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Evaluation of commercially available assays for the measurement of equine insulin

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Abstract

Determining circulating equine insulin concentrations is becoming increasingly important in equine clinical practice and research. Most available assays are optimised for human medicine, but there is strong equine cross-reactivity due to the highly conserved nature of insulin. To identify an accurate and reliable assay for equine insulin, six commercial immunoassays were evaluated for precision, accuracy and specificity. Only one assay initially reached the requisite standard: Mercodia Equine Insulin enzyme-linked immunosorbent assay (ELISA). Plasma matrix interferences were identified when the provided assay buffer was used with the Siemens Count-a-Coat Insulin Radioimmunoassay (RIA) but not when charcoal-stripped equine plasma was used as the diluent. This modified RIA plus the Mercodia Equine Insulin ELISA were evaluated further by directly examining accuracy by comparing their results for 18 equine plasma samples with values obtained using liquid chromatography and high resolution/high accuracy mass spectrometry (LC-MS). Compared to LC-MS measurements, the modified Siemens Insulin RIA rendered a moderate Lin's Concordance Coefficient (ρ_c) of 0.41, whereas the Mercodia Equine Insulin ELISA rendered a very poor ρ_c of 0.06. This suggests that the Siemens Insulin RIA is appropriate to use for routine evaluations when LC-MS is not available.

Keywords: immunoassay, assay validation, accuracy, precision, hyperinsulinaemia

Introduction

Hyperinsulinaemia has been associated with a number of disease states in the the horse, including laminitis (Johnson, 2002; Asplin et al., 2007), insulin resistance (Treiber et al., 2006) and osteochondrosis (Ralston et al., 1998). Therefore, determining circulating equine insulin concentrations is becoming increasingly important in equine clinical practice and research. Most commercial kits used for this purpose are human diagnostic assays, as cross-reactivity is anticipated, based on the highly-conserved nature of insulin among mammalian species (Conlon, 2001). Recently, several assays specifically developed to measure equine insulin have become available. However, they utilize either porcine standards or antibodies, or both. Various international research groups have utilised different immunoassays to measure insulin in equine samples, resulting in different insulin concentrations being accepted as 'normal' and 'hyperinsulinaemic', and confounding the comparison of results between laboratories (McGowan et al., 2008).

However, cross-reactivity alone is not sufficient to determine the suitability of an assay. Thorough validation should examine precision, accuracy and specificity (Deshpande, 1996). Precision, or reproducibility, is evaluated by examining the intra-assay co-efficient of variation (CV) based on the variation of repeated determinations on the same sample, whereas accuracy is the fundamental ability of the immunoassay to measure the true value of an analyte (Deshpande, 1996) and is contingent on specificity (Kemeny and Challacombe, 1989). Indirect assessments of accuracy and specificity are made using studies of recovery-on-addition and dilutional parallelism (Deshpande, 1996). However, ideally, the accuracy of an assay should also be measured directly by using an independent, unequivocal method to measure the analyte concentration, then comparing it with immunoassay values (Bangham and Cotes, 1974; Deshpande, 1996). This can be done using gas (GC) or liquid chromatography (LC), with or without mass spectrometry (MS), depending on

the analyte in question (Bangham and Cotes, 1974; Deshpande, 1996). However, few equine laboratories have access to GC or LC-MS to perform this work.

There are several commercial human insulin immunoassays currently available, which have been used to measure equine insulin. These include the Siemens Coat-A-Count (CAC) Insulin Radioimmunoassay (RIA), widely used to quantify insulin in equine plasma (Freestone et al., 1991; Treiber et al., 2005; Bailey et al., 2007; Vick et al., 2007; Frank et al., 2008), the DSL-1600 Insulin RIA (Asplin et al., 2007) and the Mercodia Insulin enzyme-linked immunosorbent assay (ELISA) (Hodavance et al., 2007). Two ELISAs have recently been marketed specifically for the horse: the Mercodia Equine Insulin ELISA, that utilises monoclonal antibodies to porcine insulin, using porcine standards; and the Shibayagi Equine Insulin ELISA which also utilises monoclonal antibodies to porcine insulin but has equine insulin standards.

The objective of this study was to apply the same validation protocol to all of the insulin immunoassays currently available in order to identify which are accurate and reliable for the measurement of equine insulin concentrations in equine plasma samples.

Materials and Methods

Immunoassays

Six commercial insulin immunoassays were evaluated using equine plasma samples held in our laboratory. Table 1 summarises the assays used. All assays were used according to manufacturers' instructions.

The Siemens CAC Insulin RIA was a gift from Siemens Medical Solutions Diagnostics; the DSL-1600 Insulin RIA and the DSL 10-1600 Insulin ELISA were gifts from Diagnostic Systems

Laboratories, Inc; the Mercodia Insulin ELISA and the Mercodia Equine Insulin ELISA were gifts from Mercodia AB; and the Shibayagi Equine Insulin ELISA was a gift from Shibayagi Co.

Samples

Equine plasma collected during glucose tolerance testing of different horses/ponies were analysed. All samples had been collected into lithium-heparin vacutainers (Vacutainer, Becton-Dickinson), kept on ice, centrifuged at 4 °C (1,000 x g for 10 min) within 20 min of collection and stored at -20°C until assayed.

Immunoassay evaluation

In evaluating each immunoassay, precision, accuracy and specificity were initially assessed as described below. Where feasible, the same samples were used for each immunoassay and were assayed in duplicate. Standards were assayed in triplicate to ensure the production of a reliable standard curve.

Precision was evaluated by examining the intra-assay CV for each immunoassay. The same internal laboratory quality control (QC) of pooled plasma from multiple equine samples was assayed at the start, middle and end of each immunoassay. A $CV \leq 10\%$ was accepted as being adequate (Deshpande, 1996). Where an assay kit was used several times, inter-assay variation was also determined.

Studies of recovery-on-addition and dilutional parallelism (or recovery-on-dilution) were used to determine accuracy and specificity. To test recovery-on-addition, plasma samples from 4 ponies were incubated overnight in a 1:1 ratio with the range of insulin standards provided with the kits,

then assayed. To test parallelism, plasma samples from 4 ponies were diluted with assay diluent in fractional dilutions of 3:4, 1:2 and 1:4 and assayed together with the undiluted samples.

To investigate interference by plasma matrix effects, recovery-on-addition and dilutional parallelism studies were repeated with the Siemens CAC RIA, using charcoal-stripped plasma (CSP) as diluent. Adapted from Carter (1978), 5% w/v activated charcoal (Sigma-Aldrich) was added to equine plasma, incubated at room temperature on an orbital shaker for 3 h, then centrifuged (1000 x g for 15 min) to remove the charcoal. The process was repeated with the supernatant, and the resulting supernatant further clarified by filtering through a 5 µm filter and frozen at -20°C until used. The technique was reported to remove 99% of free insulin, without affecting plasma proteins (Clarke and Freeman, 1968; Albano et al., 1972; Carter, 1978). The CSP was then used to reconstitute the insulin standards to spike the equine plasma samples for recovery-on-addition and to dilute the samples for dilutional parallelism.

To further investigate the need to use CSP as diluent for reconstituting the standards, we assayed 18 independent equine plasma samples using CSP or distilled water to reconstitute the insulin standards, and compared the concentrations obtained from each assay using Lin's correlation analysis (Lin, 1989).

To directly assess the accuracy of the immunoassays satisfying the initial evaluation, 18 independent samples, varying widely in apparent insulin concentrations (Table 3), were measured with each immunoassay and compared with the insulin concentration of the same samples measured using LC-MS.

LC-MS methods

The LC-MS analyses were performed by Thevis and Thomas, modified from Thevis et al. (2005). The 18 plasma samples were divided and lyophilised prior to analysis in each laboratory. The samples were managed identically for the LC-MS and the immunoassays. The LC-MS assays were performed with the analysts having no knowledge of the other assay results.

Insulin Aspart was used as the internal standard (ISTD) in all samples used in the LC-MS assay. Its stock solution contained 10 μM Insulin Aspart dissolved in 2% acetic acid (Merck), and a working solution (0.1 μM) was prepared freshly with each sample batch. All working solutions contained 0.1 μM Insulin Aspart/L to avoid loss of target analytes during the dilution process. Stock solutions were stored at 4 $^{\circ}\text{C}$ for < 1 week.

Plasma samples were prepared for LC-MS analysis using a method developed for urinary insulin determination (Thomas et al., 2009). In brief, to 500 μL of sample reconstituted in distilled water, 1 pmol ISTD and 1 mL of acetonitrile (Merck HPLC grade) were added, and vortexed for 20 s. The mixture was centrifuged (6000 $\times g$ for 5 min), and the supernatant transferred into a polypropylene tube prior to evaporation of solvent by vacuum centrifuge at 40 $^{\circ}\text{C}$. The residue was reconstituted in 500 μL of phosphate-buffered saline (PBS), followed by the addition of 2.5 μL of anti-insulin-antibodies (Monoclonal IgG, anti-mouse, CER-groupe) and 35 μL of Dynal magnetic beads suspension (anti-mouse IgG, Invitrogen). After incubation with gentle stirring for 1 h at 20 $^{\circ}\text{C}$, the supernatant was discarded using a magnetic separator and the residual beads were washed twice by consecutive adding, mixing and discarding of 300 μL PBS. Finally the antigen-antibody aggregate of the target analytes were dissolved by addition of 50 μL of acetic acid (2%) and incubation for 1 min at room temperature. After removing the beads, the supernatant was transferred to another polypropylene tube for LC-MS analysis.

The LC-MS was performed using high resolution/high accuracy MS with an Exactive mass spectrometer (Thermo) after liquid chromatographic separation using an Accela UPLC (Thermo) equipped with a Zorbax SB300 analytical column, (0.3 x 50 mm, 5 µm particle size; Agilent) and a Zorbax SB300 guard column (1x17 mm, 5 µm particle size). Liquid chromatography was performed with a gradient program (Start: 85% A, in 10 min to 25 % A, re-equilibrate for 15 min at 85 %A, flow 25 µL/min) using formic acid (0.2 %) as aqueous solvent (A) and acetonitrile as organic solvent (B). The MS was equipped with a Nanomate (Advion) nano-electrospray ion (NSI) source in LC-coupling mode. The LC-split conditions were set to an approximate flow to the NSI-chip of 800 nL/min with 1.8 kV ionisation voltage and a capillary temperature of 175 °C. The MS operated positive in full scan mode with a resolution power of 50,000 FWHM and reliable mass accuracies were ensured by mass calibration using the manufacturer's calibration kit. The chromatograms were evaluated by monitoring the five-fold protonated molecules of equine insulin at $m/z = 1150.3$ and at $m/z = 1165.9$ for the ISTD.

Statistical analyses

In evaluating precision, CV was calculated from the mean and standard deviation (SD) of the six QC samples, using the equation: $CV (\%) = (SD/mean) \times 100$. For an immunoassay to be acceptable, CV was required to be $\leq 10 \%$ (Deshpande, 1996).

Recovery-on-addition was deemed acceptable at $100 \pm 10 \%$. Linearity plots were constructed to enable further interpretation of recovery-on-addition results, by comparing the observed insulin concentration with the expected insulin concentration. Accuracy was evaluated by examining the correlation coefficient (r^2) value, where an r^2 approaching 1 was desirable (Deshpande, 1996).

Dilutional parallelism was inferred from an intercept of 0 and a slope of 1 after plotting the expected analyte concentration against the observed analyte concentration interpolated from the standard dose-response curve. A variation of ≤ 5 was deemed acceptable for both the intercept and the slope (Deshpande, 1996).

Direct assessment of the accuracy was calculated by comparison of the insulin concentration in 18 samples measured with each of the immunoassays and with that measured using LC-MS, using Lin's correlation analysis (Lin, 1989).

Results

The validation results of the six commercial immunoassays are summarised in Table 2. All immunoassays, except the Mercodia Human ELISA, displayed acceptable precision with a CV ≤ 10 %. The inter-assay variations displayed by the Siemens CAC RIA and the Mercodia Equine ELISA improved as the operator became more practiced, becoming 7.02% and 7.44%, respectively.

Three immunoassays, the Mercodia Equine ELISA, the Siemens CAC RIA and the DSL-10-1600 ELISA, displayed acceptable recovery-on-addition, with recoveries close to 100% and a SE ≤ 10 . The DSL-1600 RIA and the Mercodia ELISA underestimated insulin concentrations, whereas the Shibayagi Equine ELISA overestimated insulin concentrations in the spiked equine plasma samples.

Four immunoassays displayed adequate linearity, with r^2 approaching 1. Despite being widely used for equine samples, Siemens CAC RIA showed poor linearity, even though the results for recovery were acceptable. Samples spiked with the lowest standard were biasing the results.

Initially, only two assays displayed adequate parallelism with the slopes of the plot of expected against observed analyte concentrations approaching 1, and the y-intercept close to the origin. Results support the inaccuracies in the recovery-on-addition data demonstrated by the DSL-1600 RIA, the Shibayagi Equine ELISA and the Merckodia Human ELISA. Additionally, the Merckodia Human ELISA also showed poor CV, so these assays were excluded from further study. Despite demonstrating good CV and recovery-on-addition results, the DSL-10-1600 ELISA did not display satisfactory parallelism, with both the slope and the y-intercept unacceptable, and was also excluded from further study.

Although the Siemens CAC RIA did not display parallelism on initial evaluation, the exclusion of samples diluted at 1:4 did render parallelism. As the samples were increasingly diluted, the recovery of the analyte escalated, such that at 1:4 dilution, the measurement of insulin exceeded 200%. As this immunoassay had been used in many equine studies, being previously reported as having been validated for equine serum (Freestone et al., 1991), and other validation parameters were acceptable, further investigations were instigated in order to determine the possible cause for this failure of parallelism.

Using CSP as diluent for the standards provided with the Siemens CAC RIA improved both recovery and parallelism. Recovery-on-addition was approximately 100%, regardless of the concentration of the standard used to spike the sample so linearity was improved with $r^2=0.97$. A similar situation was observed for parallelism. Initially, samples diluted at 1:4 consistently biased the results (Figure 1A), resulting in a slope of 0.65 ± 0.04 and an intercept of 37.14 ± 4.91 . However, dilution of samples with CSP greatly improved parallelism (Figure 1B), with the slope consistently approaching 1 (0.99 ± 0.05) and the intercept close to 0 (2.85 ± 0.74).

Modifying the assay, using CSP as diluent for reconstituting standards showed the effect was negligible. Lin's Concordance Correlation Coefficient (ρ_c) was 0.99, (Lin, 1989) as shown in Figure 2. These findings suggest that the standards can be reconstituted with water, but that samples requiring dilution should be diluted with CSP.

Now, the modified Siemens CAC RIA validated equally as well as the Mercodia Equine ELISA. However, the two assays gave different insulin concentrations for the same sample (ρ_c of 0.18). Comparison to LC-MS measurements of 18 equine samples revealed that the Siemens CAC RIA achieved a moderate ρ_c of 0.41, and the Mercodia Equine ELISA rendered a very poor ρ_c of 0.06, as shown in Figure 3. The data is presented in Table 3.

Discussion

The use of human diagnostic assays to determine hormone concentrations in other species is commonplace, however it is imperative that any assay used for such a purpose is thoroughly validated.

In this study, five assays failed the initial validation tests, demonstrating poor CV, recovery and/or parallelism. One of these was the Shibayagi Equine ELISA which overestimated recovery-on-addition and did not display dilutional parallelism. The assay may have suffered from interference due to lack of specificity or poor affinity of the antibodies, which were raised against porcine insulin. Plasma matrix interference was unlikely, as the standards were derived from pancreatic equine insulin (Shibayagi Co., 2008). Reasons for its poor performance however were not explored. The poor performance of the DSL-1600 RIA, DSL-10-1600 ELISA and the Mercodia ELISA are likely explained by a lack of specificity or poor affinity of the antibodies and/or plasma matrix interference. Again, reasons for their poor performance were not explored.

On initial examination, the Siemens CAC RIA failed linearity and dilutional parallelism tests, with spurious results for samples with low insulin concentrations. However, this assay has been widely used by equine researchers, being reported as having been validated previously for use with equine serum (Freestone et al., 1991), so further investigation for use with equine plasma was warranted. It is not unusual for hormone assays to suffer from interference, often due to cross-reactivity with related hormones or hormone binding proteins (Noble and Sillence, 2001), or differences in plasma matrix between the standards and the sample (Deshpande, 1996). Insulin circulates as a free hormone in plasma, unlike its related hormone, insulin-like growth factor-1, so binding protein interference could be excluded (Noble and Sillence, 2001). We speculated that either a lack of specificity, low affinity of the antibodies or plasma matrix interference was attributable. Plasma matrix effects occur when the composition of the reconstituted standards differs markedly from that of the samples, such that there are differences in the antibody-antigen binding kinetics (Deshpande, 1996). Research into hyperinsulinaemia and insulin resistance is concerned with high plasma insulin concentrations, and so measurements at lower concentrations are of little importance. However, matrix effects are critical when samples with insulin concentration exceeding the highest standard require dilution. For example, it is likely that a threshold exists above which the development of laminitis occurs (Asplin et al., 2007). While this threshold is still unknown, it is imperative for clinicians to have full confidence in insulin concentrations measured in at-risk horses and ponies. The standardization of measurement techniques will potentially quell debate over the significance of equine insulin measured with disparate techniques.

The standards supplied with the Siemens CAC RIA are reconstituted with distilled water. Originally, linearity calculations for this RIA showed that in samples with expected low insulin, the assay overestimated insulin concentrations by up to 200% (Borer et al., 2009; Tinworth et al., 2009), giving an $r^2=0.11$. A mild discrepancy between the matrices may not be important when

measuring high concentrations of analyte, but becomes more prominent as the analyte concentration is reduced. Reconstituting standards with CSP and using these to spike equine plasma samples greatly improved linearity. It appears that using CSP to reconstitute the standards alters the standard matrix to match the sample matrix, improving antigen-antibody binding kinetics. The improvement in dilutional parallelism using CSP was also quite marked. Thus, the accuracy of the assay was maintained for all measured concentrations.

Using this methodology, the Siemens CAC RIA was found to be comparable to the Mercodia Equine ELISA in terms of validation. The Mercodia Equine ELISA for the authors was easier to use, since there is no requirement for radiation licensing or facilities, but the Siemens CAC RIA currently is more cost effective.

To establish which assay was more accurate, and in the absence of an international equine insulin standard, a direct method of measuring insulin was used, namely LC-MS. LC-MS is not prone to the matrix interferences that plague immunoassays (Bangham and Cotes, 1974; Deshpande, 1996), but the use of a non-equine insulin antigen/antibody complex in the sample preparation may mean that antibody specificity may be an issue. However, designating LC-MS as the superior technique, the Siemens CAC RIA was found to underestimate equine insulin concentration by approximately 2.8 times, and the Mercodia Equine ELISA by approximately 8.3 (Table 3). Thus, based on this comparison the Siemens CAC RIA would appear to be the more appropriate immunoassay to use for equine plasma, but either assay could be used if a correction factor for insulin estimation is applied to the results.

Conclusions

With hyperinsulinaemia implicated in the development of laminitis, the accurate measurement of equine plasma insulin is important. Several commercial assays used in human medicine have been utilised by various equine researchers, and specific equine insulin assays are also available. However, the reproducibility of measurements between different assays is not assured. This means that comparisons of results between research groups are not possible and may limit advancement in this research. This study demonstrates that the Siemens CAC Insulin RIA is the most accurate assay for measuring equine insulin concentrations, with the proviso that samples exceeding the highest standard should be diluted with CSP, rather than the manufacturer's diluent.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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References

- Albano, J.D., Ekins, R.P., Maritz, G., Turner, R.C., 1972. A sensitive, precise radioimmunoassay of serum insulin relying on charcoal separation of bound and free hormone moieties. *Acta Endocrinologica* 70, 487-509.
- Asplin, K.E., Sillence, M.N., Pollitt, C.C., McGowan, C.M., 2007. Induction of laminitis by prolonged hyperinsulinaemia in clinically normal ponies. *The Veterinary Journal* 174, 530–535.
- Bailey, S.R., Menzies-Gow, N.J., Harris, P.A., Habershon-Butcher, J.L., Crawford, C., Berhane, Y., Boston, R.C., Elliott, J., 2007. Effect of dietary fructans and dexamethasone administration on the insulin response of ponies predisposed to laminitis. *Journal of the American Veterinary Medical Association* 231, 1365-1373.
- Bangham, D.R., Cotes, P.M., 1974. Standardization and Standards. *British Medical Bulletin* 30, 12-17.
- Borer, K.E., Berhane, Y., Menzies-Gow, N.J., Harris, P.A., Elliott, J., 2009. Measurement of concentrations of insulin in equine serum that exceed the working range of radioimmunoassay kits. In: *British Equine Veterinary Association Congress, Birmingham, United Kingdom*.
- Carter, P., 1978. Preparation of ligand-free human serum for radioimmunoassay by adsorption on activated charcoal. *Clinical Chemistry* 24, 362-364.
- Clarke, H.G., Freeman, T., 1968. Quantitative immunoelectrophoresis of human serum proteins. *Clinical Science* 35, 403-413.
- Conlon, J.M., 2001. Evolution of the insulin molecule: insights into structure-activity and phylogenetic relationships. *Peptides* 22, 1183-1193.
- Deshpande, S.S., 1996. *Enzyme Immunoassays: From Concept to Product Development*. Chapman & Hall, New York.
- Frank, N., Elliott, S.B., Boston, R.C., 2008. Effects of long-term oral administration of levothyroxine sodium on glucose dynamics in healthy adult horses. *American Journal of Veterinary Research* 69, 76-81.
- Freestone, J.F., Wolfsheimer, K.J., Kamerling, S.G., Church, G., Hamra, J., Bagwell, C., 1991. Exercise induced hormonal and metabolic changes in Thoroughbred horses: effects of conditioning and acepromazine. *Equine Veterinary Journal* 23, 219-223.
- Hodavance, M.S., Ralston, S.L., Pelczer, I., 2007. Beyond blood sugar: the potential of NMR-based metabolomics for type 2 human diabetes, and the horse as a possible model. *Analytical & Bioanalytical Chemistry* 387, 533-537.
- Johnson, P.J., 2002. The equine metabolic syndrome peripheral Cushing's syndrome. *Veterinary Clinics of North America - Equine Practice* 18, 271-293.

- Kemeny, D., Challacombe, S. (Eds.), 1989. ELISA and Other Solid Phase Immunoassays: Theoretical and Practical Aspects. John Wiley & Sons, Chichester.
- Lin, L.I., 1989. A concordance correlation coefficient to evaluate reproducibility. *Biometrics* 45, 255-268.
- McGowan, T., Geor, R., Evans, H., Sillence, M., Munn, K., McGowan, C., 2008. Comparison of 4 assays for serum insulin analysis in the horse. In, ACVIM Forum, Texas, City.
- Noble, G.K., Sillence, M.N., 2001. Technical aspects of measuring IGF-1 in horses. In: The 13th International Conference of Racing Analysts and Veterinarians, Cambridge U.K. .
- Ralston, S., Black, A., Suslak-Brown, L., Schoknecht, P., 1998. Postprandial insulin resistance associated with osteochondrosis in weanling fillies. *Journal of Animal Science* 76, 176.
- Shibayagi Co., 2008. Porcine Insulin ELISA Package Insert. Shibayagi Co., Gunma.
- Thevis, M., Thomas, A., Delahaut, P., Bosseloir, A., Schanzer, W., 2005. Qualitative determination of synthetic analogues of insulin in human plasma by immunoaffinity purification and liquid chromatography-tandem mass spectrometry for doping control purposes. *Analytical Chemistry* 77, 3579-3585.
- Thomas, A., Schanzer, W., Delahaut, P., Thevis, M., 2009 Sensitive and fast identification of urinary human, synthetic and animal insulin by means of nano-UPLC coupled with high-resolution/high-accuracy mass spectrometry. *Drug Testing and Analysis* 1, 219-227.
- Tinworth, K.D., Wynn, P.C., Harris, P.A., Sillence, M.N., Noble, G.K., 2009. Optimising the Siemens Coat-A-Count radioimmunoassay to measure insulin in equine plasma. *Journal of Equine Veterinary Science* 29, 411-413.
- Treiber, K.H., Kronfeld, D.S., Geor, R.J., 2006. Insulin resistance in equids: possible role in laminitis. *Journal of Nutrition* 136, 2094S-2098S.
- Treiber, K.H., Kronfeld, D.S., Hess, T.M., Boston, R.C., Harris, P.A., 2005. Use of proxies and reference quintiles obtained from minimal model analysis for determination of insulin sensitivity and pancreatic beta-cell responsiveness in horses. *American Journal of Veterinary Research* 66, 2114-2121.
- Vick, M.M., Adams, A.A., Murphy, B.A., Sessions, D.R., Horohov, D.W., Cook, R.F., Shelton, B.J., Fitzgerald, B.P., 2007. Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. *Journal of Animal Science* 85, 1144-1155.

Table 1. Description of the commercial assays evaluated for the measurement of equine insulin.

Assay	Standards	Capture Antibody	2nd Antibody
DSL-1600 Insulin RIA	Human Insulin	Guinea Pig Anti-Insulin	Goat Anti-Guinea Pig IgG
DSL-10-1600 Insulin ELISA	Human Insulin	Anti-Insulin	Anti-Insulin
Mercodia Equine Insulin ELISA	Porcine Insulin	Mouse Monoclonal Anti-Insulin	Mouse Monoclonal Anti-Insulin
Mercodia Insulin ELISA	Recombinant Human Insulin	Mouse Monoclonal Anti-Insulin	Mouse Monoclonal Anti-Insulin
Shibayagi Equine Insulin ELISA	Equine Insulin	Mouse Monoclonal Anti-Porcine Insulin against A1 to A10	Mouse Monoclonal Anti-Porcine Insulin against B20 to B30
Siemens CAC Insulin RIA	Recombinant Human Insulin	Polyclonal Guinea Pig Anti-Insulin	

Table 2. Validation results of six commercial insulin immunoassays evaluated using equine plasma samples, showing intra-assay coefficient of variation (CV), recovery-on-addition, linearity, dilutional parallelism slope and intercept. Values shown are mean \pm standard error.

Immunoassay	Intra-assay CV (%)	Recovery-on-addition (%)	Linearity correlation (r^2)	Dilutional Parallelism slope	Dilutional Parallelism y-intercept	Validated
DSL-1600 Insulin RIA	8.81	88.05 \pm 20.2	0.19	1.03 \pm 0.08	1.61 \pm 2.3	No
DSL-10-1600 Insulin ELISA	5.16	98.16 \pm 10.4	0.96	1.18 \pm 1.4	-1.86 \pm 1.4	No
Mercodia Equine Insulin ELISA	8.40	97.00 \pm 4.3	0.99	0.995 \pm 0.05	-0.71 \pm 0.5	Yes
Mercodia Insulin ELISA	15.20	89.95 \pm 3.7	0.97	0.52 \pm 0.04	12.07 \pm 0.9	No
Shibayagi Equine Insulin ELISA	8.09	116.70 \pm 19.2	0.96	0.77 \pm 0.12	10.59 \pm 4.6	No
Siemens CAC Insulin RIA	9.71	101.86 \pm 5.7	0.11	0.65 \pm 0.04	37.14 \pm 4.9	No

Table 3. Comparative values recorded for equine insulin concentration in 18 independent plasma samples using three different methods.

Sample	Liquid Chromatography-Mass Spectrometry ($\mu\text{U/mL}$)	Siemens Count-a-Coat Insulin RIA ($\mu\text{U/mL}$)	Mercodia Equine Insulin ELISA ($\mu\text{U/mL}$)
1	177.42	71.41	19.72
2	454.16	220.58	35.97
3	373.04	192.46	35.55
4	114.17	47.08	10.65
5	224.88	90.00	20.58
6	421.94	164.00	41.19
7	249.16	88.50	23.71
8	80.03	29.36	16.21
9	112.39	58.20	8.57
10	58.55	22.52	15.33
11	50.72	17.53	8.12
12	161.43	79.37	18.87
13	158.15	29.90	14.52
14	92.07	23.24	14.85
15	116.37	24.47	11.45
16	272.33	130.87	75.93
17	378.93	194.87	73.32
18	86.08	27.27	48.67

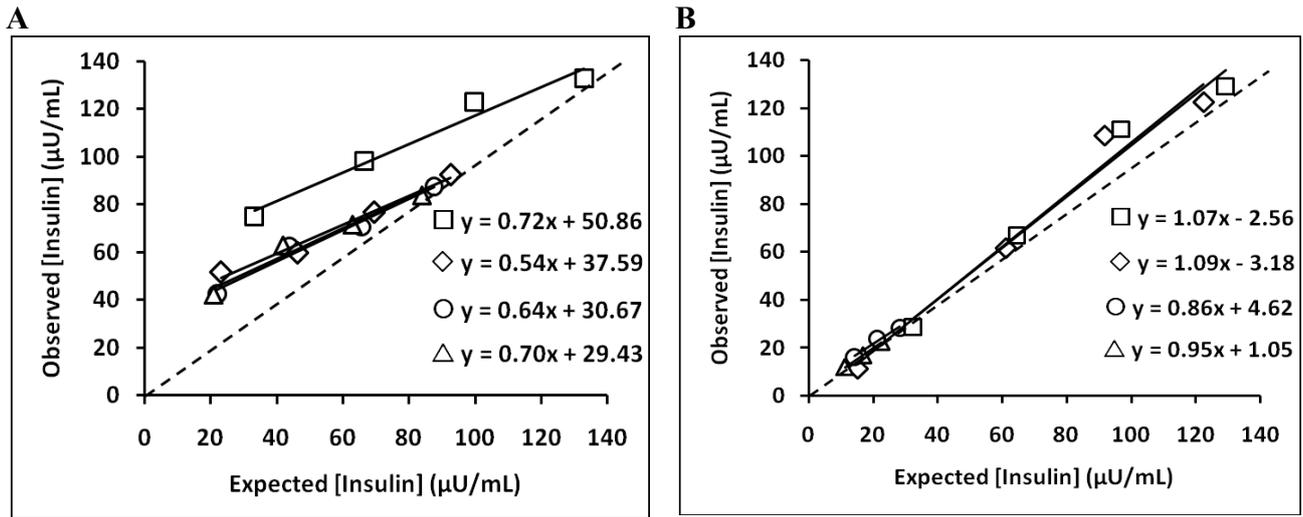


Figure 1. Parallelism studies of the Siemens Coat-A-Count Insulin RIA, using as diluent (A) the zero standard reconstituted with water, and (B) the zero standard reconstituted with charcoal-stripped plasma. Parallelism was inferred from an intercept of 0 and a slope approaching 1. The linear regression equations from each of the 4 samples are displayed. The dashed line represents a perfect linear regression.

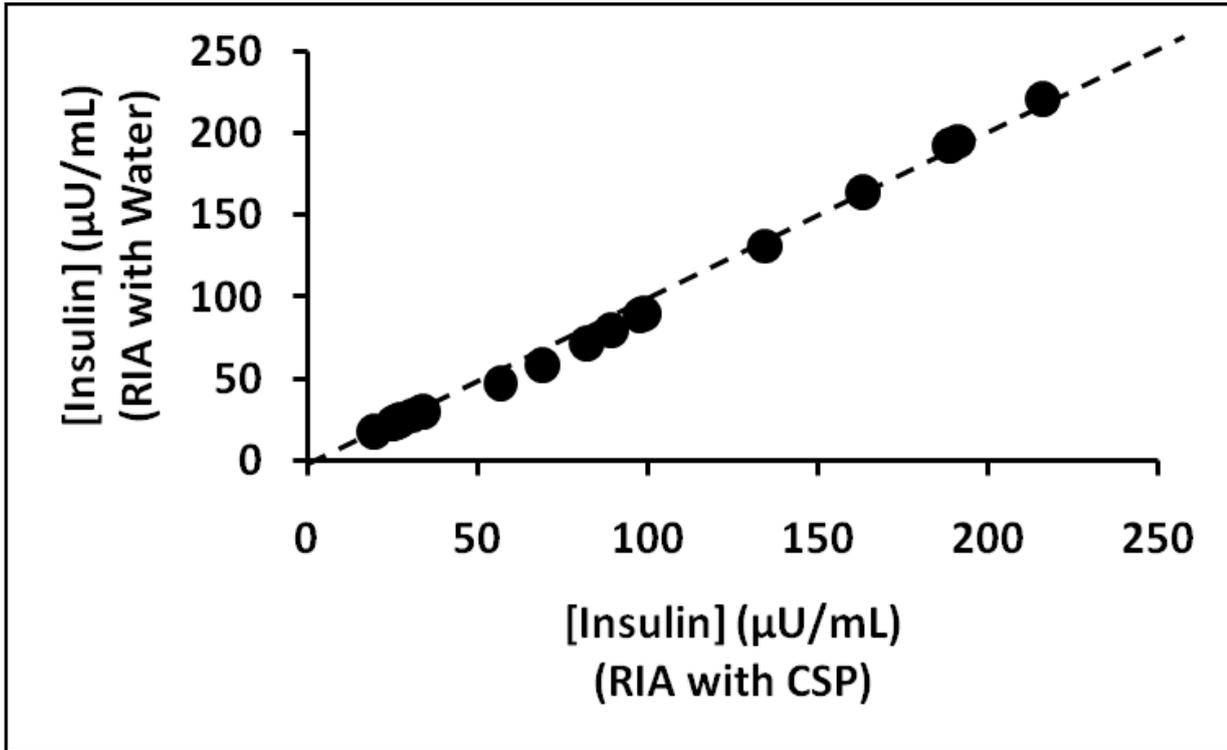


Figure 2. Plot of insulin concentrations in 18 equine plasma samples where the assay standards were reconstituted with water or charcoal-stripped plasma (CSP). The effect of standard diluent was negligible, with Lin's Concordance Correlation Coefficient (ρ_c) a value of 0.99. The dashed line represents a perfect correlation.

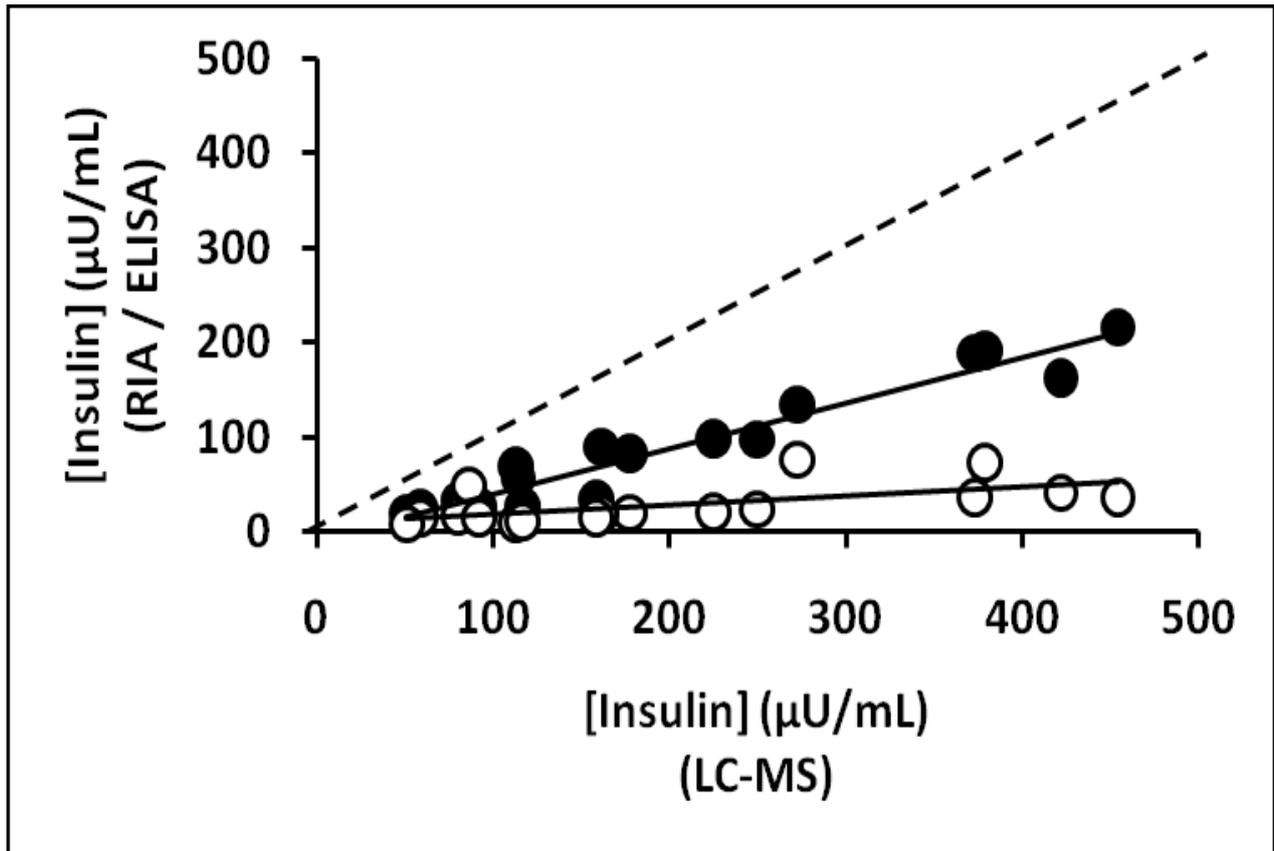


Figure 3. Plot of insulin concentrations measured in 18 equine plasma samples using immunoassays or liquid chromatography and mass spectrometry (LC-MS). The Lin correlations of the Siemens Coat-A-Count Insulin RIA (●) show a moderate correlation of $\rho_c = 0.41$ and the Mercodia Equine Insulin ELISA (○) show a very poor correlation of $\rho_c = 0.06$ to LC-MS. The dashed line represents a perfect correlation.