

Evaluation of a commercially available radioimmunoassay and species-specific ELISAs for measurement of high concentrations of insulin in equine serum

Kate E. Borer-Weir, BVSc, PhD; Simon R. Bailey, BVMS, PhD; Nicola J. Menzies-Gow, VetMB, PhD; Patricia A. Harris, VetMB, PhD; Jonathan Elliott, VetMB, PhD

Objective—To evaluate a human radioimmunoassay (RIA) and equine and high-range porcine (hrp) species-specific ELISAs for the measurement of high serum insulin concentrations in ponies.

Samples—Serum samples from 12 healthy nonobese ponies (7 clinically normal and 5 laminitis prone; 13 to 26 years of age; 11 mares and 1 gelding) before and after glucose, insulin, and dexamethasone administration.

Procedures—Intra- and interassay repeatability, freeze-thaw stability, dilutional parallelism, and assay agreement were assessed.

Results—Assay detection limits were as follows: RIA, $< 389 \mu\text{U/mL}$; equine ELISA, $< 175 \mu\text{U/mL}$; and hrp ELISA, 293 to 8,775 $\mu\text{U/mL}$. Mean \pm SD intra- and interassay repeatability were respectively as follows: RIA, $6.5 \pm 5.1\%$ and $7.4 \pm 3.4\%$; equine ELISA, $10.6 \pm 11.0\%$ and $9.0 \pm 4.6\%$; and hrp ELISA, $19.9 \pm 17.2\%$ and $17.3 \pm 16.6\%$. Freezing and thawing affected measured concentrations. Dilutional parallelism in the RIA was only evident when insulin-depleted equine serum was used as a diluent (percentage recovery, $95.7 \pm 27.4\%$); in the ELISAs, dilutional parallelism was observed when a zero calibrator was used. Agreement between RIA and equine ELISA results was good for samples containing concentrations $< 175 \mu\text{U}$ of insulin/mL (bias, $-18.5 \pm 25.5 \mu\text{U/mL}$; higher in RIA). At higher concentrations, assay agreement was poor between RIA and equine ELISA results (bias, $-185.3 \pm 98.7 \mu\text{U/mL}$) and between RIA and hrp ELISA results (bias, $25.3 \pm 183.0 \mu\text{U/mL}$).

Conclusions and Clinical Relevance—Agreement among results of the 3 assays was variable, and dilutional parallelism was only evident with the RIA when insulin-depleted equine serum was tested. Caution is recommended when evaluating high insulin concentrations measured with the RIA or ELISAs. (*Am J Vet Res* 2012;73:1596–1602)

Insulin resistance in horses and its links to endocrine and metabolic diseases, including laminitis and so-called equine metabolic syndrome, are receiving

Received July 21, 2011.

Accepted October 12, 2011

From the Department of Veterinary Clinical Sciences, Royal Veterinary College, North Mymms, Hatfield, Hertfordshire, AL9 7TA, England (Borer, Menzies-Gow); Faculty of Veterinary Science, University of Melbourne, Melbourne, VIC 3031, Australia (Bailey); Waltham Centre For Pet Nutrition, Freeby Ln, Waltham-on-the-Wolds, Leicestershire, LE14 4RT, England (Harris); and Department of Veterinary Basic Sciences, Royal Veterinary College, University of London, London, NW1 0TU, England (Elliott).

Dr. Borer was in receipt of a Biotechnology and Biological Sciences Research Council Collaborative Awards in Science and Engineering studentship in association with the Waltham Centre for Pet Nutrition.

Presented in oral form at the 48th British Equine Veterinary Association Congress, Birmingham, England, September 2008.

Mercodia provided the ELISA kits; the authors have no other financial relationship with Mercodia, and Mercodia had no role in the collection of data, interpretation of the results, or writing of the manuscript.

Address correspondence to Dr. Borer (kborer@rvc.ac.uk).

ABBREVIATIONS

CV	Coefficient of variation
I ¹²⁵	Sodium iodide I 125
IDS	Insulin-depleted equine serum
LC-MS	Liquid chromatography–mass spectrometry
RIA	Radioimmunoassay

ing increasing attention. A recent consensus statement outlines criteria for the diagnosis of equine metabolic syndrome that includes confirmation of the presence of insulin resistance.¹ Most insulin-resistant horses have compensatory insulin resistance with resulting hyperinsulinemia.^{2,3}

Accurate measurement of serum insulin concentrations is central to diagnosis of insulin resistance and is challenging in horses and ponies. No gold standard diagnostic test is available, and the various available assays are not comparable, making interpretation of values and determination of laboratory reference limits

difficult.¹ A cutoff value of 20 $\mu\text{U}/\text{mL}$ has been recommended as the upper reference limit for serum insulin concentration,¹ although values as high as 700 $\mu\text{U}/\text{mL}$ have been demonstrated in laminitis-prone ponies.² Thus, a wide range of serum insulin concentrations is possible in horses, and an ideal assay should be able to yield accurate results across this range. It is particularly important to accurately measure serum insulin concentrations in equids with hyperinsulinemia.

Laminitis has been induced in clinically normal horses and ponies that received a continuous rate infusion of insulin for 48 to 72 hours. In those studies,^{4,5} serum insulin concentrations increased to approximately 1,000 $\mu\text{U}/\text{mL}$, as measured with an RIA, which exceeds the upper detection limit of the assay. Validation of the assay once samples are diluted is required to determine the precision and accuracy of these measured concentrations. It is also important to determine whether ponies in natural conditions consistently have high serum insulin concentrations, which have been hypothesized to induce laminitis. For this reason, accurate measurement of serum insulin concentrations $\geq 1,000 \mu\text{U}/\text{mL}$ is necessary. Furthermore, treatment protocols for equids with insulin resistance include administration of drugs that decrease serum insulin concentrations,⁶ making it clinically important to accurately detect slight changes in these concentrations, even when they remain high.

Serum insulin concentrations in horses are commonly measured with RIAs. Although RIAs have been validated for this purpose, the validation process primarily involved measurement of low serum insulin concentrations.⁷⁻⁹ Problems have been reported with the use of insulin RIAs in horses, particularly when the serum samples contain high insulin concentrations (as high as 2,000 $\mu\text{U}/\text{mL}$).^{10,11} The commercially available assays are solid-phase RIAs based on competitive binding between either insulin labeled with radioactive iodine (I^{125}) or the horse's insulin to polyclonal antibody bound to the wall of a polypropylene tube.^a The antibody used in the assay was changed in 1995^b after the previous validation studies⁷⁻⁹ were performed. Problems with assay reproducibility also occur when RIAs are used to measure serum insulin concentrations in humans, and these problems are associated with the use of kits containing different lots of antibody.¹² It has consequently been recommended that revalidation be performed whenever a new lot of antibody is used.^{8,12}

Insulin is formed within the beta cells of the pancreas by cleavage of proinsulin, releasing the connecting peptide (C-peptide) in the process.¹³ Insulin is a polypeptide hormone comprised of an A chain and B chain, which are covalently joined by disulfide links.^{13,14} The molecular weight of human insulin is 5,808 Da, and that of equine insulin is 5,748 Da.¹⁵ Two amino acid differences distinguish equine insulin from human insulin. Amino acid 9, which is in the center of the A chain, is serine in humans and glycine in equids.¹⁴ This region of the insulin molecule is considered to be hypervariable among mammalian species and is not usually considered important for biological activity.¹⁶ Amino acid 30, which is located at the carboxy (C) terminus of the B chain, is threonine in humans and alanine in equids.¹⁴ This region is also considered hypervariable,¹⁶ although

substitutions therein may affect the 3-D structure of the molecule and are known to affect the rate of self-association of insulin.¹⁴ The different amino acid located at position 30 in equine and human insulin could affect the molecule's binding to the polyclonal antibody in an RIA tube, resulting in different assay characteristics between humans and equids.

An equine-specific ELISA designed to measure low to moderate insulin concentrations has been developed and is based on a direct sandwich technique involving 2 monoclonal antibodies against insulin.^c The results of this ELISA are in good agreement with those of an RIA and ELISA designed to measure insulin concentrations in humans^d; however, the validity of the equine-specific ELISA has not been determined for samples containing high insulin concentrations. A porcine ELISA^e is available with a higher upper detection limit than that of the equine ELISA, which would eliminate the need to dilute samples containing a high insulin concentration, although this assay has not been validated for use with equine serum either. Porcine insulin differs from equine insulin in only 1 amino acid: the glycine at position A9 in equids is serine in swine.¹⁵ Therefore, assays that involve antibody against porcine insulin may be more accurate for measuring serum insulin concentrations in horses than are those that involve antibody against human insulin.

The objectives of the study reported here were to evaluate the currently available insulin RIA and equine and high-range porcine ELISAs in the measurement of high equine serum insulin concentrations and to compare serum insulin concentrations in horses as measured with all 3 assays. The hypothesis was that the RIA and species-specific ELISAs would be comparable and valid for measurement of high serum insulin concentrations in horses.

Materials and Methods

Animals—Twelve ponies (7 clinically normal and 5 previously laminitic) were used; all were mixed UK-native breeds (primarily New Forest, Welsh, and cross breeds) and were not of the same lineage. The clinically normal ponies had no history of laminitis within 5 to 10 years prior to the study. The previously laminitic ponies had recurrent bouts of pasture-associated laminitis within the previous 5 years. The laminitis had been diagnosed by experienced equine surgeons on the basis of clinical signs and, when required, digital radiography findings. None of the ponies developed clinical signs of active laminitis during the study.

All clinically normal ponies were mares, and the previously laminitic ponies included 4 mares and 1 gelding. The mean \pm SD age and body weight for the clinically normal ponies were 19 ± 4 years and 341 ± 71 kg, respectively, and for the previously laminitic ponies were 16 ± 3 years and 289 ± 60 kg, respectively. The mean \pm SD body condition score of the clinically normal ponies on a 9-point scale¹⁷ was 5.4 ± 1.1 and of the previously laminitic ponies was 5.0 ± 1.0 ; body condition did not vary over the study period. All procedures involving the ponies received institutional ethical approval prior to study commencement.

Sample collection—Blood samples for insulin analysis were obtained on multiple occasions during other studies involving IV injection of glucose and insulin, IM injection of dexamethasone, and feeding of glucose. Samples were collected in evacuated tubes and allowed to clot at 37°C for 20 minutes followed by centrifugation (3,000 × g) at 4°C for 10 minutes. Serum was harvested and stored at –80°C pending analysis.

Insulin assays—Serum insulin concentrations were analyzed with a commercially available RIA kit.^a All samples remained undiluted in accordance with the manufacturer's instructions. Briefly, 200 µL of each serum sample was assayed in duplicate in antibody-coated polypropylene tubes and incubated with 1,000 µL of I¹²⁵-labeled insulin for 20 hours at room temperature (approx 18° to 20°C). Afterward, the liquid was decanted and counts of I¹²⁵ bound to the tube were determined with a gamma counter.

Samples were also analyzed with 2 commercially available ELISA kits: one specifically designed to measure equine insulin^c (detection limits, 0.02 to 1.5 µg/L [2.3 to 175.5 µU/mL]) and the other designed to measure high concentrations of porcine insulin^c (detection limits, 2.5 to 75 µg/L [293 to 8,775 µU/mL]). The conversion factor from µg/L to µU/mL of 117 was supplied by the manufacturer, as determined on the basis of their analysis of 116 serum samples from clinically normal horses with the equine-specific ELISA, as calibrated against the first human international reference preparation 66/304.^f For the equine-specific ELISA, duplicate 25-µL serum samples were incubated for 2 hours with an anti-insulin antibody conjugated to a peroxidase enzyme in wells coated with another anti-insulin antibody. Unbound enzyme-conjugated antibody was then washed away, and the antibody-bound enzyme remaining in the wells was conjugated to 3,3'-5,5'-tetramethylbenzidine to provide a detectable colorimetric endpoint followed by sulfuric acid to stop the reaction. The principle of the procedure was the same for the high-range porcine ELISA, but 10 µL of serum was incubated for 1 hour with the anti-insulin antibody in the wells.

Intra- and interassay repeatability—For the RIA, intra- and interassay repeatability were measured by use of 9 pooled equine serum samples, which were each assayed twice within the same assay and in 2 different assays. The pooled samples were created to contain low, medium, and high insulin concentrations within the limits of detection of the RIA (approx 10, 100, and 300 µU/mL, respectively). In the equine insulin ELISA, 10 serum samples within the limits of detection of the ELISA (concentration range, 10 to 150 µU/mL) were measured twice with each of 2 assays. Twenty samples (concentration range, 300 to 1,500 µU/mL) were evaluated twice in each of 4 different assays to determine intra- and interassay repeatability of the high-range porcine ELISA.

Stability of insulin after freezing and thawing—The RIA was used to determine the effect of freezing and thawing on equine insulin measurement by analyzing 13 serum samples that had been stored at –80°C for

2 to 4 months and that contained insulin concentrations from 5 to 420 µU/mL after the first and second thaw. The calculated insulin concentrations before and after second thawing were compared. The high-range porcine ELISA was used to measure insulin concentrations in 22 serum samples (concentration range, 380 to 1,950 µU/mL) after their first and second thaw, and results were compared between thaws.

Dilutional parallelism—For the RIA, equine serum samples were diluted from 2 to 32 times with 4 diluents: distilled water, PBS solution, the zero standard supplied by the manufacturer, and IDS previously prepared in the authors' laboratory. The IDS was prepared by the incubation of 1 mL of equine serum with 50 mg of activated charcoal^g for 20 hours at 20°C with continuous mixing. Serum was then centrifuged twice (3,000 × g) at 4°C for 20 minutes, with the supernatant decanted after each centrifugation. Finally, the supernatant was filtered through a 0.2-µm syringe filter^h and stored at –80°C. A sample of IDS was analyzed with each assay to confirm the absence of insulin. Additionally, the 2 most concentrated standards supplied by the manufacturer (insulin concentrations, 389 and 201 µU/mL) were serially diluted from 2 to 16 times with IDS to assess dilutional parallelism of the standards in the equine serum matrix.

The percentage recovery of insulin in diluted equine serum samples on dilution was calculated for the various diluents. To assess dilutional parallelism, the insulin concentration within the sample to be diluted was required to be within the detection limits of the assay (approx 14% to 80% binding). For some samples, this required predilution of the original sample with the same diluent to decrease the concentration to within the detection limits of the assay. Percentage recovery of insulin after dilution was then calculated for each subsequent dilution by reference to the concentration of insulin measured in the first dilution (between 14% and 80% binding).

For the ELISAs, serum samples were diluted with the zero calibrator supplied with the respective ELISA kit and with IDS from 2 to 8 times and assayed. Percentage recovery after dilution was calculated as described.

Comparison of RIA standard curves—To assess the matrix effects when the various diluents were used to reconstitute the freeze-dried standards supplied by the manufacturer, different RIA standards from the same lot were reconstituted with distilled water (as recommended by the manufacturer) or IDS. The standards were then assayed with the same RIA, and the total radioactivity counts and percentage binding of each standard were compared.

Cross-reaction or interference by C-peptide—Human C-peptideⁱ was added to IDS or the RIA zero standard, serially diluted as many as 8 times. Percentage recovery after dilution was calculated after these dilutions were assayed with the RIA.

Statistical analysis—The Kolmogorov-Smirnov test was used to assess normality of data distribution.^j A 1-sample *t* test or Wilcoxon signed rank test was used to compare percentage recovery on dilution to the expected 100% recovery. Insulin concentrations mea-

Table 1—Percentage recovery of insulin on dilution of equine serum samples (initial insulin concentrations indicated in parentheses) with various diluents that were subsequently assayed with a commercially available RIA designed for use in humans.

Diluent	Range of dilutions (X)	Recovery (%)	
		Mean ± SD	Range
PBS solution (n = 6; 41–1,135 μU/mL)	2–16	48.0 ± 18.5	15.1–62.8
Distilled water (n = 11; 34–993 μU/mL)	2–32	57.0 ± 30.4	10.6–106.7
Zero standard (n = 7; 28–1,721 μU/mL)	2–32	72.1 ± 15.2	52.2–91.3
IDS (n = 12; 13–2,460 μU/mL)	2–32	95.7 ± 27.4	69.0–149.8

Percentage recovery was only calculated for samples in which the percentage binding was within the detection limits of the RIA (up to an insulin concentration of 389 μU/mL; 14% to 80% binding). The samples with initial measured insulin concentrations higher than this range required predilution with the same diluent to decrease the concentrations to within the detection limits of the RIA. The first value for insulin concentration with percentage binding within the detection limits of the RIA was used to calculate percentage recovery for further dilutions.

sured with the RIA and ELISAs were compared through Bland-Altman analysis. Values are reported as mean ± SD when normally distributed or median (range) when not normally distributed. Values of $P < 0.05$ were considered significant for all analyses.

Results

RIA—The mean ± SD intra-assay CV was $6.5 \pm 5.1\%$, and interassay CV was $7.4 \pm 3.4\%$. The intra- and interassay CVs were similar for pooled serum samples containing low ($7.3 \pm 7.1\%$ and $6.7 \pm 4.4\%$, respectively), medium ($3.3 \pm 1.0\%$ and $7.0 \pm 0.1\%$, respectively), and high ($7.5 \pm 2.6\%$ and $8.7 \pm 3.8\%$, respectively) insulin concentrations and for samples obtained before and after ponies had received glucose, insulin, and dexamethasone in other studies.

The median difference in insulin concentration within the RIA after freezing and thawing was $0.94 \mu\text{U/mL}$, although the difference ranged from -128.4 to $83.2 \mu\text{U/mL}$, which represents a percentage difference of -62% to 62% . The percentage difference was smaller for samples containing measured insulin concentrations $> 100 \mu\text{U/mL}$ (range, -36% to 28%).

When serum samples were diluted with PBS solution, distilled water, or zero standard, the percentage recovery of insulin decreased with increasing dilution and dilutional parallelism was not observed (Table 1). Samples diluted with IDS yielded the most consistent recovery of insulin after dilution, and the mean percentage recovery when IDS was used was not significantly ($P = 0.60$) different from 100%.

Dilution of the 2 highest concentration RIA standards supplied by the manufacturer with IDS resulted in mean percentage recoveries of $104.2 \pm 21.6\%$ and $99.7 \pm 11.7\%$. Standard curves constructed from insulin values obtained with RIA standards reconstituted with distilled water or IDS were graphically displayed (Figure 1). The percentage binding of the standards reconstituted with the various diluents was similar, particularly for the higher concentration standards. When different amounts of human C-peptide were added to IDS or the RIA zero standard, the percentage binding of I^{125} within the RIA (range, 98% to 135%) was similar to or greater than that of the zero standard (assigned an arbitrary value of 100% binding).

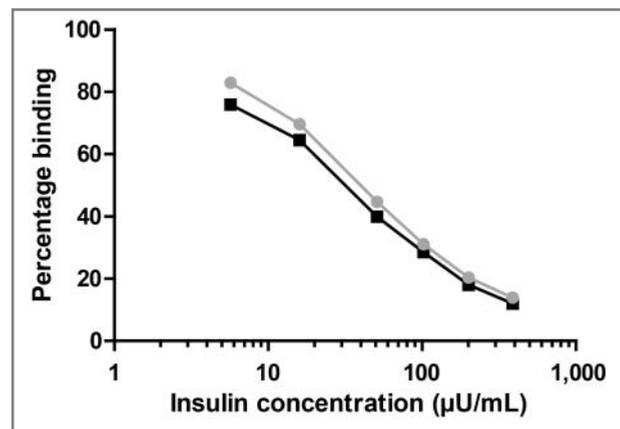


Figure 1—Standard curves for an insulin RIA constructed on the basis of values for standards reconstituted with distilled water (squares) or IDS (circles). Percentage binding represents the radioactivity count of the standard in relation to the radioactivity count of the zero standard (assigned an arbitrary value of 100% binding). The standards contained the following insulin concentrations (set by the manufacturer of the RIA): 5.7, 16, 51, 102, 201, and 389 μU/mL. For IDS-reconstituted standards, 80% maximum binding was 7 μU/mL and 20% maximum binding was 224 μU/mL. For distilled water-reconstituted standards, 80% maximum binding was 5 μU/mL and 20% maximum binding was 195 μU/mL. Notice that the x-axis is a logarithmic scale.

ELISA—For the equine-specific ELISA, the mean intra-assay CV was $10.6 \pm 11.0\%$ and interassay CV was $9.0 \pm 4.6\%$. For the high-range porcine ELISA, the mean intra-assay CV was $19.9 \pm 17.2\%$ and interassay CV was $17.3 \pm 16.6\%$. The mean difference in insulin concentration within the high-range porcine ELISA after freezing and thawing was $52.3 \pm 221.0 \mu\text{U/mL}$ (median difference, $15.6 \mu\text{U/mL}$; range, -343.1 to $514.4 \mu\text{U/mL}$).

In the equine-specific ELISA, dilution of serum samples with the zero calibrator yielded a mean percentage recovery of $102.4 \pm 20.5\%$ (range, 70.7% to 137.1%), which was not significantly ($P = 0.65$) different from 100%. In the high-range porcine ELISA, dilution of samples with the zero calibrator yielded a median percentage recovery of 99.9% (range, 34.5% to 328.9%), which was not significantly ($P = 0.22$) different from 100%. When serum samples were diluted with IDS in the high-range porcine ELISA, the mean

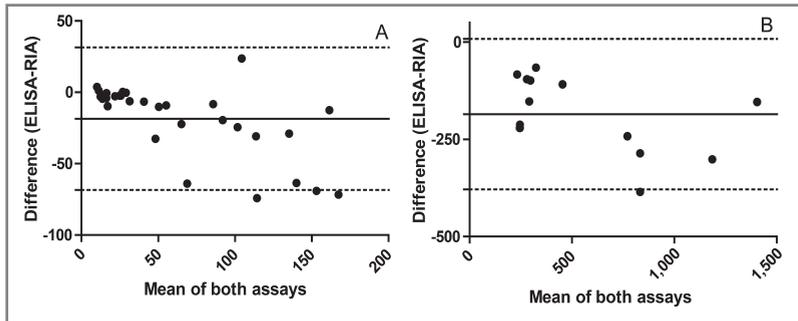


Figure 2—Bland-Altman plots showing agreement between insulin concentrations ($\mu\text{U/mL}$) in equine serum samples as measured with an equine-specific ELISA and a human RIA. A—Samples used had initial insulin concentrations as measured with the ELISA of $< 175 \mu\text{U/mL}$ (within the working range of the ELISA). B—Samples used had initial insulin concentrations that were higher than the upper detection limit of the ELISA, which required serum dilution to decrease the insulin concentrations to within the detection limits of the assay. In each panel, the solid line represents the bias (mean \pm SD, $-18.5 \pm 25.5 \mu\text{U/mL}$ in A; $-185.3 \pm 98.7 \mu\text{U/mL}$ in B) and the dotted lines indicate the 95% limits of agreement (-68.5 to $31.4 \mu\text{U/mL}$ in A; -378.8 to $8.2 \mu\text{U/mL}$ in B).

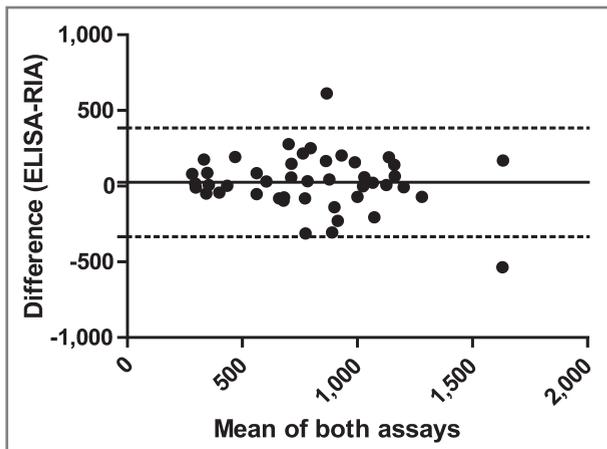


Figure 3—Bland-Altman plot showing agreement between insulin concentrations ($\mu\text{U/mL}$) in equine serum samples measured with a high-range porcine ELISA and a human RIA. The solid line represents the bias ($25.3 \pm 183.0 \mu\text{U/mL}$) and the dotted lines indicate the 95% limits of agreement (-333.5 to $384.0 \mu\text{U/mL}$).

percentage recovery was $182.9 \pm 112.9\%$ (range, 57.2% to 431.0%), which was significantly ($P = 0.01$) different from 100%.

Comparison between the RIA and ELISAs—The comparisons for the equine-specific ELISA were divided into those samples with initial measured insulin concentrations in the ELISA $< 175 \mu\text{U/mL}$ (within the detection limits of the ELISA) and those samples with initial measured concentrations $> 175 \mu\text{U/mL}$ that required dilution before concentrations were within the detection limits of the ELISA. For those samples with measured insulin concentrations $< 175 \mu\text{U/mL}$, the mean bias between the ELISA and RIA was $-18.5 \pm 25.5 \mu\text{U/mL}$ (concentrations higher in the RIA) and 95% limits of agreement were -68.5 to $31.4 \mu\text{U/mL}$ (Figure 2). For serum samples analyzed with the equine-specific ELISA that had initial measured insulin concentrations $> 175 \mu\text{U/mL}$ (thus diluted with zero standard before measurement), the mean bias was $-185.3 \pm 98.7 \mu\text{U/mL}$ (concentrations higher in the RIA) and 95% limits of agreement were -378.8 to $8.2 \mu\text{U/mL}$. The difference

in insulin concentrations measured with the high-range porcine ELISA and the RIA ranged from -535.5 to $611.8 \mu\text{U/mL}$ (Figure 3). The mean bias was $25.3 \pm 183.0 \mu\text{U/mL}$ (concentrations higher in the ELISA), and 95% limits of agreement were -333.5 to $384.0 \mu\text{U/mL}$.

Discussion

When the RIA designed for use in humans is used to measure high serum insulin concentrations in equids, serum samples should be diluted with IDS to obtain the most precise, reliable results. When a species-specific ELISA is used, the zero standard supplied with the kit should be used for dilution. The study reported here showed the 3 assays evaluated yielded comparable results when used to measure low serum insulin concentrations (up to $175 \mu\text{U/mL}$) in equine serum, but were not comparable at higher concentrations. None of the assays accurately measured high insulin concentrations, although dilution of samples for use in the RIA appeared to produce the most consistent results.

The intra- and interassay repeatability were good for the RIA (both $< 7.5\%$) but were only moderate for the equine-specific ELISA (approx 10%) and were poor for the high-range porcine ELISA (17% to 20%).¹⁸ This variability may be attributable to the small sample volume ($10 \mu\text{L}$) required for the porcine assay, resulting in inaccuracies in dispensing the sample into the wells. Intra- and interassay repeatability for the high-range porcine ELISA were assessed in 4 assay sessions, and samples with high CVs were detected in each. The measured insulin concentrations in these samples with the highest CVs (up to 67%) ranged from 225 to $1,200 \mu\text{U/mL}$. Conversely, other samples with measured insulin concentrations in this range yielded CVs of $< 1\%$, and there was no consistent relationship between the measured insulin concentration and the CV. These large intra- and interassay variabilities limit the accuracy of insulin concentrations measured with the high-range porcine ELISA.

Freezing and thawing had different effects on samples analyzed with the RIA and the high-range porcine ELISA. Although the effect on measured median insulin concentration when the RIA was used was small, the measured range was large, representing a difference in insulin concentrations of approximately $200 \mu\text{U/mL}$. The difference was equally large between samples in which measured insulin concentration was lower after freezing and thawing and those in which it was higher. Measured insulin concentration was more likely to be lower in the high-range porcine ELISA after freezing and thawing than before freezing and thawing, but again the range of differences was large. Some of this difference might have been due to high interassay variation in the high-range porcine ELISA. In view of this, we recommend that samples not be subjected to repeated freezing and thawing before measurement of insulin concentrations. One of the limitations of the

present study was that insulin concentrations were not measured in any of the samples prior to freezing, so the effect of the initial freeze is unknown.

For analysis of samples expected to contain insulin concentrations that exceed the detection limits of the RIA, the manufacturer recommends diluting the samples with the zero standard supplied with the kit. In the present study, dilutional parallelism was not observed with equine serum samples diluted with zero standard or with distilled water or PBS solution. However, use of IDS to dilute equine serum samples resulted in parallelism. Additionally, standards diluted with IDS demonstrated parallelism. The mean percentage recovery for equine serum samples (with a wide range of initial measured insulin concentrations) serially diluted up to 32 times with IDS was 96%. There was a wide range in percentage recovery (69% to 150%), which suggested that the RIA may have detected substances other than insulin in serum. For these reasons, caution is necessary when interpreting the accuracy of measured insulin concentrations in only 1 sample after dilution. The circumstances also make it difficult to define specific cutoff concentrations that may be associated with laminitis induced after prolonged insulin infusions in experiments.^{4,5} Standard curves constructed from standards reconstituted with IDS or distilled water were similar; thus, there are unlikely to be significant matrix effects when diluents other than distilled water (eg, IDS) are used to reconstitute the standards.¹⁹ In contrast, when IDS was used to dilute serum samples for use in the ELISAs, parallelism was not evident. However, dilutional parallelism was observed when the zero calibrator supplied in the ELISA kit was used for dilution.

When insulin concentrations measured with the RIA were compared with those measured with the equine-specific ELISA, results for serum samples that were < 175 $\mu\text{U}/\text{mL}$ with the ELISA agreed reasonably well with the insulin concentrations measured in the same samples with the RIA. However, Bland-Altman plots showed a systematic increase in bias between the 2 methods as the measured insulin concentration increased, compared with the mean measured concentration. Results for samples determined to contain initial measured insulin concentrations > 175 $\mu\text{U}/\text{mL}$ with the equine-specific ELISA that were thus diluted to ensure the sample absorbance was within the detection limits of the assay did not agree well with the insulin concentrations measured with the RIA (mean bias, -185 $\mu\text{U}/\text{mL}$, with values much lower with the ELISA than with the RIA). These samples were not diluted for measurement with the RIA because concentrations were within the detection limits of that method (up to 389 $\mu\text{U}/\text{mL}$). It is difficult to know whether the observed bias represents an underestimation of serum insulin concentration by the ELISA or overestimation by the RIA.

In another study,²⁰ equine plasma samples were analyzed with the equine-specific ELISA, human RIA, and LC-MS, showing large variability in concentrations measured via the 3 techniques. For example, the sample with the lowest concentration yielded a value of 50.72 $\mu\text{U}/\text{mL}$ when LC-MS was used, 17.53 $\mu\text{U}/\text{mL}$ when the RIA was used, and 8.12 $\mu\text{U}/\text{mL}$ when the ELISA was used.²⁰ The sample with the highest concentration

yielded a value of 454.16 $\mu\text{U}/\text{mL}$ via LC-MS, 220.58 $\mu\text{U}/\text{mL}$ via RIA, and 35.97 $\mu\text{U}/\text{mL}$ via ELISA.²⁰ Overall, the RIA underestimated concentrations measured by LC-MS by 2.8-fold and the ELISA underestimated concentrations measured by LC-MS by 8.3-fold.²⁰ These findings are consistent with those of the present study, suggesting that measured concentrations when the RIA is used are much higher than when the equine-specific ELISA is used. Additionally, both assay techniques underestimate concentrations measured through LC-MS, suggesting they may be measuring nonspecific substances.

The mean bias between serum insulin concentrations measured with the high-range porcine ELISA and the RIA was small (25 $\mu\text{U}/\text{mL}$); however, there was an extremely large range of differences between the 2 methods. The wide limits of agreement suggested the 2 methods are not directly comparable, particularly for use on a small number of samples. Again, it is difficult to identify the method that yields the most accurate insulin concentration because neither can be considered a gold standard assay or verified against a known purified equine insulin concentration.

A potential explanation for overestimation of serum insulin concentrations by the RIA could be antibody cross-reaction with proinsulin or its metabolites. In humans with type II diabetes, a large increase in serum proinsulin concentration is common.²¹ This increase is believed to be a result of the increased stimulation of pancreatic insulin release, which overwhelms the intracellular mechanisms for converting proinsulin to insulin and results in release of proinsulin into the circulation.²¹ High concentrations of proinsulin and conversion intermediates can cause an overestimation of measured insulin concentrations by the RIA,²² which could be responsible for the discrepancy between the measurements obtained with the RIA and the equine-specific ELISA in the present study. It is not known whether this release of proinsulin into the circulation also exists in insulin-resistant horses with extremely high serum insulin concentrations that are thus likely to have high pancreatic release of insulin. Equine proinsulin or other intermediary conversion metabolites may cross-react with the polyclonal antibodies in the RIA designed to bind human insulin.²¹ This cross-reaction could explain some of the problems associated with validation of the RIA for use in horses. Purified equine proinsulin is not available to confirm or refute this hypothesis.

Additionally, C-peptide is released when proinsulin is converted to insulin. Serum C-peptide concentrations increase in insulin-resistant horses and following IV glucose infusion in clinically normal horses.³ Purified human C-peptide was analyzed in the RIA in the present study to confirm that it was not cross-reacting with the polyclonal insulin antibodies or interfering with the binding of insulin to the antibody, but the percentage binding of samples containing C-peptide was close to that of the zero standard, indicating no considerable cross-reaction in the RIA. Purified equine C-peptide is also not available; therefore, it remains possible that this protein may cross-react with the polyclonal antibodies.

For serum samples containing insulin concentrations < 175 µU/mL in the present study, the agreement between the RIA and equine-specific ELISA appeared to be good. The ELISA was quicker to perform and did not require use of radioactive materials. Consequently, it may be the preferred method for analyzing samples containing low insulin concentrations, although this assay is more expensive than the RIA. On the other hand, agreement was poor between the 2 methods for serum samples containing insulin concentrations > 175 µU/mL. For samples containing high insulin concentrations, dilution with IDS resulted in dilutional parallelism within the RIA and should thus produce consistent results. However, the wide range in percentage recoveries after samples are diluted raises concern about the absolute accuracy of the insulin concentration measured and the validity of making specific clinical diagnoses on the basis of cutoff values in individual equids. The high-range porcine ELISA used had poor intra- and interassay repeatability, lowering any confidence in the accuracy of results obtained with this method. Larger sample volumes may improve the consistency of this assay. Additional research is required to develop improved assays for accurate measurement of high serum insulin concentrations in equids. The availability of purified equine insulin standards would greatly facilitate the development of such assays and help in determining their accuracy.

- a. Coat-A-Count Insulin RIA, Siemens, Camberley, Surrey, England.
- b. McCarthy T, Siemens, Camberley, Surrey, England: Personal communication, 2009.
- c. Mercodia equine insulin ELISA, Mercodia, Uppsala, Sweden.
- d. Oberg J, Lilliehook I, Wattle O, et al. Validation of a species specific enzyme-linked immunosorbent assay for measurement of serum insulin in horses, in *Proceedings* (abstr). Am Coll Vet Intern Med Forum, 2009;8.
- e. Mercodia high-range porcine insulin ELISA, Mercodia, Uppsala, Sweden.
- f. Johansson H, Mercodia, Uppsala, Sweden: Personal communication, 2010.
- g. Norit, Sigma-Aldrich Co Ltd, Gillingham, Dorset, England.
- h. Anachem Supatop, Anachem Instruments Ltd, Luton, Bedfordshire, England.
- i. C-peptide (3-33), human, GenScript, Piscataway, NJ.
- j. Prism, version 5, GraphPad Software Inc, San Diego, Calif.

References

1. Frank N, Geor RJ, Bailey SR, et al. Equine metabolic syndrome. *J Vet Intern Med* 2010;24:467–475.
2. Bailey SR, Habershon-Butcher JL, Ransom KJ, et al. Hypertension and insulin resistance in a mixed-breed population of ponies predisposed to laminitis. *Am J Vet Res* 2008;69:122–129.
3. Toth F, Frank N, Martin-Jimenez T, et al. Measurement of C-peptide concentrations and responses to somatostatin, glucose infusion, and insulin resistance in horses. *Equine Vet J* 2010;42:149–155.
4. Asplin KE, Sillence MN, Pollitt CC, et al. Induction of laminitis by prolonged hyperinsulinaemia in clinically normal ponies. *Vet J* 2007;174:530–535.
5. de Laat MA, McGowan CM, Sillence MN, et al. Equine laminitis: induced by 48 h hyperinsulinaemia in Standardbred horses. *Equine Vet J* 2010;42:129–135.
6. Durham AE, Rendle DI, Newton JR. The effect of metformin on measurements of insulin sensitivity and β cell response in 18 horses and ponies with insulin resistance. *Equine Vet J* 2008;40:493–500.
7. Freestone JF, Wolfsheimer KJ, Kamerling SG, et al. Exercise induced hormonal and metabolic changes in Thoroughbred horses: effects of conditioning and acepromazine. *Equine Vet J* 1991;23:219–223.
8. Reimers TJ, Cowan RG, McCann JP, et al. Validation of a rapid solid-phase radioimmunoassay for canine, bovine, and equine insulin. *Am J Vet Res* 1982;43:1274–1278.
9. Freestone JF, Shoemaker K, Bessin R, et al. Insulin and glucose response following oral glucose administration in well-conditioned ponies. *Equine Vet J Suppl* 1992;(11):13–17.
10. Borer KE, Berhane Y, Menzies-Gow NJ, et al. Measurement of concentrations of insulin in equine serum that exceed the working range of radioimmunoassay kits, in *Proceedings*. 48th Br Equine Vet Assoc Cong 2009;103.
11. Tinworth KD, Wynn PC, Harris PA, et al. Optimising the Siemens Coat-A-Count radioimmunoassay to measure insulin in equine plasma. *J Equine Vet Sci* 2009;29:411–413.
12. Burge MR, McLeod J, Bowsher RR, et al. Validity of Coat-A-Count insulin RIA kit for quantifying total and free Humalog (lett). *Clin Chem* 1996;42:777.
13. Stryer L. *Biochemistry*. 3rd ed. East Lansing, Mich: Freeman, 1988.
14. Kuuranne T, Thomas A, Leinonen A, et al. Insulins in equine urine: qualitative analysis by immunoaffinity purification and liquid chromatography/tandem mass spectrometry for doping control purposes in horse-racing. *Rapid Commun Mass Spectrom* 2008;22:355–362.
15. Ho EN, Wan TS, Wong AS, et al. Doping control analysis of insulin and its analogues in equine plasma by liquid chromatography-tandem mass spectrometry. *J Chromatogr A* 2008;1201:183–190.
16. Conlon JM. Evolution of the insulin molecule: insights into structure-activity and phylogenetic relationships. *Peptides* 2001;22:1183–1193.
17. Henneke DR, Potter GD, Kreider JL, et al. Relationship between condition score, physical measurements and body fat percentage in mares. *Equine Vet J* 1983;15:371–372.
18. Gosling JP. *Immunoassays*. Oxford, England: Oxford University Press, 2000.
19. Wood WG. “Matrix effects” in immunoassays. *Scand J Clin Lab Invest Suppl* 1991;205:105–112.
20. Tinworth KD, Wynn PC, Boston RC, et al. Evaluation of commercially available assays for the measurement of equine insulin. *Domest Anim Endocrinol* 2011;41:81–90.
21. Kippen AD, Cerini F, Vadas L, et al. Development of an isotope dilution assay for precise determination of insulin, C-peptide, and proinsulin levels in non-diabetic and type II diabetic individuals with comparison to immunoassay. *J Biol Chem* 1997;272:12513–12522.
22. Deberg M, Houssa P, Frank BH, et al. Highly specific radioimmunoassay for human insulin based on immune exclusion of all insulin precursors. *Clin Chem* 1998;44:1504–1513.