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Evaluation of an equine-optimized enzyme-linked immunosorbent assay for serum insulin measurement and stability study of equine serum insulin

Josefine Öberg · Johan Bröjer · Owe Wattle ·
Inger Lilliehöök

Abstract This study aims to evaluate a commercially available equine-optimized insulin assay and to evaluate the stability of equine insulin. In addition, serum insulin concentrations before and after feeding are also presented. Samples were taken before and after feeding from 40 healthy horses and from 15 equine patients visiting the University Equine Hospital. Insulin was analysed with the equine ELISA and with two human methods (one ELISA and one RIA). Precision was determined by repeated analysis of samples on one assay run and from one sample analysed on 15 different assays. Recovery from two dilution series and from an additional study was evaluated. Stability of equine insulin was evaluated in samples with and without haemolysis stored at 18–20°C, 6–8°C for 30 days and at −20°C for 1 year. The equine assay correlated well with both human assays ($r^2=0.97$ for both assays). The intra-assay coefficient of variance (CV) was 2.0–6.5%, and the inter-assay CV was 10.7%. Recovery upon dilution was 82–100%, and recovery upon addition was 102–115%. There was no significant decrease in insulin concentrations for non-haemolyzed samples when stored at 6–8°C for 30 days or at −20°C for 1 year. Mean insulin concentration was significantly higher (347 ng/L) after feeding compared with before feeding (123 ng/L). The equine assay correlated well with the previously used assays. The assay had good precision and recovery after dilution and addition. A significant increase in mean insulin concentration was seen in horses after feeding.

Keywords Metabolic syndrome · Insulin resistance · Validation · ELISA · Horses · Diabetes mellitus

Abbreviations
IR   Insulin resistance
RIA   Radioimmunoassay
CV   Coefficient of variance

J. Öberg (✉) · I. Lilliehöök
Clinical Pathology Laboratory,
Swedish University of Agricultural Sciences,
Box 7038, 75007 Uppsala, Sweden
e-mail: josefine.oberg@uds.slu.se

J. Bröjer · O. Wattle
Department of Clinical Sciences,
Swedish University of Agricultural Sciences,
Uppsala, Sweden

J. Öberg
Bagarmossen Referral Animal Hospital,
128 48 Bagarmossen, Sweden

Introduction

Equine metabolic syndrome is a clinical syndrome recognized in horses and ponies. The components of this syndrome include obesity, regional adiposity, insulin resistance (IR) and increased risk of laminitis (Frank et al. 2010; Johnson 2002; Treiber et al. 2006a, b). Insulin resistance is characterized by hyperinsulinaemia or abnormal glycaemic and insulinaemic responses to glucose or insulin tolerance tests. Diagnosis of IR is dependent on measurement of glucose and insulin concentrations in a single pre-prandial blood sample or in some cases dynamic tests to assess insulin sensitivity (Lyphochek Immunoassay Plus Control

I–III, Bio-Rad Laboratories, Irvine, USA). Several other abnormalities have been recognized with metabolic syndrome in horses, for example hypertriglyceridaemia and dyslipidaemia (Carter et al. 2009; Treiber et al. 2006a, b), arterial hypertension (Bailey et al. 2008; Treiber et al. 2006a, b) and reproductive disturbances (Sessions et al. 2004; Vick et al. 2006). Secondary, type 2, diabetes mellitus has also been associated with prolonged insulin resistance in horses (Jeffrey 1968; Myulle 1986; Riggs 1972; Ruoff et al. 1986; Tasker 1966).

The diagnosis of metabolic syndrome in horses is dependent on accurate insulin measurements. Correct insulin measurement is also important in several research fields. Until now, insulin has been measured with different radioimmunoassays (RIA) or enzyme-linked immunosorbent assays (ELISA) prepared for humans (Coat-A-Count RIA, DPC, Siemens Medical Solutions Diagnostics, Los Angeles, USA; Immulite insulin solid-phase chemiluminescent assay, Siemens Medical Solutions Diagnostics, Los Angeles, USA; DSL-1600 insulin radioim-munoassay, Diagnostic Systems Laboratories Inc., Webster, Texas, USA) (Reimers et al. 1982a, b). When measuring insulin in samples from animals with methods prepared for humans, the cross-reactivity may vary depending on the immunoglobulin used in the test. In addition, antibodies can vary between tests and batches (Karol et al. 1978). In a species-optimized assay, the immunoglobulins used are prepared for the intended species. The buffers, calibrator, detection limit and analytical range of the assay are also optimized. During the last few years, species-specific insulin ELISA assays for canine, feline, porcine and equine samples have been developed (Mercodia Insulin Equine, Mercodia AB, Uppsala, Sweden). The antibodies used in the equine assay are originally made against porcine insulin, but as the porcine and equine insulin molecules differ only in two amino acids (Smith 1966), the antibodies also recognize the equine insulin molecule. According to the manufacturer, the antibodies have been isosterically changed to optimize binding with the equine insulin molecule. They are directed to different parts of the insulin molecule, on locations that are not accessible in the proinsulin molecule, making the assay more specific for insulin and not sensitive to interference with proinsulin or c-peptide (Directions for use, Mercodia Equine Insulin ELISA, 10-1205-01).

The aim of this study was to evaluate the equine insulin ELISA assay in terms of precision (intra- and inter-assay coefficient of variance, CV), recovery after dilution and addition as well as to compare it with previously used assays. The stability of equine insulin was evaluated both in haemolyzed and non-haemolyzed samples. Serum insulin concentrations in blood samples taken from 40 horses before and after feeding are also presented.

Materials and methods

Samples

For the method comparison study, blood samples were taken from 40 clinically healthy 3–15-year-old horses and ponies (12 Swedish warm bloods, 10 Icelandic horses, 8 Shetland ponies and 10 Connemara ponies). The sampling of the horses was approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden. The samples were taken with a Vacutainer system from the jugular vein in serum tubes without additives (Vacu-tainer tube, Hettich, Sweden). The horses were in their regular home environment, and samples were collected twice. The first sample was taken in the evening, 60 min after feeding, and the second sample was taken the next morning before feeding. The feeding among the horses varied. Feeding of forage in the evening consisted of a bolus of hay or haylage. Seven of the horses (Icelandic horses) had access to hay overnight. Small amounts of concentrated feed or beet pulp were given in the evening as a treat to most of the Connemara and Shetland ponies and the Icelandic horses. The Swedish warm bloods were fed 0.5–1 kg concentrated feed in the evening.

The samples were centrifuged for 5 min at 3,700×g within 1 h after collection. Serum was separated, kept cold and frozen to −20°C within 4 h. Samples were first analysed, within 21 days, with the human insulin assays used in the Clinical Pathology Laboratory, Swedish University of Agri-cultural Sciences, during that time (ELISA (Insulin ELISA, Mercodia AB, Uppsala, Sweden) and RIA (Coat-A-Count RIA, DPC, Siemens Medical Solutions Diagnostics, Los Angeles, USA)) and then stored at −20°C for 1.5 years until they were analysed with the equine insulin assay, when it was introduced into the veterinary market. All samples were analysed in duplicate by well-trained staff at the Clinical Pathology Laboratory, Swedish University of Agricultural Sciences, according to the written recommendations for the respective assay.

For the precision, addition and stability studies, 15 equine serum samples from clinical patients were used. The blood samples were taken from patients with different medical conditions visiting the animal hospital during the last weeks of the study. The blood samples were centrifuged for 5 min at 3,700×g within 1 h, and serum was collected. Samples used in the stability study were analysed within 2 h after collection. Thereafter, the serum samples were divided into aliquots and stored under different storage conditions. The procedures for the haemolysis and stability study are further explained later in the manuscript. Samples for the precision and addition studies were either analysed within 2 h after collection or frozen at −20°C until analysis within a maximum of 5 days. When a sample was used

several times in the precision study, it was kept in the refrigerator between analyses.

The insulin assays

The equine-optimized insulin ELISA is a solid-phase two-site enzyme immunoassay. It is based on a direct sandwich technique in which two monoclonal antibodies are directed against two separate antigenic determinants on two different parts of the insulin molecule. Insulin in the sample reacts with anti-insulin antibodies bound to a microtitre plate and with peroxidase-conjugated free anti-insulin antibodies during an incubation of 120 min at 18–20°C. Samples were incubated on a plate shaker, Grant-bio PHMP-4 (Grant-bio PHMP-4, Grants Instruments Ltd., Cambridge, Great Britain). After incubation, unconjugated antibodies were removed with an automatic washer (Columbus Pro Washer, Tecan, Group Ltd., Männedorf, Austria), and the substrate 3.3′5.5′-tetramethylbenzidine was added. After a second incubation in 18–20°C for 15 min, an acid (0.5 M H_2SO_4) was added to the enzyme reaction. The amount of the reaction, reflecting the insulin concentration in the sample, was measured spectrophotometrically with a Multi-scan RC (Multiscan RC, Labsystem, Helsinki, Finland). The results were processed with an immunoassay data management programme, Multi Calc 2000, version 2.7 (Multi Calc 2000, version 2.7, Wallac Oy, Turku, Finland). Insulin measurement was performed according to the instructions from the manufacturer. Each kit contains one calibrator 0 and five different ready-to-use calibrators (20, 50, 150, 500 and 1,500 ng/L). The calibrators are made of porcine insulin, as there are no equine insulin standards available (Directions for use, Mercodia Equine Insulin ELISA, 10-1205-01). We used three human controls (Lyphochek Immunoassay Plus Control I–III, Bio-Rad Laboratories, Irvine, USA). The calibrators, controls and all samples were analysed in duplicates in all three assays, and the mean results of the duplicates were used. Samples in the addition study were analysed in four replicates. The results are reported in nanogrammes per litre, and the analytical range for the assay is 20–1,500 ng/L (Directions for use, Mercodia Equine Insulin ELISA, 10-1205-01). The assay capability of detection (defined according to the International Organization for Standardization 11843) is the minimum insulin concentration that is clearly distinguishable from the insulin-free calibrator. According to the manufacturer, the capability of detection for the equine ELISA is 10 ng/L (Directions for use, Mercodia Equine Insulin ELISA, 10-1205-01).

The human insulin ELISA assay (Insulin ELISA, Mercodia AB, Uppsala, Sweden) used for the comparison study was from the same manufacturer as the equine ELISA. It is also a solid-phase, two-site enzyme immuno-assay with a direct sandwich technique, similar to the equine serum insulin assay. The calibrators in the human kit are recombinant human insulin expressed in yeast. It is calibrated with an international standard, 1st international reference preparation 66/304 (Directions for use. Mercodia Insulin ELISA, 10-1203-01). The results from the human insulin assay are reported in milliunits per litre, and the analytical range is 3–200 mU/L (Directions for use. Mercodia Insulin ELISA, 10-1203-01). The detection limit is 1 mU/L, calculated as 2 standard deviations above calibrator 0 (Directions for use. Mercodia Insulin ELISA, 10-1203-01). The same controls, shaker, washer, reader and data management programme were used as for the equine ELISA.

The human insulin RIA assay (Coat-A-Count RIA, DPC, Siemens Medical Solutions Diagnostics, Los Angeles, USA) is a solid-phase ^{125}I radioimmunoassay. Seven human calibrators are used in the assay (0, 5, 15, 50, 100, 200 and 400 mU/L) (Lyphochek Immunoassay Plus Control I–III, Bio-Rad Laboratories, Irvine, USA). Calibrators and samples are added to the test tubes in the assay followed by ^{125}I-labelled insulin. The labelled samples are incubated at 18–20°C for 18–24 h before counting in a gamma counter (Gamma Counting System, Packard Instrument Company, Meriden, USA). The same controls used in the ELISA assays were used.

Validation study

For the method comparison study (equine insulin ELISA compared to the human insulin ELISA and the human insulin RIA), blood samples from 40 healthy horses were obtained before and after feeding. Precision of the equine insulin ELISA was evaluated by calculating intra- and inter-assay CV from eight equine serum samples (clinical patients) and three human controls (Multiscan RC, Labsystem, Helsinki, Finland). The intra-assay CV was calculated from four serum samples with a mean insulin concentration of 17, 200, 350 and 1,565 ng/L analysed in eight replicates within one run and from three serum samples with a mean insulin concentration of 53, 381 and 934 ng/L analysed in 26 replicates within one run. The inter-assay CV was calculated from one serum sample with a mean insulin concentration of 116 ng/L analysed in duplicate in 15 different assay runs during five consecutive days. Inter-assay CV from the three human controls (Multi Calc 2000, version 2.7, Wallac Oy, Turku, Finland) was calculated from duplicate analyses on 18 different assays runs. Serum insulin concentrations in samples obtained from the 40 healthy horses before and after feeding were compared to achieve information about insulin concentrations in pre- and post-prandial healthy horses.

Dilution series were performed using samples with high and medium-high insulin concentrations (1,414 and 998 ng/L). The high-concentration sample was diluted with physiologic saline in five twofold steps down to a measured concentration of 36 ng/L. The medium-high-concentration sample was diluted in four twofold steps down to a measured concentration of 64 ng/L. The measured insulin concentrations were compared with the mathematically calculated insulin concentrations, and recovery was calculated. For the addition study, a standard volume of 20 μl of blank (calibrator 0) or 20 μl of one of two purified porcine insulin preparations (extracted from porcine pancreas by Mercodia AB, Uppsala) with different insulin concentrations was added to 200 μl of three different equine serum samples. These mixtures were called, B (blank added) and A_1 and A_2 (porcine insulin preparation 1 or 2 added). In order to correctly quantify the insulin concentration of the two porcine insulin preparations, 20 μl of each preparation was added to 200 μl blank before analyses with the equine insulin ELISA (called C_1 and C_2; insulin concentration 207 and 754 ng/L, respectively, when measured with the equine insulin ELISA). Each mixture was analysed in four replicates with the equine insulin assay, and the mean insulin concentrations for the replicates were used in the recovery calculations. The measured insulin concentrations from each mixture were compared with mathematically predicted concentrations, and recovery in percent was calculated $(A/(B+C) \times 100)$.

Stability and haemolysis study

Four serum samples with insulin concentrations of 129, 195, 358 and 1,401 ng/L were used for the stability study. After analysis in four replicates on the first day (day 0), each sample was divided into three different aliquots. One aliquot from each sample was kept at room temperature (18–20°C), whereas the second aliquot from each sample was kept refrigerated (6–8°C). Samples were analysed in duplicate after 1, 3 and 30 days. The third aliquot from each sample was frozen at −20°C and analysed in duplicate on day 30 and after 1 year.

The effect of haemolysis on the insulin concentration in stored samples was evaluated in two samples. The samples were centrifuged at 3,000×g for 5 min. After serum was collected, the cell pellet in the serum tube was frozen overnight at −20°C, and after thawing, the tube was centrifuged at 3,000×g for 10 min. Analyses of the two haemolysed supernatants with Advia 2120 (Advia 2120, Siemens Healthcare Diagnostics, USA) yielded concentrations of 246 and 188 g/L Hb. To obtain samples with moderate haemolysis with approximately 5 g/L haemoglobin, 10 and 15 μL of the respective haemolysates were

added to 500-μL serum samples. Insulin concentrations were measured with the equine insulin assay in the haemolysed samples on day 0. The samples were divided into three aliquots each. One was kept in room temperature (18–20°C), one in a refrigerator (6–8°C) and one in a freezer (−20°C). Samples kept at room temperature and in the refrigerator were analysed in duplicate after 1 and 3 days. Samples kept in the freezer were only analysed on day 30, one in duplicate and the other one as a single analysis because of shortage of sample.

Statistics

The agreement between the assays was evaluated with Bland–Altman plots (Bland and Altman 1986) and linear regression. Pearson's correlation was used to determine correlation between the equine ELISA and the two human assays. In order to compare the results from the different assays, which were expressed in different units, the results were logarithmically transformed, $\log_{10}$. A paired t test was used to evaluate differences between the pre-feeding and post-feeding samples in the horses. Examining the stem-and-leaf plots for both methods assessed normality of the differences between the pre-feeding and post-feeding samples. A p value of <0.05 was considered statistically significant. To evaluate the precision of the equine ELISA, intra- and inter-assay CV in percent was calculated by dividing the standard deviation (SD) with the mean value from repeated measurement and multiplying with 100. Linearity was evaluated in a linearity plot, and recovery upon dilution was calculated by dividing each measured value by calculated value and multiplying by 100. Recovery upon addition was calculated by dividing spiked samples by the sum of sample and calibrator 0 (blank) and the spiking preparation together with calibrator 0 (blank). To get the results in percent, the values were multiplied by 100 (Table 2). Mean recovery, from the dilution and addition studies, was calculated, and the results are presented as mean and range. To evaluate the stability of insulin during storage under different conditions, relative changes from the baseline value day 0 were calculated. An observed change (in percent) was considered statistically significant if it exceeded the critical value $k\sqrt{\mathrm{CV}_e^2\left(\frac{1}{m}+\frac{1}{n}\right)+2\mathrm{CV}_b^2}$, where CV_e is the intra-assay CV, CV_b the inter-assay CV, m the number of replicates at day 0 and n the number of replicates for the stored sample. The factor k is chosen to give a significance level of about 5%. In this study, CV_b was estimated from 15 runs, and the value of k was set to 2.2. The relative difference between insulin concentrations at day 30 in the samples stored in refrigerator (6–8°C) and room temperature (18–20°C) and the samples stored in the freezer (−20°C) was

also calculated and compared. An observed difference (in percent) is considered as statistically significant if it exceeds the critical value $k\sqrt{CV_e^2\left(\frac{1}{m}+\frac{1}{n}\right)}$. CV_e was estimated with 14 degrees of freedom, and k was set to 2.2 (Dybkaer 1995).

Statistical calculations were performed using the JMP package (JMP® version 5.1 package; SAS Institute, Inc., Cary, NC, USA), Microsoft Office Excel 2003 and Analyse-it (Analyse-it Software Ltd, Leeds, England).

Results

Comparison of insulin concentrations measured with the equine ELISA and with the human ELISA or RIA is shown in Figs. 1 and 2. The square of the correlation (r^2) was 0.97 when the equine ELISA was compared with the human ELISA as well as when it was compared with the RIA. The results from the regression analysis and the difference plots showed that the equine insulin ELISA gave results with good agreement with the two human assays; however, as the result had different units of insulin in the different assays (nanogrammes per litre vs. milliunits per litre), the results were on different levels. There was a small proportional error detected with the difference plot between the equine ELISA and the human RIA in samples with high insulin concentrations.

The mean serum insulin concentration was significantly higher in samples taken after feeding ($p<0.001$) compared to those taken before feeding (Table 1). Insulin concentrations were higher in samples taken after feeding in 37 of the 40 horses.

The equine serum insulin ELISA had an inter-assay coefficient of variation of 10.7%, and the intra-assay coefficient of variation was below 7% at all levels (Table 2). Inter-assay CV from three human controls were 13.3% for the low concentration control (mean 83 ng/L), 2.2% for the medium-high concentration control (mean 532 ng/L) and 4.3% for the high concentration control (mean 1,495 ng/L). Mean recovery after dilution of equine samples with insulin concentrations of 1,414 and 998 ng/L was 82% (range 74–90%) and 100% (range 82–104%), respectively (Fig. 3a and b). The mean recovery upon addition was 107% (range 102–115%) (Table 3).

There was no significant decrease in serum insulin concentrations over time when samples were stored at 6–8°C for 1 month or at −20°C for 1 year (Table 4 and Fig. 4). At room temperature (18–20°C), the insulin concentration was stable for 3 days. After 30 days storage at room temperature (18–20°C), the insulin concentration was significantly lower (21–28%) compared with the aliquots stored in the freezer (−20°C). When the concentrations in samples stored at room temperature were compared with their respective baseline value at day 0, the decrease after
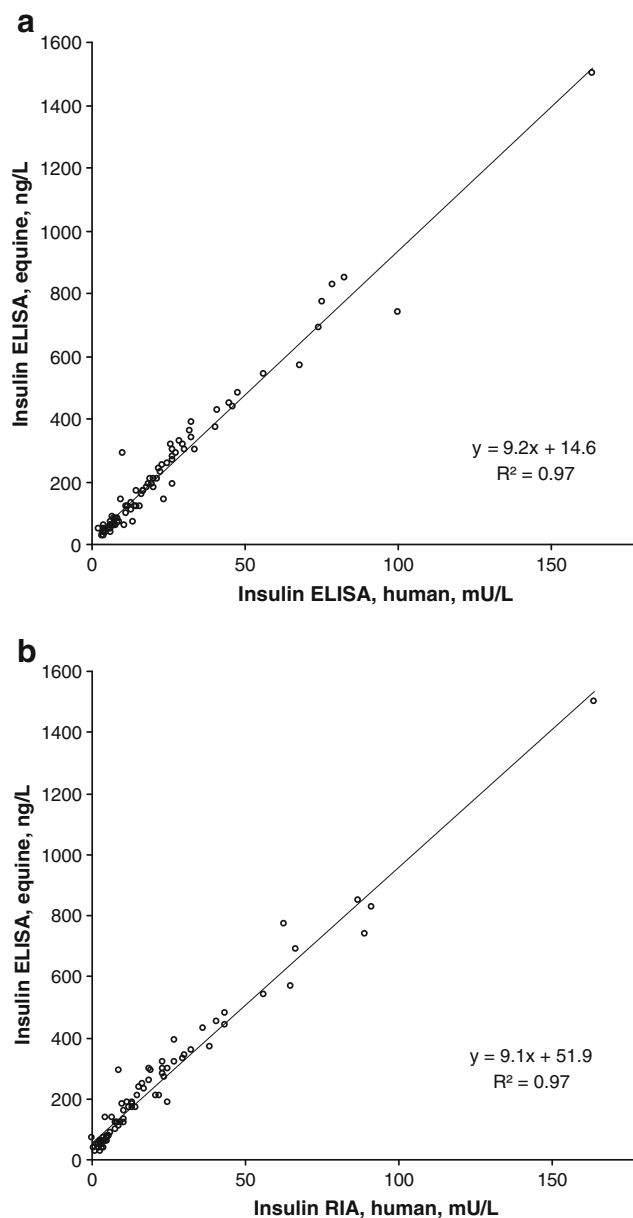

Fig. 1 Comparison of insulin concentrations measured with the equine ELISA and with the human ELISA (**a**) and human RIA (**b**). The results in milliunits per litre from the human ELISA and RIA are plotted on the *x*-axis, and the results in nanogrammes per litre from the equine ELISA on the *y*-axis. *n*=80 (samples from 40 horses before and after feeding). *Grey lines* indicate line of best fit. Equation of linear regression and Pearson's correlation coefficient are shown in the figures

30 days was not significant, mainly due to the fact that the inter-assay CV also has to be included in the statistics. Insulin concentration decreased more rapidly in the haemolysed samples and was significantly lower after 30 days storage in both the refrigerator and the freezer (Table 5). There was no change in measured insulin concentration when insulin was measured in one sample before haemolysate was added (129 ng/L) compared to when it was analysed directly after haemolysate was added (123 ng/L).

Fig. 2 Difference plots of insulin concentration analysed with the equine insulin ELISA and comparing it with the human ELISA (**a**) and RIA (**b**). The results have been logarithmically transformed. *Blue line* indicates mean bias, and *black line* represents $x=y$. Dashed blue line indicates limits of agreement. $n=80$ (samples from 40 horses before and after feeding)
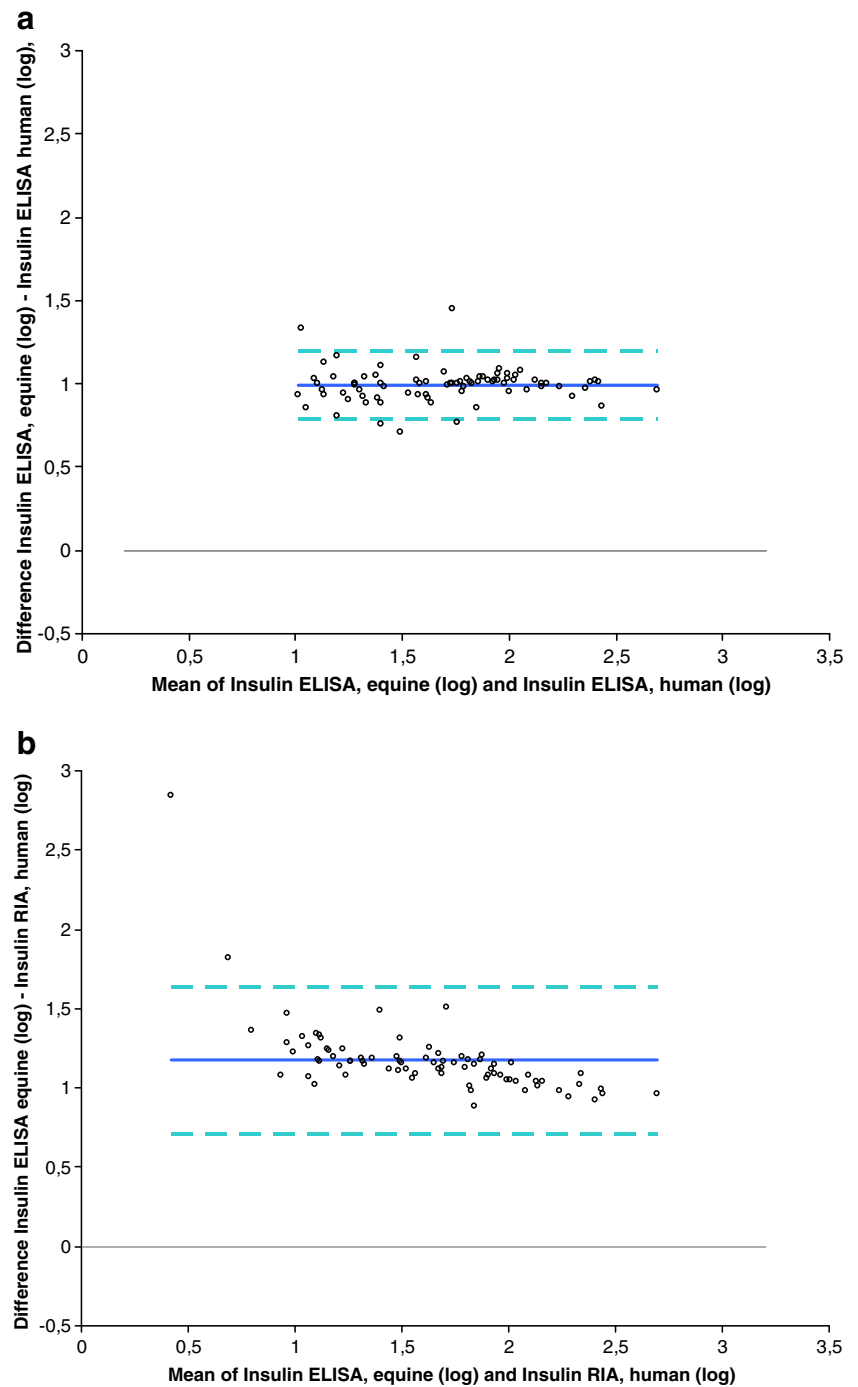
Discussion

The insulin molecule is highly conserved between species; therefore, some human assays for serum insulin have been used with good results for equine samples (Reimers et al. 1982a, b). However, when measuring insulin in samples from animals with methods developed for a human hormone, cross-reactivity may vary depending on the antibody used (Karol et al. 1978), and antibodies can vary between tests and batches. The advantage with an equine-

optimized ELISA is that the antibodies, calibrators, buffer and analytical range have been optimized for equine samples.

Before a new assay that has been introduced on the veterinary market can be used in clinical practice and in research, it should be validated independently of the producer. This is often done by evaluating the precision of the assay in a different part of the analytical range, preferably in terms of intra- and inter-assay precision as well as by evaluating the accuracy and specificity of the

Table 1 Insulin concentrations before and after feeding in 40 horses analysed with the equine ELISA, the human ELISA and the human RIA

	Before feeding mean (range)	After feeding mean (range)
Equine ELISA	123 ng/L (30–740)	347 ng/L (30–1,500)*
Human ELISA	13 mU/L (2.3–100)	35 mU/L (4.2–164)
Human RIA	10 mU/L (0.1–89)	31 mU/L (2.5–163)

*$p < 0.001$ (significant increase, t test)

assay (dilution and addition studies). When validating a new assay, the assay is also often compared to previously used assays or, if available, to a golden standard method, to further investigate the accuracy of the assay. Interference with different substances, for example haemoglobin, as well as analytical range and detection limit should also be evaluated (Westgard and Hunt 1973).

The equine insulin ELISA validated in this study had good agreement with two earlier-used human assays, a RIA and an ELISA, even if the results of the equine assay are presented with different units. A small proportional error was noted for samples with high insulin concentrations when the equine assay was compared to the RIA. This might be due to loss of linearity in the higher range for one of the assays. The equine assay was linear up to an insulin concentration of 1,400 ng/L, which is near the upper measuring range of the assay. Therefore, decreased correlation between the assays probably is due to loss of linearity of the RIA in the upper measuring range. This earlier frequently used Coat-and-Count RIA from DPC, Siemens Health Diagnostics assay, is no longer available on the market, and the linearity could therefore not be tested.

The equine ELISA assay had acceptable precision. To the authors' knowledge, there is only one published

Table 2 Intra- and inter-assay CV from equine samples analysed with the equine ELISA assay

Variable	Intra-assay							Inter-assay
n	26[□]	26[□]	26[□]	8[a]	8[a]	8[a]	8[a]	30[b]
Mean (ng/L)	53	381	934	17	200	350	1,565	116
SD (ng/L)	3.6	12.4	19.0	0.8	7.8	14.9	87.5	12.4
CV (%)	6.5	3.3	2.0	4.6	3.9	4.3	5.6	10.7

Intra-assay CV calculated from three samples with different insulin concentrations analysed in 26 replicates within the same assay

n number of replicates

[a] Intra-assay CV was also calculated from four samples with different insulin concentrations analysed in eight replicates within an assay run

[b] For inter-assay CV, one sample was analysed in duplicate in 15 different assays during five consecutive days

validation study on equine serum insulin (Reimers et al. 1982a, b). In that study, the human RIA, used for method comparison in this study, was validated for use in horses. The assay had an inter-assay precision of 6.7–20.1% and an intra-assay precision of 4.4–10.7% (Reimers et al. 1982a, b). Different immunological assays for insulin measurements have been validated for use in dogs and cats, with CV ranging from 3.8 to 56.8 (Hoier and Jensen 1993; Lutz and Rand 1993; Öberg et al. 2011). Compared to those assays, the precision of the equine assay in this study was deemed to be good, indicating an acceptable precision of this ELISA. Generally, a CV of 10% or lower has been deemed to be good for immunological assays (Graham 2010).

To evaluate whether the equine assay is able to correctly quantify the equine insulin molecule, an equine standard solution should be used. As there is no such equine standard available, we used two different porcine insulin preparations. Porcine insulin was chosen since the equine and porcine insulin molecules differ only in two amino acids, and the molecular weight therefore has been assumed to be the same (Smith 1966). The recovery after porcine insulin addition was close to 100% (i.e. 102–115%) indicating good specificity and no constant error. No proportional bias, such as interference with different substances, for example proinsulin and c-peptide, was detected in the dilution study where the calculated recovery was 82–100%.

In equine practice, insulin measurement is mainly used to investigate insulin resistance in horses with suspected metabolic syndrome. High serum insulin concentrations can be expected when insulin is measured in insulin-resistant horses during dynamic glucose tolerance tests (Schezenmier 1996; Frank et al. 2010; Carter et al. 2009). The analytical range must therefore be wide enough to encompass these high levels. The measuring range in this assay was good for samples from healthy horses; however, for samples obtained during dynamic testing for insulin resistance from horses with metabolic syndrome, a higher analytical range might be more optimal, or some samples have to be diluted.

Equine serum insulin was stable for 3 days at room temperature, for 1 month in the refrigerator and 1 year in the freezer. Human and canine insulin have also been shown to be very stable in serum samples (Reimers et al. 1982a, b; Vogaser and Parhofer (2005; Jeffrey 1968). In our study, insulin concentrations decreased more in haemolyzed samples than in non-haemolyzed samples after 30 days even when stored in the refrigerator or freezer which is in agreement with previous studies (Pasic et al. 1991; Yonezawa et al. 1988).

Insulin concentration was higher after feeding in 37 of 40 healthy horses. Seven of the horses (Icelandic horses) had access to hay during the night. Despite this, these

Fig. 3 Linearity plot of insulin concentrations measured with the equine ELISA after dilution of a high equine sample (1,414 ng/L, **a**) diluted in five equal steps and a median high sample (998 ng/L, **b**) diluted in four equal steps. The *blue lines* indicate $x=y$
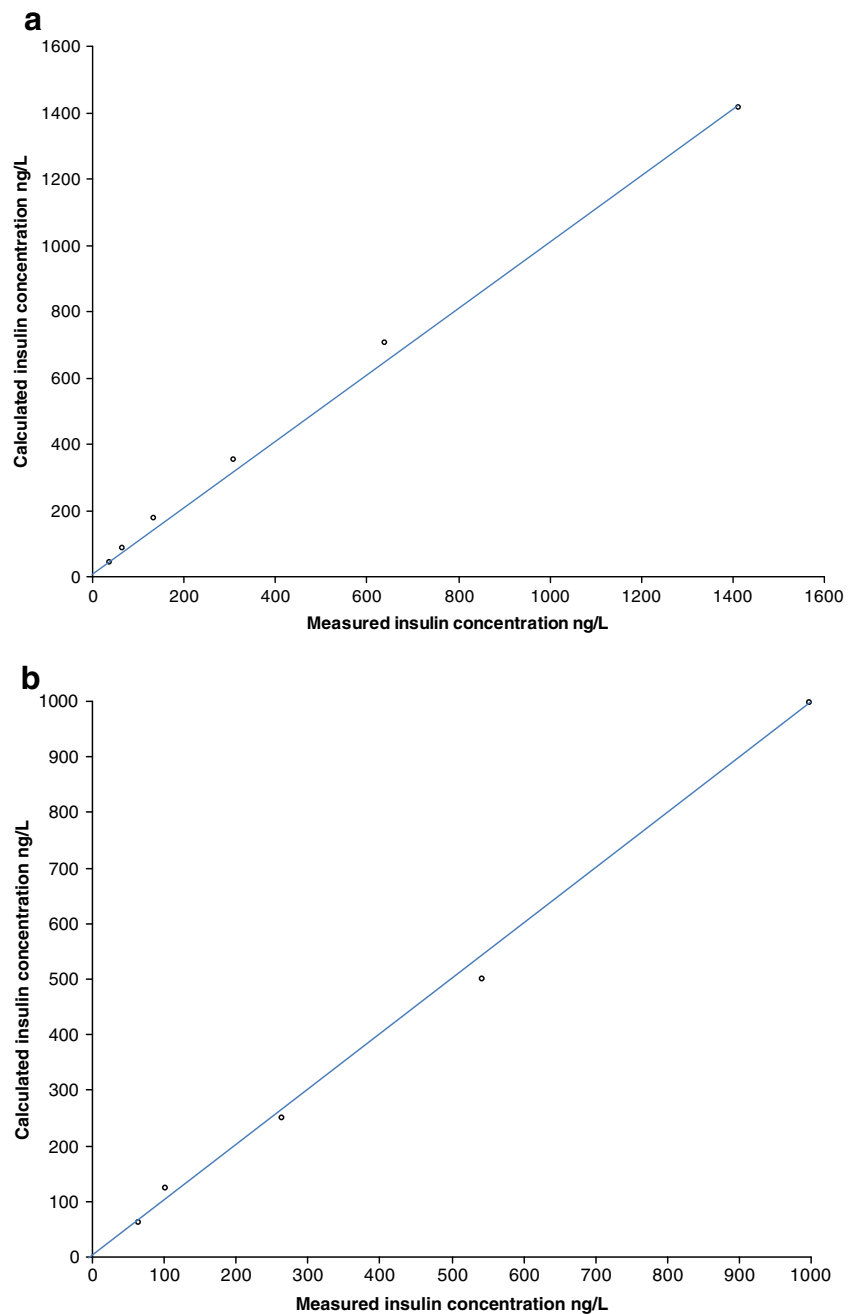

Table 3 Recovery after addition of porcine insulin

	A_1	A_2	B	C_1	C_2	A_1 recovery (%)	A_2 recovery (%)	Mean recovery (%)
Sample 1	334	905	71			120	110	115
Sample 2	548	1,214	348			99	110	104
Sample 3	1,019	1,601	801			101	103	102
Insulin preparation				207	754			

Porcine insulin was added to three equine patient samples (samples 1–3) analysed in duplicates. Columns A_1 and A_2 show results from the three equine samples spiked with one of two commercial purified porcine insulin preparations with different concentrations. Results in column B are from equine samples 1–3 to which a blank without insulin was added. C_1 and C_2 are the two porcine insulin preparations used to spike the samples analysed after addition of the blank. All results are in nanogrammes per litre. Recovery in percent was calculated by $A/(B+C)\times100$

Table 4 Insulin concentrations in nanogrammes per litre in four equine samples analysed with the equine insulin ELISA on day 0 and after 1, 3 and 30 days of storage in room temperature (18–20°C) and in refrigerator (6–8°C)

Sample	Day 0 ng/L	Storage	Day 1 ng/L	Day 3 ng/L	Day 30 ng/L	1 year ng/L
1		18–20°C	140	122	99[a]	ND
	129	6–8°C	135	127	125	ND
		−20°C	ND	ND	125	124
2		18–20°C	200	182	140[a]	ND
	195	6–8°C	198	199	194	ND
		−20°C	ND	ND	191	183
3		18–20°C	337	336	264[a]	ND
	358	6–8°C	343	357	360	ND
		−20°C	ND	ND	356	340
4		18–20°C	1,601	1,496	1,111[a]	ND
	1,401	6–8°C	1,545	1,521	1,517	ND
		−20°C	ND	ND	1,462	1,380

The frozen samples (−20°C) were analysed on day 30 and after 1 year

[a] The insulin concentration in the sample stored in room temperature was significantly lower than that of the samples stored in the freezer, however not significantly low compared with baseline values of the same samples analysed on day 0

Table 5 Insulin concentrations in nanogrammes per litre in two haemolysed equine samples analysed with the equine insulin ELISA

Samples	Day 0 ng/L	Storage	Day 1 ng/L	Day 3 ng/L	Day 30 ng/L
1		18–20°C	113	107	ND[a]
	123	6–8°C	126	116	45[b]
		−20°C	ND	ND	38[b]
2		18–20°C	940	878	ND[a]
	1,071	6–8°C	1,067	1,042	364[b]
		−20°C	ND	ND	325[b]

Samples stored in room temperature were analysed after 1 and 3 days, and samples stored in refrigerator or freezer were analysed after 1, 3 and 30 days

[a] Insulin concentration was not analysed because of shortage of sample

[b] There was a significant decrease in insulin concentrations in haemolysed samples stored in refrigerator or freezer after 30 days compared with day 0

horses had pre-feeding serum insulin concentrations at the lower end of the range of pre-feeding insulin concentrations for all 40 horses. Horses with constant access to forage most likely consume the feed over an extended period of time, and therefore, this feeding regime does not affect the insulin concentration. Continuous feeding with forage has shown not to affect insulin concentrations in previous studies (Nadal et al. 1997; Ralson 2002; Williams et al. 2001). The magnitude of the increase in insulin concen-
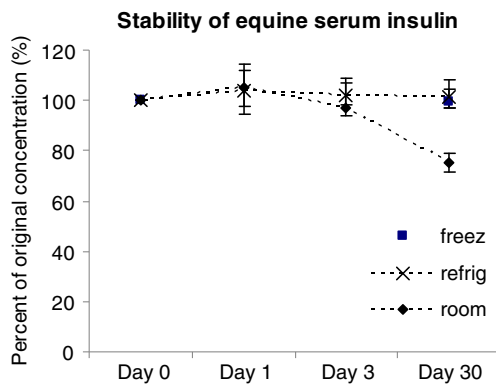

Fig. 4 Stability study of insulin analysed with the equine ELISA. Four equine samples were kept in room temperature (18–20°C), in refrigerator (6–8°C) and in freezer (−20°C). Samples were analysed in four replicates on day 0 and 30 and in duplicate on day 1 and 3. The sample stored in freezer was analysed on day 0, day 30 and after 1 year (last time point not in the figure). Relative change in percent from the values on day 0 is shown as mean and as range

trations after feeding varied between individuals. Due to the limited numbers of horses, the high variation of serum insulin concentrations among the individual horses and the different feeding regimes between the breeds, it was not possible to draw any conclusions of the effects of different feeds on the post-prandial serum insulin concentrations.

The results from this assay are given in nanogrammes per litre. Traditionally, insulin has been measured in an activity unit, units per litre, which is based on the activity of the human insulin molecule. It is not known whether the equine insulin molecule has the same activity as the human insulin molecule, and therefore, it is preferable to use a quantitative unit. An immunologic assay, as this insulin ELISA, detects the presence of the equine insulin molecule, not its activity. As different antibodies are used in different assays, results cannot be compared without calculating a conversion factor between the assays. A better way to judge results from a new assay is to set up new reference values. Results from 40 healthy horses before and after feeding are reported in the study, but the numbers of samples are too small to be used as a reference interval (Yong 1992).

A shortcoming of this study is the time between insulin analyses in the method comparison study. The samples were first analysed to establish pre- and post-feeding insulin concentration intervals in the assay used in routine work (RIA). As a human ELISA was available and ELISA assays often are preferable to use, the samples were also analysed on that assay in order to evaluate if it could be used for equine samples. After another year, the equine-optimized assay was introduced, and the previously used samples were used again in this validation study, with good results in spite of the long storage time. In this study, equine serum insulin was stable for 1 year.

References

Bailey SR, Habershon-Butcher JL, Ransom KJ et al (2008) Hypertension and insulin resistance in a mixed-breed population of ponies predisposed to laminitis. Am J Vet Res 69:122–129

Bland JM, Altman DG (1986) Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 8:307–310

Carter RA, Treiber KH, Geor RJ et al (2009) Prediction of incipient pasture-associated laminitis from hyperinsulinemia, hyperleptinemia and generalised and localised obesity in a cohort of ponies. Equine Vet J 41:171–178

Dybkaer R (1995) Result, error and uncertainty. Scand J Clin Lab Invest 55:97–118

Frank N, Geor RJ, Bailey AE et al (2010) Equine metabolic syndrome, ACVIM consensus. J Vet Intern Med 24:467–475

Graham P (2010) Impact of analytical method on endocrine diagnosis. Proceedings 20th ECVIM-CA Congress. pp 45–47

Hoier R, Jensen AL (1993) Evaluation of an enzyme linked immunosorbent assay (ELISA) for determination of insulin in dogs. J Vet Med 40:26–32

Jeffrey JR (1968) Diabetes mellitus secondary to chronic pancreatitis in a pony. J Am Vet Med Association 153:1168–1175

Johnson PJ (2002) The equine metabolic syndrome peripheral Cushing's syndrome. Vet Clin North Am Equine Pract 18:271–293

Karol R, Reichlin M, Noble RW (1978) Idiotypic cross-reactivity between antibodies of different specificities. J Exp Med 148:1488–1497

Lutz TA, Rand JS (1993) Comparison of five commercial radioimmunoassay kit for the measurement of feline insulin. Rec vet Soc 55:64–69

Myulle E (1986) Non-insulindependent diabetes mellitus in a horse. Equine Vet J 18:145–146

Nadal MR, Thompson DL Jr, Kincaid LA (1997) Effect of feeding and feed deprivation on plasma concentrations of prolactin, insulin, growth hormone, and metabolites in horses. J Anim Soc 75:736–744

Pasic J, Bhatanger MK, Pickup JC (1991) Self-collection by diabetic patients of capillary blood for free insulin monitoring; reduction by diameide of haemolysis-induced insulin loss. Diabet Med 8:140–145

Ralson SL (2002) Insulin and glucose regulation. Vet Clin North Am Equine Pract 18:295–304

Reimers TJ, Cowan RG, McCann JP et al (1982a) Validation of a rapid solid-phase radioimmunoassay for canine, bovine and equine insulin. Am J Vet Res 43:1274–1278

Reimers TJ, McCann JP, Cowan RG et al (1982b) Effects of storage, hemolysis, and freezing and thawing on concentrations of thyroxine, cortisol, and insulin in blood samples. Proc Soc Exp Biol Med 170:509–516

Riggs WL (1972) Diabetes mellitus secondary to chronic necrotizing pancreatitis in a pony. The Southwestern Veterinarian 25:149–152

Ruoff WW, Baker DC, Morgan SJ et al (1986) Type II diabetes mellitus in a horse. Equine Vet J 18:143–144

Schezenmier J (1996) Hyperinsulinemia, hyperproinsulinemia and insulin resistance in the metabolic syndrome. Experentia 52:426–432

Sessions DR, Reedy SE, Vick MM et al (2004) Development of a model for inducing transient insulin resistance in the mare: preliminary implications regarding estorus cycle. A Soc Anim Sci 82:2321–2328

Smith LF (1966) Species variation in the amino acid sequence of insulin. Am J Med 40:662–666

Tasker (1966) Diabetes mellitus in the horse. J Am Vet Med Assoc 149:393–399

Treiber KH, Kronfeld DS, Geor RJ (2006a) Insulin resistance in equids: possible role in laminitis. J Nutr 136:2094–2098

Treiber KH, Kronfeld DS, Hess TM et al (2006b) Evaluation of genetic and metabolic predispositions and nutritional factors for pasture-associated laminitis in ponies. J Am Vet Med Assoc 228:1538–1545

Vick MM, Sessions DR, Murphy BA et al (2006) Obesity is associated with altered metabolic and reproductive activity in the mare: effects of metformin on insulin sensitivity and reproductive cyclicity. Reprod Fertil Dev 18:609–617

Vogaser M, Parhofer KG (2005) Limited preanalytical requirements for insulin measurement. Clin Biochem 38:572–575

Westgard JO, Hunt MR (1973) Use and interpretation of common statistical tests in method-comparison studies. Clin Chem 19:49–57

Williams CA, Kronfeld DS, Staniar WB et al (2001) Plasma glucose and insulin responses of Thoroughbred mares fed a meal high in starch and sugar or fat and fibre. J Am Sci 79:2196–2201

Yonezawa K, Yokono K, Shii K et al (1988) Insulin-degrading enzyme is capable of degrading receptor-bound insulin. Biochem Biophys Res Commun 150:605–614

Yong D (1992) Determination and validation for reference intervals. Arch Path Lab Med 116:700–709

Öberg J, Fall T, Lilliehöök I (2011) Validation of a species-optimized enzyme linked immunosorbent assay (ELISA), for detemination of serum insulin concentrations in dogs. Vet clin path 40:66–73