Comparison and validation of ELISA assays for plasma insulin-like growth factor-1 in the horse

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Abstract

Insulin-like growth factor-1 (IGF-1) plays several important physiological roles, and IGF-related pathways have been implicated in developmental osteochondral disease and endocrinopathic laminitis. This factor is also a downstream marker of growth hormone activity and its peptide mimetics. Unfortunately, previously used assays for measuring equine IGF-1 (radioimmunoassays and ELISAs) are no longer commercially available, and many of the kits on the market give poor results when used on horse samples. The aim of the present study was to compare three different ELISA assays (two human and one horse-specific). Plasma samples from six Standardbreds, six ponies and six Andalusians were used. The human IGF-1 ELISA kit from Immunodiagnostic Systems (IDS) proved to be the most accurate and precise of the three kits; the other two assays gave apparently much lower concentrations, with poor recovery of spiked recombinant human IGF-1 and unacceptably poor intra-assay coefficients of variation (CV). The IDS assay gave an intra-assay CV of 3.59 % and inter-assay CV of 7.31%. Mean percentage recovery of spiked IGF-1 was 88.82%, and linearity and dilutional parallelism were satisfied. The IGF-1 plasma concentrations were 123.21 ±8.24 ng/mL for Standardbreds, 124.95 ±3.69 ng/mL for Andalusians and 174.26 ±1.94 ng/mL for ponies. Therefore of the three assays assessed, the IGF-1 ELISA manufactured by IDS was the most suitable for use with equine plasma samples and may have many useful applications in several different research areas. However, caution should be used when comparing equine studies where different analytical techniques and assays may have been used to measure this growth factor.

Keywords: Equine, Insulin-like growth factor, Ponies.

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Introduction

Insulin-like growth factor (IGF-1) is a 70 amino acid peptide, produced by the liver, which plays a role in cell growth and turnover in several different mammalian tissues. Receptors for this growth factor are found in skeletal muscle, bone, cartilage and some epithelial cells, and increased concentrations of this mediator have been associated with developmental osteochondral lesions in growing horses (Verwilghen *et al*., 2009).

Investigators have suggested that IGF-1 plays a critical role in the expression and synthesis of collagen type II as well as protecting chondrocytes from apoptosis. Therefore, changes in concentrations of IGF-1 could modulate the biological mechanisms that regulate joint and bone development (Lejeune *et al*., 2007). Serum IGF-1 concentrations appear to peak in horses at 10 months of age correlating with the beginning of reproductive maturity and then begin to decrease, reaching a steady adult concentration at approximately 450 days old (Fortier *et al*., 2005).

The biological activity of circulating IGF-1 is modulated by a family of IGF-binding proteins, which may promote its interaction with the receptor as well as prolonging its half-life (Kostecka and Blahovec, 1999). Furthermore, the major stimulus for the secretion of IGF-1 from the liver is growth hormone; therefore an 'IGF-1/GH axis' has been described (De Palo *et al*., 2001).

The measurement of plasma IGF-1 concentration can be useful clinically as an indicator of growth hormone deficiency in humans (Faust *et al*., 2012), and in horses measuring IGF-1 concentrations has been found to be a reliable indicator to monitor plasma GH concentrations (Fortier *et al*., 2005). This is helped by the fact that plasma IGF-1 concentrations typically remain relatively stable throughout the day. Certain performance enhancing agents including some peptides may mimic the effects of growth hormone, and so an accurate IGF-1 ELISA assay for use in equine blood samples may be useful in testing performance horses as well as monitoring age related orthopaedic disorders (Popot *et al*., 2001). Furthermore, the role of the IGF-1 signalling pathway is being investigated in equine laminitis (de Laat *et al*., 2013).

Currently the most sensitive means for detecting IGF-1 in equine plasma is LC electrospray ionisation mass spectrometry (Popot *et al*., 2008). However, cost,

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sample preparation time and the availability of equipment may limit the use of this technique. Radioimmunoassays (RIAs) have also been widely used to measure IGF-1.

Comparisons across species have shown that the equine IGF-1 nucleotide sequence is highly homologous to that of other mammals including humans, suggesting that human IGF-1 immunoassays should be appropriate for use in the horse (Ropp *et al*., 2003). De Kock *et al*. (2001) determined the effect of GH administration on IGF-1 concentrations in the horse, validating an RIA assay.

In a study by Noble *et al*. (2007), IGF-1 was measured in a large population of 1,880 Thoroughbred horses, also by RIA. The mean concentration was 310 ng/mL, with a similar magnitude between geldings and mares but increased magnitude in intact males.

Unfortunately these RIAs, as well as a number of previously-used ELISA assays, are no longer commercially available. There are a number of IGF-1 ELISA assays on the market (mostly developed as human IGF-1 assays, with human specific reagents). However, many of these assays give poor results when used for horse samples. There is currently no standard recommended assay for equine IGF-1 that is widely used and this makes it difficult to compare values obtained between different studies.

It is therefore important to validate and compare available assays to determine the most acceptable assay to use in the horse. Also, since there are breed differences in metabolism associated with different concentrations of the important related peptide hormone insulin (Bamford *et al*., 2014), it may be important to consider different breeds when measuring IGF-1.

The aim of this study was to compare three different ELISA assays (two human and one horse-specific).

Materials and Methods

A total of eighteen horses were used in this study: six Standardbreds (mean age: 9.8 years; range 6-16 years), six ponies of various breed (10.2 years; 6-17 years) and six crossbreed Andalusians (10.0 years; 7-14 years). EDTA and heparinised blood samples were collected from each subject via aseptic venipuncture using 10-mL BD Vacutainer tubes with 20 gauge needles. After collection, samples were placed on ice immediately and transported to the laboratory. Plasma was separated and stored in 1-mL aliquots at -80ºC and was not thawed until assayed (within 3 months).

Three assays were evaluated: Assay A: Human IGF-1 ELISA kit (ab100545; Abcam Inc, Massachusetts, USA) (developed for measuring human IGF-1), Assay B: Horse insulin-like growth factor 1 (somatomedin C; IGF-1) ELISA kit (Cusabio Biotech, Hubei Province, China) (equine specific), and Assay C: IGF-1 ELISA-Immunoenzymometric assay for the quantitative

determination of Insulin-like growth factor 1 (IGF-1) in human serum or plasma (Immunodiagnostic Systems (IDS), Boldon, UK) (developed for measuring human IGF-1 in serum or plasma).

Reagents and standards were prepared according to the manufacturers' instructions. The manufacturers' instructions in regards to assay procedures were also followed; however, the addition of an IGF-1 extraction step for Assay B was also implemented to dissociate IGF-1 from the insulin-like growth factor binding proteins (IGFBPs). All assays were read using an automated microplate reader (Synergy H1 Hybrid Reader, BioTek, Vermont, USA), with Gen5 Microplate Reader Software (BioTek, Vermont, USA). Assay A was a commercially available sandwich ELISA kit based on a human IGF-1 antibody-coated plate. Samples were treated with acid-ethanol extraction solution prepared as per the manufacturer's instructions and incubated for 30 minutes. Samples were then centrifuged and 100 µL of supernatant was added to 200µL of Tris-buffer (pH 7.6). Following this, 300µL of sample diluent was added to each tube and 100µL of each sample was pipetted into appropriate wells. Samples were then washed, treated with 100µL of biotinylated antibody and incubated for 1 hour. The sample plate was then washed again and treated with 100µL of streptavidin. TMB was added and the reaction was stopped after 30 minutes incubation and read immediately at 450nm.

Assay B was a commercially available competitive ELISA kit without extraction based on an equine specific IGF-1 fragment. In addition to performing the assay without extraction, an acid-ethanol extraction was performed to dissociate IGF-1 from the IGFBPs; 120µL of commercially purchased acid-ethanol solution was added to 30µL of sample in 3mL polyethylene centrifuge tubes, the samples were then vortexed and incubated at room temperature for 30 minutes. Samples were centrifuged for 5 minutes at 10,000 rpm after which 100µL of supernatant was removed and placed in fresh centrifuge tubes and mixed with 200µL of neutralising solution (2M Tris buffer, pH 7.6).

All samples were then processed as per the manufacturer's instructions. Briefly, samples were diluted with sample diluent and 50µL was added to appropriate wells, 50µL of HRP-conjugate solution was then added and incubated for 40 minutes. The samples were then washed, 90µL of TMB substrate was added to each well and the reaction was stopped after 20 minutes incubation. Absorbance was read immediately at 450nm.

Assay C was a commercially available sandwich ELISA, based on a human IGF-1 antibody-coated plate, and suitable for assay of either human serum or plasma samples.

Rather than an acid-ethanol extraction solution, Assay C employs a proprietary releasing reagent that inactivates IGFBPs. All samples were processed as per the manufacturer's instructions. Briefly, 25μL of sample was added to plastic tubes and incubated with 100μL of releasing agent for 10 minutes. Sample diluent was then added to each tube and 50μL of the diluted sample was added, with 200μL of enzyme conjugate added before a two-hour incubation. The samples were washed and 200μL of TMB substrate was added to each well and stopped after a 30-minute incubation. Absorbance was read immediately at 450nm with a reference wavelength of 650nm.

The coefficient of variation (CV) was determined for each assay using pooled samples from each breed group. Each heparinised plasma sample was processed three times within the same analytical run for each assay to determine intra-assay variation. EDTA plasma samples were also processed for comparison with heparinised plasma.

The target acceptance measure for intra-assay variation was <10%. Inter-assay variation was subsequently determined where an acceptable intra-assay CV was obtained; the acceptable target was also <10%. The accuracy of each assay was determined by investigating parallelism of the standard curve with serial dilutions. Pooled samples from each breed group were diluted (1:2, 1:4, 1:8 and 1:16) with phosphate-buffered saline (PBS) in 3mL polyethylene centrifuge tubes. Expected concentrations of IGF-1 were calculated from the mean concentrations observed for each breed previously and the ratio of observed vs expected concentrations was determined for each dilution. Linearity was determined by plotting observed vs expected concentrations, and linear regression was used to calculate the r^2 value for these plots.

Pooled plasma samples from each breed group were assayed for IGF-1 after they had been spiked with human recombinant IGF-1. The amount of recombinant IGF-1 added to each sample was based on the detection range for each assay so that the expected final concentration did not exceed the working range (Assay A: 18.2 ng/mL, Assay B: 40ng/mL and Assay C: 190ng/mL). Samples were incubated overnight with the recombinant IGF-1 to allow equilibration with binding proteins and then processed as described above. Percentage recovery was then calculated.

Results and Discussion

Assay C proved to be the most accurate and precise of the three kits (Table 1), giving concentrations in the 100-200 ng/mL range for normal plasma, plus it gave the best recovery and validation characteristics. Assay A in comparison gave very low IGF-1 concentrations in the samples and poor recovery. Assay B was slightly better; however, the intra-assay coefficients of variation for both Assays A and B were unacceptably poor.

Assay A yielded the lowest IGF-1 concentrations in the plasma samples of 1.0 ±1.25 ng/mL (mean ±SD) for Standardbreds and 1.05 ±1.69 ng/mL for Andalusians. Ponies showed slightly higher concentrations (2.25 ±3.90 ng/mL). Assay B gave a mean concentration of 11.97 ±2.10 ng/mL for Standardbreds, 15.71 ±7.84 ng/mL for Andalusians and 17.25 ±9.14 ng/mL for ponies. In the absence of an extraction step, all concentrations were below 9 ng/mL in this assay.

Assay C recovered much higher concentrations in the samples, with a mean concentration of 123.21 ±8.24 ng/mL for Standardbreds, 124.95 ±3.69 ng/mL for Andalusians and 174.26 ±1.94 ng/mL for ponies.

The intra-assay coefficient of variation obtained from the three kits ranged from 1.14% - 173.21%, with Assay C being the only kit to have a mean CV less than the acceptable 10% range (3.59 ±1.63 %; Table 1). Percentage recovery from the spiked samples was the lowest for Assay A with a mean of only 28.86% and Assay B had a mean percentage recovery of 79.97%. Assay C had the highest mean % recovery of 88.82%. Dilutional parallelism could only be satisfied for Assays B and C. Since Assay C was the only one of the kits to give IGF-1 plasma concentrations approaching those obtained in previous studies using RIAs (Noble *et al*., 2007), and was the only one with an acceptable intra-assay coefficient of variation, further investigation and validations were only performed for this kit. Firstly, assay characteristics were repeated for Assay C using EDTA plasma samples for comparison with heparinised plasma. Ponies were again found to have the highest IGF-1 concentration of 121.12 ±14.82 ng/mL, followed by Andalusians with 94.30 ±4.15 ng/mL and then Standardbreds with an IGF-1 concentration of 70.36 ±0.60 ng/mL. The mean CV of 5.83% was again acceptable and the mean % recovery was found to be 87.10%. Parallelism performed on pooled samples was found to be linear (Fig. 1). Values obtained for heparinised plasma conducted at the same time were found to be comparable to the previous values with a mean IGF-1 concentration for ponies of 201.95 ±14.6 ng/mL, Andalusians 146.16 ±3.64 ng/mL and Standardbreds 138.74 ±5.24 ng/mL. Again parallelism was satisfied and % recovery was 90.65%. Therefore values obtained from heparinised plasma were consistently higher than values from the same animals using EDTA plasma, even though recovery appeared to be similar. The inter-assay variation across analytical runs on different days (and on different plates) was also determined for Assay C only, and found to be 7.31%. This study evaluated the performance of three ELISA assays for the measurement of IGF-1 in the horse, using plasma samples obtained from three different breeds/types; namely ponies (of mixed breed), Andalusian horses and Standardbred horses.

Table 1. Comparison between different assay kits for the measurement of IGF-1 in heparinised equine plasma from ponies, Andalusian horses and Standardbred horses. Intra-assay coefficients of variation (CV) were calculated from the standard deviation (SD) expressed as a % of the mean; recovery was calculated by using spiked samples and linearity was determined by linear regression from plots of observed vs expected values.

	Mean (ng/mL)	SD	CV %	% Recovery	Linearity (r^2)	Parallelism (yes/no)
Assay A (Abcam)						
Pony	2.25	3.90	173.21	43.20	0.77	No
Andalusian	1.05	1.69	160.99	18.68	0.47	No
Standardbred	1.00	1.25	124.90	24.70	0.84	No
Mean			**153.00**	**28.86**		
SEM			**14.50**	**7.38**		
Assay B (Cusabio)						
Pony	17.25	9.14	52.98	72.98	0.99	Yes
Andalusian	15.71	7.84	49.90	81.93	0.97	Yes
Standardbred	11.97	2.10	17.57	73.01	0.99	Yes
Mean			**40.15**	**75.97**		
SEM			**11.30**	**2.98**		
Assay C (IDS)						
Pony	174.26	1.94	1.14	90.40	0.99	Yes
Andalusian	124.95	3.69	2.95	82.92	0.99	Yes
Standardbred	123.21	8.24	6.69	93.13	0.99	Yes
Mean			**3.59**	**88.82**		
SEM			**1.63**	**3.05**		

Table 2. Comparison between EDTA and heparinised plasma samples. IGF-1 concentrations were measured in EDTA and heparinised plasma samples from ponies and horses. Intra-assay coefficients of variation (CV) were calculated from the standard deviation (SD) expressed as a % of the mean; recovery was calculated by using spiked samples and linearity was determined by linear regression from plots of observed vs expected values.

	Mean (ng/mL)	SD	CV %	% Recovery	Linearity (r^2)	Parallelism (yes/no)
EDTA plasma						
Pony	121.12	14.82	12.24	83.69	0.99	Yes
Andalusian	94.30	4.15	4.40	90.85	-	No
Standardbred	70.36	0.60	0.85	86.75	-	No
Mean			**5.83**	**87.10**		
SEM			**3.36**	**2.07**		
Heparin plasma						
Pony	201.95	14.16	7.01	93.25	1.00	Yes
Andalusian	146.16	3.64	2.49	90.79	0.99	Yes
Standardbred	138.74	5.24	3.77	87.92	1.00	Yes
Mean			**4.42**	**90.65**		
SEM			**1.35**	**1.54**		

These types were selected because Standardbreds represent most typical athletic breeds of horse, while ponies and Andalusian horses tend to demonstrate metabolic characteristics including hyperinsulinaemic responses to dietary carbohydrates, regional obesity and also an increased risk of laminitis (Bamford *et al*., 2014). Assay A was found to be totally unsuitable for use in the horse, since it gave a recovery of less than 30% of spiked human recombinant IGF-1 in equine plasma, and parallelism could not be satisfied. Clearly there were substances in the plasma which interfered with the detection of the target peptide that resulted in very low apparent concentrations of IGF-1 in the assay of normal equine plasma samples. The factors interfering with IGF-1 detection are unknown. The IGFBPs may well be involved, although the acid-ethanol extraction method used in this assay to dissociate IGF-1 from its binding proteins is typical of many other kits, both ELISAs and RIAs. An acid-ethanol extraction method was also used in Assay B, although recovery was much improved compared with Assay A.
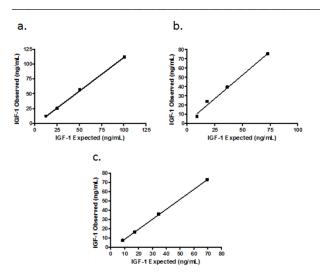
Fig. 1. Observed vs. expected concentrations of IGF-1 measured by Assay C, following dilution of pony (a), Andalusian (b) and Standardbred (c) heparinised plasma. R^2 values were 0.99 in all cases. The plasma assayed for each breed group was a pooled sample containing 6 individual animals.

However, recovery from spiked samples was still not optimal even though parallelism appeared to be satisfied, and relatively low values were also measured in the normal plasma samples. The intra-assay coefficient of variation was certainly not acceptable, and therefore either the extraction of IGF-1 from binding proteins or the degree of binding to the capture antibody may have been very variable from sample to sample or well to well.

Of the three IGF-1 assays evaluated, Assay C was the most suitable for measuring IGF-1 concentrations in the horse. This assay had an excellent coefficient of variation, both intra- and inter-assay, showed good dilutional parallelism and the plots of expected to observed concentrations in the diluted samples clearly demonstrated a linear relationship. The assay results in normal equine plasma were in the range of approximately 100-200 ng/mL which was at least an order of magnitude higher than the other two assays. The percentage recovery after spiking samples (88.42%) was slightly lower than the recovery reported by the manufacturer (95%), and this was acceptable although still not ideal. The improved recovery compared with the other two kits may have been related to the extraction method, since the other kits used the acid-ethanol technique; however, acid-ethanol extraction has been considered as an acceptable method in previous assays, including the very useful and sensitive RIAs (Noble *et al*., 2007). The range of normal concentrations for IGF-1 in equine plasma reported using radioimmunoassay techniques is around 300 ng/mL (Noble *et al*., 2007). That study measured concentrations in Thoroughbreds, which presumably would have IGF-1 concentrations similar to Standardbreds, which had the lowest IGF-1 concentrations of the breeds in our study. Therefore assuming the RIA is accurate, ELISA assay C may tend to under-report the true plasma concentration, and this might be associated with the recovery being less than 100%. However, in the studies by Noble *et al*. (2007) and Ropp *et al*. (2003), it is notable that they measured IGF-1 in samples of serum rather than plasma. It could be argued that plasma samples might be more appropriate for the measurement of IGF-1, because platelets contain significant quantities of IGF-1 (bound to IGFBP-3) within their alpha granules (Chan and Spencer, 1998). This additional IGF-1 would be released during the clotting process, accounting for a considerably higher apparent concentration. Therefore the concentrations found in the present study, using heparinised plasma, may represent more accurately the actual circulating concentration of this hormone. Serum samples were not available in the present study for comparison. Heparinised plasma consistently gave higher values than EDTA plasma. It should be noted that EDTA has occasionally been found to interfere with some types of ELISA (Assink *et al*., 1983).

It was interesting to note that the plasma IGF-1 concentrations recorded in samples from ponies appeared to be higher than those observed in the horse breeds, although this was not the primary objective of the current study. Further studies would be necessary to confirm whether there are breed differences. If there are, then this would not appear to be related to the hyperinsulinaemia and insulin resistance seen in ponies, because the IGF concentrations in Andalusian horses (which also show this tendency) were not different to Standardbreds (Bamford *et al*., 2014). These findings confirm the suitability of the IGF-1 ELISA-Immunoenzymometric assay manufactured by IDS for use in the horse and this kit seems to provide a convenient, straightforward and cost effective method for measuring this growth factor.

It may have applications for detecting the use of growth hormone or its mimetics and in the investigation of developmental osteochondral disease and endocrinopathic laminitis. Caution should be used when comparing equine studies where different analytical techniques have been used to measure concentrations of IGF-1.

Conflict of interest

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