland. It incorporates a functional maleimido group at the C-terminus that allows it to covalently bind plasma proteins such as serum albumin. These CJC-1295-protein conjugates have a much greater half-life compared to the unconjugated peptide and are capable of stimulating GH production for more than six days in humans after a single administration. Conjugated CJC-1295 is difficult to detect in blood by mass spectrometry due to its low abundance, high molecular weight and conjugation to a range of different protein substrates. Previously we described a screening procedure for the detection of CJC-1295 in equine plasma using an immuno-PCR assay. Here we demonstrate the confirmation of CJC-1295 in equine plasma by LC/MS/MS after immuno-affinity capture and tryptic digestion. Using this method, CJC-1295 was identified down to concentrations as low as 180 pg/mL in 1ml of equine plasma.

Introduction

CJC-1295 is a 30 amino acid peptide analogue of the growth hormone releasing hormone (GHRH)[1,2]. Compared to the native protein sequence, CJC-1295 has four amino acid substitutions which improve protease resistance, and an additional lysine residue at the C-terminus that incorporates a maleimidopropionic acid group. This maleimidopropionic acid, sometimes called the drug affinity complex (DAC), forms stable covalent linkages with free thiol groups on the surface of plasma proteins such as serum albumin (figure 1) [3]. The conjugation of CJC-1295 to plasma proteins dramatically increases the biological stability of the drug, extending plasma half-life up to 6 – 8 days in humans [3].

While the extended plasma half-life of CJC-1295 makes it an attractive drug screening target compared to other peptide-based drugs, which typically have short half-lives, conjugated CJC-1295 is not readily detected by mass spectrometry based peptide screens [4,5] due to its high molecular weight and similarity to abundant plasma proteins. The high molecular weight of the

conjugated CJC-1295 means that the drug needs to be analysed as a protein rather than a peptide-based drug. Accordingly, we have demonstrated the successful detection of CJC-1295-protein conjugates in equine plasma with an immuno-PCR (IPCR) screen [6].

Whilst antibody based assays are excellent screening tools, they cannot unequivocally identify a performance enhancing drug in a biological sample due to the possibility of the antibodies interacting with other related antigens. The IPCR assay for CJC-1295, for example, appeared to detect endogenous equine GHRH in addition to CJC-1295 and other synthetic GHRH analogues [7]. To provide absolute certainty in the identification of CJC-1295, we have developed a method for confirming the presence of CJC-1295 in equine plasma by LC/MS/MS. This method operates in a manner equivalent to other mass spectrometry based confirmation methods, using immunoaffinity capture followed by trypsin digestion and targeted MS/MS to provide confirmation of CJC-1295 in equine blood at high sensitivity [8–10].

Materials and Methods

Reagents and materials

10 x phosphate buffered saline (PBS) solution, EDTA, sodium chloride, sodium hydrogen phosphate, sodium dihydrogen phosphate and Zeba desalting columns were purchased from Thermo Scientific (Waltham, MA, USA). Tween 20, glycerol, and tris base were purchased from Sigma-Aldrich (St Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Bovogen (Victoria, Australia). The RD4 monoclonal antibodies to CJC-1295 were made to order by the Monash Antibody Technology Facility (Clayton, Australia). CJC-1295 and CJC1295 minus DAC (Table 1) were synthesized by Auspep (Tullamarine, Australia). M-280 Tosyl Activated Dynabeads were purchased from Invitrogen Dynal (Oslo, Norway). Anhydrous disodium hydrogen orthophosphate was from Biolab (Victoria, Australia). All aqueous buffers were prepared in Milli Q Synergy grade water (Billerica, MA, USA).

Preparation of Equine Serum Albumin-CJC-1295 conjugate

A single 0.5 mg vial of CJC-1295 was dissolved in 300 µL of 10 mM EDTA, 100 mM sodium phosphate (pH 7.0), and added to 110 µL of equine plasma mixed with 90 µL of 10 mM EDTA, 100 mM sodium phosphate pH 7.0, to give a final volume of 500 µL. The vial was incubated for 4 h 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. The concentration of CJC-1295 conjugate in the sample was determined by IPCR testing [6].

Plasma samples

Equine blood samples were obtained from Racing Victoria Limited (Flemington, Australia), and supplied as part of regular testing. 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. For the administration study, a 10 mg

injection of CJC-1295 was delivered by intramuscular injection to a Thoroughbred gelding as described previously [6]. 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. This experiment was performed with the approval of the University of Melbourne Animal Ethics Committee (Ethics approval number 1413071.1) [6].

Preparation of RD4 labelled paramagnetic beads

RD4, the monoclonal antibody against CJC-1295, was covalently attached to M-280 Tosyl Activated Dynabeads as described in Timms *et al.*[10], M-280 Tosyl Activated Dynabeads (30 mg/mL) were resusupended by vortexing and 5 mg (165 µL) removed for transfer to a low bind 1.5 mL eppendorf microfuge tube. Dynabeads were pelleted using a rare earth magnet for all washing and recovery steps. The beads were washed with 1 mL of 1 x PBS before being mixed with 100 µg of RD4 antibody, 100 µL of 3 M ammonium sulphate and 50 µL of 100 mM sodium phosphate, pH 7.4. The tube was incubated at 37°C overnight (16-20 hours) with shaking on an Eppendorf Comfort orbital incubator. Following overnight incubation the beads were pelleted and the supernatant removed. Unreacted sites were blocked by incubation in 1 mL of 1 M Tris-HCl, pH 7.4 at 37°C for 2 hours. The Dynabeads were then recovered with a magnet and the supernatant discarded. The beads were washed with 1.0 mL of 0.1% BSA in PBS before being stored in 0.245 mL of 0.1% BSA in PBS at 4°C.

Immuno-affinity purification of CJC1295

Equine plasma (1 mL) was transferred to a 1.5 mL microfuge tube and spun at 17000 x g for 10 minutes in a benchtop centrifuge, after which the supernatant was transferred to clean 1.5 mL low bind microfuge tube. RD4 labelled Dynabeads were washed in 0.1% BSA in PBS before use and resuspended to a final concentration of 20 mg/mL in 0.1% BSA in PBS, before 1 mg was added to each sample tube. The samples were vortexed and incubated at 37°C with shaking for two hours using the Eppendorf Comfort orbital incubator. For washing, the samples were centrifuged briefly to settle the liquid and beads before the magnetic beads were captured using a rare earth magnet. The supernatant was discarded and the beads briefly washed twice in 1 mL of 1 x PBS-T and once in 1 mL of water. After discarding the supernatant from the final wash, the samples were briefly centrifuged and returned to the rare earth magnet before all residual liquid was removed from the sample. Captured CJC-1295 conjugates were eluted with 80 µL 100mM glycine, pH 2.0.

The pH of the eluent was raised by the addition of 20 µL of 1 M TEAB. Kemptide was added to a final concentration of 1 µg/mL from a 1 mg/mL stock to serve as a carrier. Kemptide was also used as an internal control for digestion efficiency (Table 2). Samples were digested at 37°C overnight with 1 µg of trypsin. The next day the digested samples were acidified with 10 µL of 10% formic acid, centrifuged at 17000 x g for 30 seconds and loaded onto 1 mL plexa SPE cartridges that had been conditioned with 800 µL of methanol, followed by 800 µL of water. The cartridges were washed with 1 mL of 0.1% formic acid before being eluted with 800 µL 0.1% formic acid in methanol. The eluted samples were dried using a Speedivac for 2

h at 60°C, and dissolved in 25 μL of 0.1% formic acid in 10% acetonitrile. Finally the samples were vortexed and centrifuged at 17000 x g for 10 minutes before transfer to polypropylene HPLC vials. A 10 μL volume of sample was injected for analysis by LC-MS/MS.

High resolution mass spectrometry

High resolution mass MS was performed on a Q-Exactive (Thermo Fisher, Bremen, Germany) equipped with a Nexera UHPLC (Shimadzu, Kyoto, Japan) and using a 2.1 x 150 mm Proteocol G203 C18 column (Trajan Scientific and Medical, Ringwood, Australia). Buffer A was 0.1% formic acid, Buffer B was 0.1% formic acid in acetonitrile. Samples were analysed on a 10–85% Buffer B gradient over 21 minutes with the Q-Exactive running a top 5 data dependent MS/MS acquisition programme in positive mode targeting singly and multiply charged precursors. The following settings were used for the MS scan; scan range: m/z 300–1500, resolution: 35 000, max ion trap fill time: 250 milliseconds, AGC target: 1e6. For the data dependent MSMS, the scan range was 100–1685, resolution: 17500, maximum ion trap fill time: 120 milliseconds, AGC target 1e6, minimum AGC target 5e3, isolation window: 2 m/z, NCE: 28 and the loop count was set to 5. For precursor selection, the intensity threshold was set to 4.2e4, the apex trigger set to between 1 and 9 seconds and the dynamic exclusion was 4 seconds. Spray voltage was 3500 V, sheath gas and auxiliary gas were 30 and 10 psi, respectively. The capillary temperature was 320°C while the gas heater temperature was 260°C. The S-lens RF level was 85. No lock mass was used and the instrument was calibrated in positive mode with caffeine, MRFA, Ultramark, and n-butylamine standards using the manufacturer's instructions.

Low resolution mass spectrometry

Chromatography was performed on an Eksigent nanoLC425 chromatography system (Sciex, Framingham, MA, USA) running at a flow rate of 20 μL/min using a 530 μm x 100 mm MyCapLC capillary column packed with 3 μm ProteCol® C18 resin with a 200 Å pore size (Trajan Scientific and Medical, Ringwood, Australia). The aqueous solvent (A) was 0.1% (v/v) formic acid in water, and the organic phase (B) was 0.1% (v/v) formic acid in 90% acetonitrile. The gradient was held at 5% B for 1.2 minutes before being increased to 40% B over 9.8 min. It was increased to 90% B over 0.5 minutes and held for 1.5 minutes before returning to 5% B over 0.5 min, where the column was allowed to re-equilibrate for 2.0 min. The overall chromatography run time was 15.5 min. Mass spectrometry was performed on an AB Sciex 6500 QTRAP equipped with a TurboV source (Sciex, Framingham, MA, USA). The declustering potential, entrance potential and exit potential were set to 70 V, 10 V and 10 V respectively. Curtain gas, source gas 1 and gas 2 were set to 20, 35 and 0 PSI respectively. Collision gas was set to low. Ion spray voltage was set to 5500 V and source temperature was set to 400°C. Identification of CJC-1295 was performed by LC-MS/MS using the MRM transitions outlined in Table 2.

Validation

The method was validated with respect to limit of confirmation, specificity, and precision. The limit of confirmation was defined as that sample giving a peak area 3 times greater than background noise for 3 MRM transitions, with retention time drift being less than 2% or 12 seconds compared to a known standard, whichever is the greater. Additionally, chromatographic peak area ratios calculated against the base peak should have a relative variance in abundance not exceeding 20% absolute or 40% relative when compared to a trypsin digest of the standard. Specificity was established using blank equine plasma samples and monitoring for interfering signals. Precision was assessed by analysing peak areas and chromatographic stability in equine plasma samples spiked at 177, 355, 710 and 1770 pg/mL. Relative standard deviation was determined using four samples tested in duplicate.

Sample Stability

Heparinised equine plasma was spiked with ESA-CJC1295 at a concentration of 700 pg/mL and stored at 4°C. One mL aliquots were removed for analysis at 0, 24, 48, 72 and 96 hours. Samples were then stored at -70°C until extracted. Extraction was performed using the RD4 monoclonal antibody as described above. Peak area was calculated using the VLAQSLAR transition 429/645. Percentage peak area was calculated against the time zero control.

Results

Identification of CJC-1295 tryptic peptides in equine plasma by high resolution mass spectrometry

The CJC-1295 peptide has 5 trypsin cleavage sites yielding three peptides large enough for MS/MS analysis: YADAIFTQSYR (T1), LLQDILSR (T3) and VLAQLSAR (T5). These peptides were BLAST searched against the equine proteome without any complete matches, indicating that they are suitable for the confirmation of CJC-1295 in equine plasma. Theoretical MS/MS spectra were generated by the UCSF Proteinprospector website (http://prospector.ucsf.edu/ prospector/mshome.htm) and all three peptides were identified by high resolution mass spectrometry in a trypsin digest of the CJC-1295-ESA conjugate (Figure 2). The most abundant sequence ions were selected and used to develop the MRMs for low resolution MS/MS analysis (Table 2).

The C-terminal CJC-1295 tryptic peptide conjugated to an endogenous equine protein was also sought. When administered to rats, 90% of CJC-1295 was found covalently attached to albumin[1] . Serum albumin is the most abundant of all plasma proteins and ESA contains a single free cysteine in the N-terminus (C34) that is capable of reacting with the DAC. We have previously shown that CJC-1295 incubated with equine plasma forms conjugates with a range of different plasma proteins [7]. To confirm that CJC-1295 formed conjugates with ESA, the drug was incubated with equine plasma, and subsequently digested with trypsin before being

analysed by high resolution LC-MS/MS. The single free cysteine in ESA is located at position 34 in the fifth tryptic (T5) peptide, GLVLVAFSQYLQQCPFEDHVK, which has a monoisotopic mass of 2421.2271 Da. The C-terminal amidated lysine from CJC-1295, including the DAC, has a molecular mass of 296.1485. The conjugation of this peptide tag to the ESA T5 peptide would produce a peptide with the monoisotopic mass of 2717.3756 Da. The conjugated ESA T5 peptide was identified as a m/z 912.4664 ion and confirmed by analysis of the MS/MS spectra (Figure 3A). This ion is consistent with the conjugated peptide in a 3+ charge state, with a hydrolysed DAC [11] and a theoretical m/z of 912.4669. Sequencing of this peptide indicated the mass difference between the y_7 and the y_8 ion to be 417.1682 Da, rather than the 103.0092 Da expected for an unmodified cysteine residue. For comparison purposes the fragmentation sequence of the unmodified T5 peptide is shown in figure 3B. Here the mass difference between the y_7 and the y_8 ion is 103.0086 Da, identifying unmodified cysteine.

Extraction of CJC-1295 from equine plasma and detection by low resolution MS/MS

In order to confirm the presence of the CJC-1295 at low levels it was necessary to purify the drug from the plasma matrix. This was accomplished by a one-step immuno-affinity procedure in which a monoclonal antibody directed to CJC-1295 was attached to paramagnetic beads. The method was validated by spiking equine plasma with varying amounts of an ESA-CJC-1295 conjugate. Antigen recovered by immuno-affinity purification was digested with trypsin and analysed by mass spectrometry using the MRM transitions outlined in table 2. Figure 4 shows the detection of three CJC-1295 peptides, YADAIFTQSYR, VLAQLSAR and LLQDILSR following affinity capture and trypsin digestion. Each peptide is detected by three individual MRMs. No peaks were detected in the blank plasma sample (Figures 4A, D, G) while peaks matching the CJC-1295 standard were detected in plasma spiked with CJC-1295-ESA conjugate at a concentration of 180 pg/mL (Figures 4B, E, H). Spectra from a tryptic digest of CJC-1295 without DAC is shown in figures 4C, F and I. The conjugated ESA T5 peptide was not detected by low resolution MS/MS with sufficient sensitivity to be useful in confirming the presence of CJC-1295 in plasma, despite is detection by high-resolution MS/MS at significantly higher concentrations. Regardless, the identification of the peptides YADAIFTQSYR, VLAQLSAR and LLQDILSR confirmed the ability of the monoclonal antibody to capture and recover CJC-1295 from an equine plasma sample.

The effectiveness of the method was further tested by analysis of a plasma sample from a thoroughbred horse after administration of CJC-1295. This sample was taken three days after administration and analysis by IPCR showed that it contained approximately 178 pg/mL of CJC-1295 (data not shown). Immunoaffinity purification with RD4 antibody followed by tryptic digestion and LC/MS/MS analysis shows clear identification of all three CJC-1295 tryptic peptides in the sample (Figure 5).

Assay Validation

Assay specificity was established on untreated equine plasma samples looking for interferences in those regions of the chromatogram in which CJC-1295 standard peptides eluted. No

interferences were seen (Figures 4A, D and G). A limit of confirmation (LOC) for CJC-1295 was established at 180 pg/mL from a 1.0 mL plasma sample (Figures 4B, E and H). In accordance with current AORC criteria [12] the LOC was defined as a sample giving a peak area 3 times greater than background noise for 3 MRM transitions, with retention time drift being less than 2% compared to a known standard. Additionally, chromatographic peak area ratios calculated against the base peak were required to have a relative variance in abundance not exceeding 20% absolute or 40% relative when compared to a trypsin digest of the standard. Table 3 shows the relative variance in abundance for those ions identifying YADAIFTQSYR, VLAQLSAR and LLQDILSR peptides. All ion ratios matched those of the CJC-1295 standard within the limits allowed under AORC criteria. Likewise the retention times of peptides in spiked samples matched those of the standards with drift being less than 2% of the standard (Table 4). The stability of ESA-CJC-1295 was assessed in heparinised equine plasma after incubation at 4°C for up to four days (figure 6). Under these conditions, which are typical for the storage of blood samples under laboratory conditions, the CJC-1295 protein conjugates had an estimated half-life of 20 days.

Discussion

Compared to other GHRH analogues such as sermorelin, mGRF1-29 and tesamorelin (Table 1), CJC-1295 has a much longer half-life and thus a longer period over which to stimulate production of GH. A single dose of CJC-1295 in humans has been shown to increase both GH levels (2-10 fold) and IGF-1 levels (1.5-3.0 fold). These levels remained elevated after administration for up to 6 days and 14 days respectively [3]. The long biological half-life of CJC-1295 makes it an attractive analytical target compared to most other peptide based-drugs which have a biological half-life of two hours or less [13,14].

While sold as a peptide-based drug, the ability of CJC-1295 to covalently bind plasma proteins means the drug behaves like a protein-based doping agent, rather than a peptide, with increased stability and reduced renal excretion. By the same logic, CJC-1295 needs to be analysed by methods developed for protein-based doping agents, rather than those typically used to detect peptide-based drugs. We have previously demonstrated the high-sensitivity detection of this drug after administration in horses using IPCR [7]. While immunoassays offer a cost efficient method for screening samples, a mass spectrometry based method for the detection CJC-1295 is still required as antibody based screening is not considered specific enough for legal prosecution. This is particularly true for the CJC-1295 IPCR screen which detected high background levels of equine GHRH in a minority of equine blood samples.

The method detailed here is derived from the rhEPO confirmation method developed by Timms et al. [10]. It uses an efficient single-step immunoaffinity purification to separate the CJC-1295-protein conjugates from abundant plasma proteins that would otherwise interfere with analysis, followed by tryptic digestion and targeted MS/MS. It should be noted that previous methods that have claimed to detect CJC-1295 [4,5] have targeted peptides without the DAC that defines CJC-1295 [3]. While these other methods have also used immunoaffinity purification to extract GHRH analogues from plasma, they did not use tryptic digestion which is required to isolate

CJC-1295 peptides that would otherwise be covalently attached to serum albumin and other plasma proteins.

The sensitive detection of an equine serum albumin peptide carrying the c-terminal lysine and the DAC group from CJC-1295 was not achieved by this method. The sensitivity of detection of this ESA peptide is likely to be limited by its relatively large size as well as the hydrolysis and oxidation of the maleimido group producing multiple different molecular species [11].

Confirmation of three tryptic CJC-1295 peptides in plasma was possible at concentrations down to 180 pg/mL in plasma, when starting with 1.0 mL of plasma. Greater confirmation sensitivity could be achieved by using higher volumes of plasma for sample preparation. The three peptides use to confirm the presence of CJC-1295 in this assay cover 90% of the primary sequence of this drug. The YADAIFTQSYR, VLAQLSAR and LLQDILSR peptides are distinct from equine and human GHRH but are common to the CJC-1295-without-DAC and mGRF 1-29 peptides. As such, the confirmation method is unable to distinguish between CJC-1295 and these two synthetic peptide analogues. This lack of specificity is not significant as the peptides CJC-1295, CJC-1295-without-DAC and mGRF 1-29 are all banned under the rules of racing as well as by WADA [16]. In addition, the poor stability and rapid excretion of unconjugated peptides like mGRF 1-29 [7] means that these peptides are much less likely to be present in a blood sample at concentrations high enough for confirmation. The assay described here now allows the confirmation CJC-1295 from protein conjugates following immunoassay screening.

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Table 1. GHRH and GHRH analogues

Peptide Hormone	Amino Acid Sequence	MH+ (mono)	$T_{1/2}$
Human GHRH 1-44	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL	5038.6504	7 min
Equine GHRH 1-44	YADAIFTNNYRKVLGQLSARKILQDIMSRQHGERNQEQGAKTRF	5151.677	N/A
Tesamorelin	N-(trans-3-hexenoyl)-YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL]-amide	5133.7239	26 min
CJC1295	Y[D-Ala]DAIFTQSYRKVLAQLSARKLLQDILSRK[Maleimido]-amide	3646.0228	6-8 days
CJC1295 minus DAC	Y[D-Ala]DAIFTQSYRKVLAQLSARKLLQDILSRK-amide	3494.9958	<10min
Modified GRF1-29	Y[D-Ala]DAIFTQSYRKVLAQLSARKLLQDILSR-amide	3366.9009	30 min
GRF1-29 (Sermorelin)	YADAIFTNSYRKVLGQLSARKLLQDIMSR-amide	3356.826	<10min

Table 2 MRMs for detection of CJC-1295 and Serum Albumin tryptic fragments

Peptide	Q1 Setting	Q3 Setting	Sequence Ion	CE (V)
YADAIFTQSYR (CJC-1295 T1)	667.82	801.39	y6	30
		350.13	b3	35
		914.47	y7	30
VLAQLSAR (CJC-1295 T3)	429.26	645.36	y6	20
		574.33	y5	25
		446.27	y4	25
LLQDILSR (CJC-1295 T5)	479.29	731.4	y6	20
		603.34	y5	25
		488.32	y4	32
GLVLVAFSQYLQQCPFEDHVK (ESA T5+CJC-1295 tag)	912.7	383.24	b4	40
		871.43	y7	50
		498.26	y4	50
KEMPTIDE	386.74	539.34	a5-NH_3	35
		409.27	b3-NH_3	35

Table 3. Ion ratios for the CJC-1295 tryptic peptides VLAQLSAR, LLQDILSR and YADAIFQSYR

Sample (n=8)	Tryptic Peptide: VLAQLSAR				Tryptic Peptide: LLQDILSR				Tryptic Peptide: YADAIFQSYR			
	Peak area ratio S2/S1 (%)	Pass/Fail Tolerance (±40% relative)	Peak area ratio S3/S1 (%)	Pass/Fail Tolerance (±40% relative)	Peak area ratio S2/S1 (%)	Pass/Fail Tolerance (±40% relative)	Peak area ratio S3/S1 (%)	Pass/Fail Tolerance (±40% relative)	Peak area ratio S2/S1 (%)	Pass/Fail Tolerance (±40% relative)	Peak area ratio S3/S1 (%)	Pass/Fail Tolerance (±40% relative)
CJC1295 Standard	19.94	19.94 (±7.98)	16.39	16.39 (±6.56)	42.95	42.95 (±17.18)	19.16	19.16 (±7.66)	35.68	35.68 (±14.27)	35.94	35.94 (±14.38)
177pg CJC-1295-BSA spike	22.90	Pass	12.64	Pass	41.71	Pass	18.49	Pass	37.26	Pass	33.00	Pass
355pg CJC-1295-BSA spike	18.40	Pass	14.32	Pass	42.60	Pass	18.87	Pass	34.04	Pass	31.58	Pass
710pg CJC-1295-BSA spike	17.61	Pass	14.93	Pass	41.20	Pass	18.39	Pass	33.42	Pass	35.21	Pass
1770pg CJC-1295-BSA spike	17.46	Pass	14.64	Pass	43.55	Pass	17.54	Pass	32.66	Pass	33.74	Pass
177pgCJC-1295-BSA spike	15.66	Pass	12.08	Pass	43.65	Pass	18.23	Pass	38.19	Pass	31.28	Pass
355pg CJC-1295-BSA spike	16.15	Pass	14.20	Pass	40.31	Pass	17.58	Pass	33.33	Pass	33.44	Pass
710pg CJC-1295-BSA spike	17.70	Pass	15.10	Pass	41.74	Pass	17.58	Pass	35.70	Pass	30.57	Pass
1770pg CJC-1295-BSA spike	19.21	Pass	15.45	Pass	43.75	Pass	18.10	Pass	34.55	Pass	31.85	Pass

One mL of equine plasma was spiked with a CJC-1295-BSA conjugate and analysed by LC-MS/MS. Peak area ratios for the VLAQLSAR peptide were derived by dividing signals 2 (S2 = transition 429/574) and 3 (S3 = transition 429/446) by signal 1 (S1 = transition 429/645). Peak area ratios for the LLQDILSR peptide were derived by dividing signals 2 (S2 = transition 479/603) and 3 (S3 = transition 479/488) by signal 1 (S1 = transition 479/731). Peak area ratios were for the YADAIFQSYR peptide were derived by dividing signals 2 (S2 = transition 667/350) and 3 (S3 = transition 667/914) by signal 1 (S1 = transition 667/801). Pass/Fail was based on ion ratios being within ±40% relative to the CJC-1295 standard.

Table 4. Retention time data for the CJC-1295 peptides VLAQLSAR, LLQDILSR and YADAIFQSYR

A range for retention time (Rt) was calculated from the retention time of the standard ± 2%.

Sample (n=9)	VLAQLSAR		LLQDILSR		YADAIFQSYR	
	Retention Time (min)	Accepted Rt range (min)	Retention Time (min)	Accepted Rt range (min)	Retention Time (min)	Accepted Rt range (min)
CJC1295_Standard	4.62	N/A	6.41	N/A	6.16	N/A
177pg CJC-1295-BSA spike	4.57	4.53-4.71	6.35	6.28-6.54	6.21	6.04-6.28
355pg CJC-1295-BSA spike	4.61	4.53-4.71	6.41	6.28-6.54	6.11	6.04-6.28
710pg CJC-1295-BSA spike	4.6	4.53-4.71	6.39	6.28-6.54	6.15	6.04-6.28
1770pg CJC-1295-BSA spike	4.59	4.53-4.71	6.4	6.28-6.54	6.14	6.04-6.28
177pg CJC-1295-BSA spike	4.6	4.53-4.71	6.39	6.28-6.54	6.21	6.04-6.28
355pg CJC-1295-BSA spike	4.59	4.53-4.71	6.39	6.28-6.54	6.14	6.04-6.28
710pg CJC-1295-BSA spike	4.61	4.53-4.71	6.4	6.28-6.54	6.13	6.04-6.28
1770pg CJC-1295-BSA spike	4.62	4.53-4.71	6.41	6.28-6.54	6.16	6.04-6.28

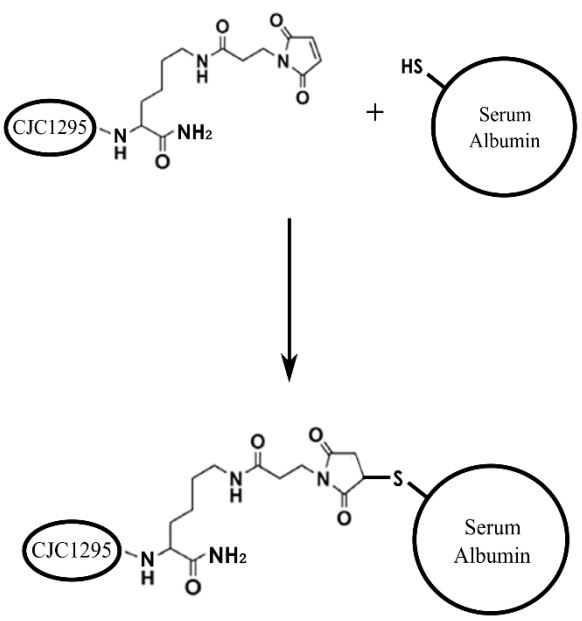

Figure 1. The CJC-1295 peptide covalently binds to plasma proteins via free cysteines.

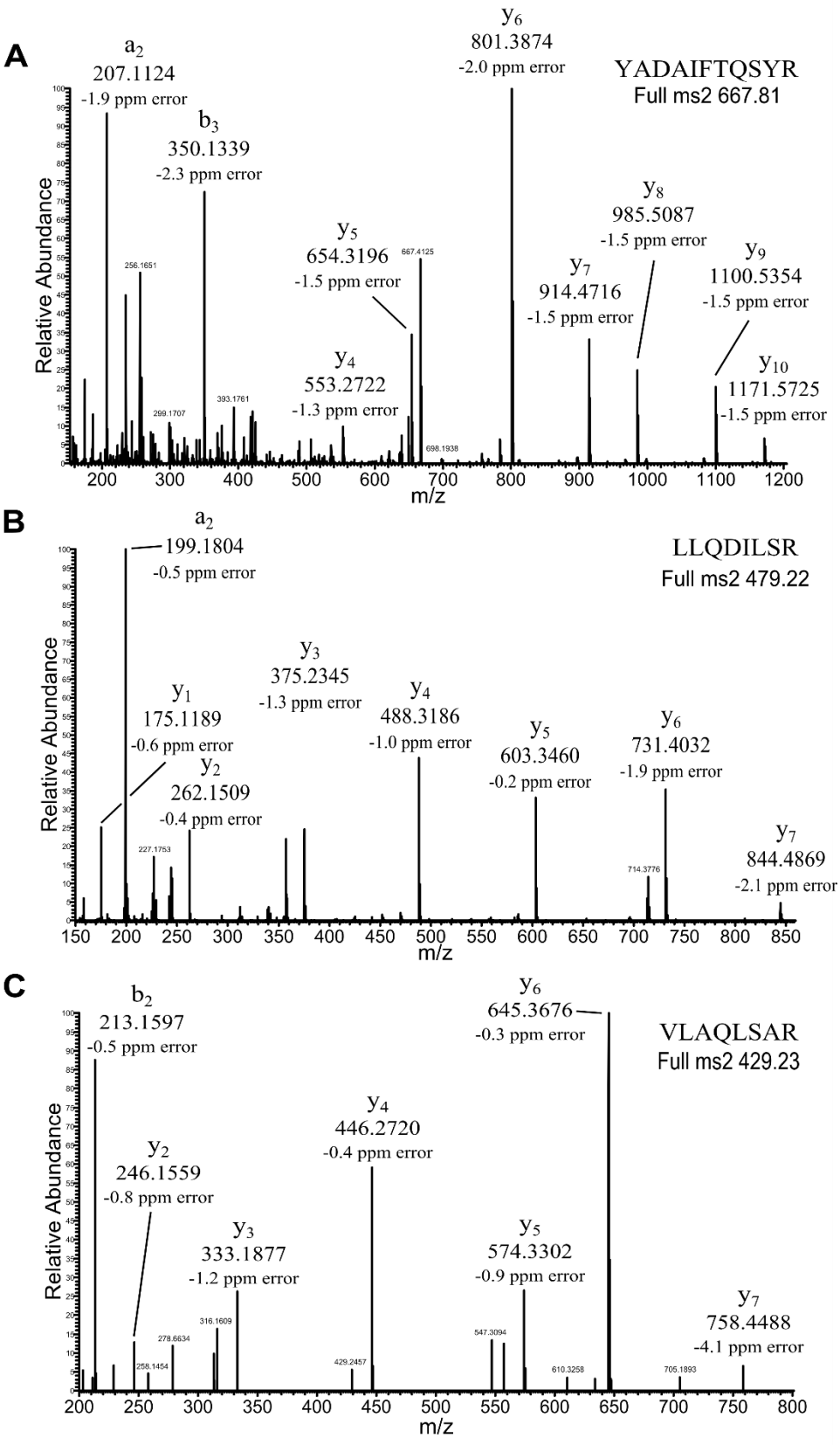

Figure 2. Fragmentation of the CJC-1295 peptides (A) YADAIFTQSYR, (B) LLDILSR and (C) VLAQLSAR identified in a CJC-1295-ESA conjugate tryptic digest.

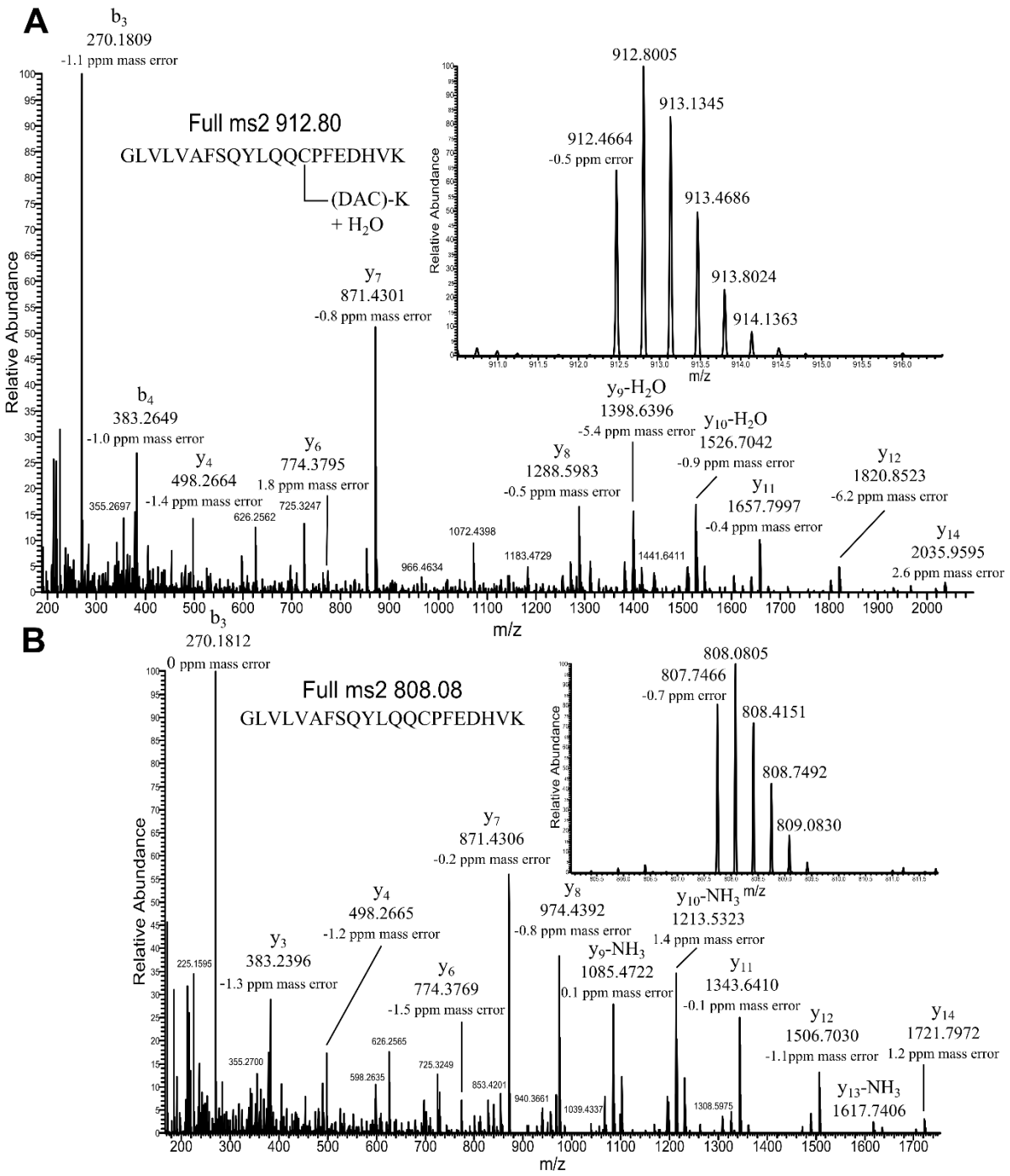

Figure 3. (A) Fragmentation of ESA T5 peptide with the hydrolysed CJC-1295 amidated lysine maleimodopropionic acid tag. (B) Fragmentation of ESA T5 peptide.

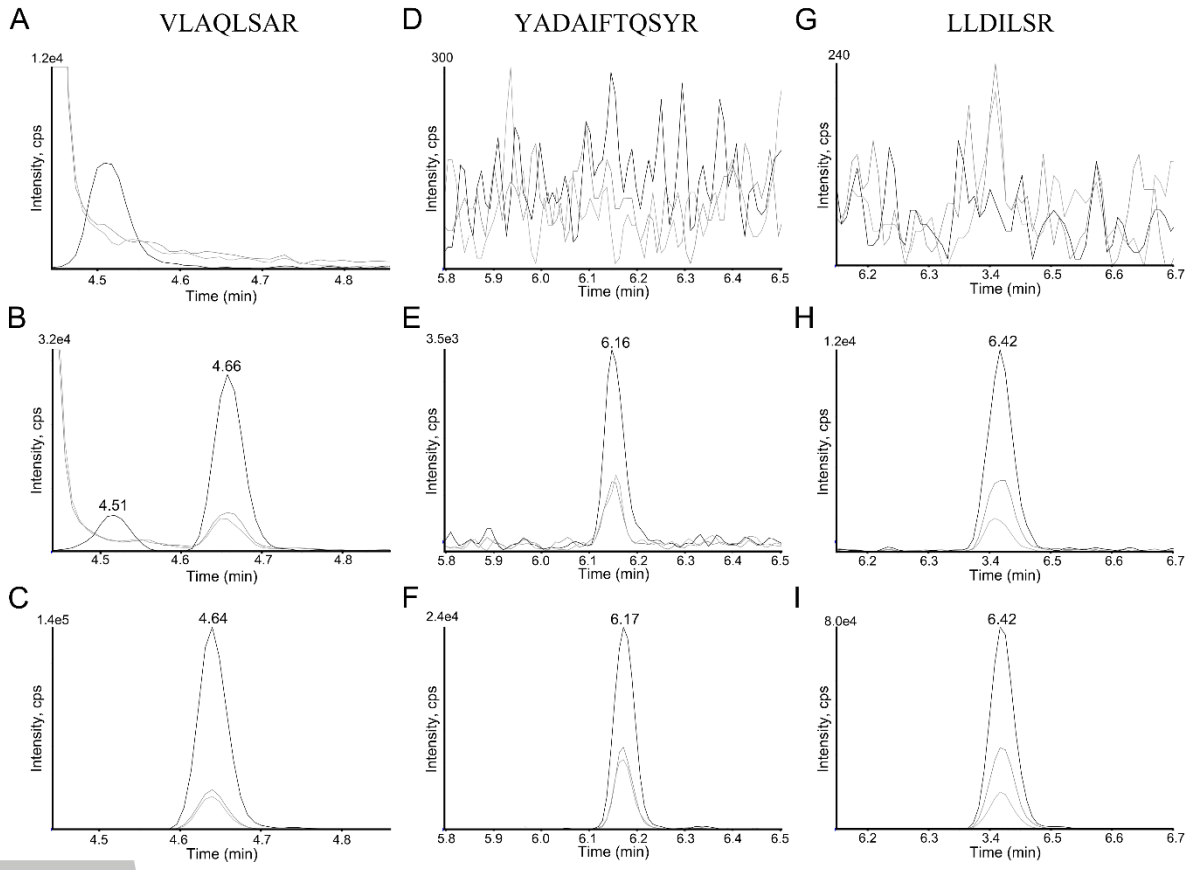

Figure 4. Extraction of CJC-1295 from equine plasma. Panels 4A-4 I show XICs for the CJC-1295 peptides VLAQLSAR, LLQDILSR and YADAIFQSYR following immunoaffinity purification and trypsin digestion. Panels A, D and G show blank plasma. Panels B, E and H show equine plasma spiked with 180pg/mL of CJC-1295 conjugate. Panel C, F and I show analysis of a CJC-1295-without DAC peptide standard.

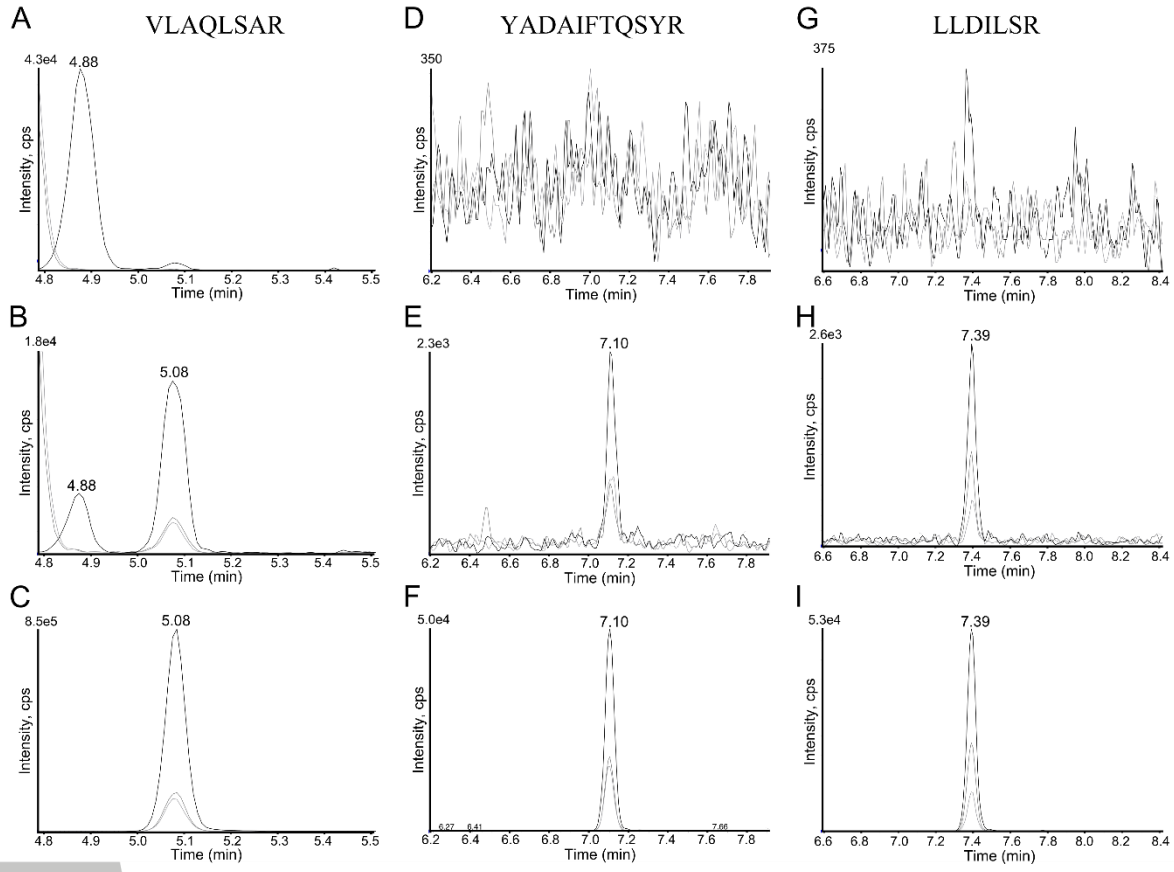

Figure 5. Confirmation of CJC-1295 in a Thoroughbred blood sample after administration of CJC-1295. Panels A, D and G show a blank plasma sample. Panels B, E and H show equine plasma from a CJC-1295 administration study. Panels C, F and I show analysis of a CJC-1295-without-DAC peptide standard.

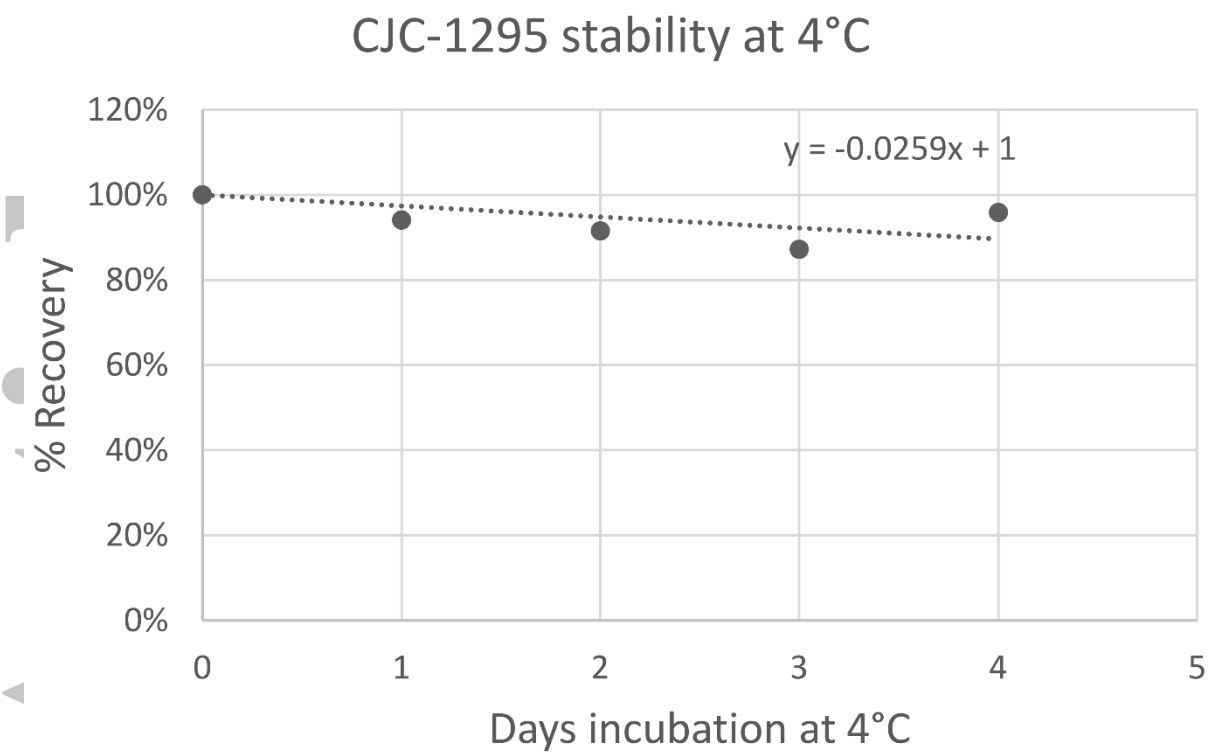

Figure 6. The stability of CJC-1295 protein conjugates in heparinised equine plasma. Equine plasma was spiked with ESA-CJC1295 to a final concentration of 700pg/mL and samples stored at 4°C for up to four days. Extraction was performed using the RD4 monoclonal antibody and the stability of CJC-1295 assessed by measuring the peak area of the VLAQSLAR transition 429/645.