**Short Communication**

**Serum insulin concentration in horses: effect of storage and handling**

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**Abstract**

**S**erum insulin concentration is commonly measured during investigation of suspected endocrinopathic disease in horses, but immediate analysis is frequently unavailable. The aim of this study was to examine the effect of storing samples at room temperature for 72h as serum and as whole blood, compared to immediate separation and freezing. Samples from 14 horses were evaluated. Correlation was excellent for all comparisons. Bland Altman plots revealed a negative bias (mean difference 2.16µIU/mL) in samples stored as whole blood compared to serum, but this difference was not considered clinically significant. At two commonly used diagnostic cut-offs there was no effect of storage on result. This study indicates that storage at room temperature for 72h, either as serum or whole blood, has minimal effect on measured serum insulin concentration in horses.

*Keywords:* Horse; Insulin; Stability; Serum; Storage

Equine metabolic syndrome (EMS) and pituitary pars intermedia dysfunction (PPID) are commonly suspected in equine practice, especially in horses presenting with laminitis (Karikoski et al., 2011; McGowan et al., 2013; Morgan et al., 2014). Both PPID and EMS have been associated with compensated insulin resistance or insulin dysregulation and measurement of serum insulin concentration basally, or following oral or intravenous glucose challenge remains an important part of the diagnosis and monitoring of affected horses (Durham et al., 2014; Morgan et al., 2015).

Following collection of the appropriate samples, most veterinary surgeons have to post them to external laboratories, in many cases unseparated due to having no immediate access to a centrifuge. Furthermore, samples taken on a Friday may take up to three days to reach a laboratory if routine postal services are used or laboratories are not staffed on the weekend. As such there is the potential for samples to be stored separated or unseparated for up to three days at room temperature.

The effect of storage and handling on a variety of clinicopathological parameters has been reported (Collicutt et al., 2015; Prutton et al., 2015; Rendle et al., 2009) but to the authors’ knowledge no published literature exists on the effect of storage prior to analysis on serum insulin concentration in horses.

The aims of this study were to examine the effect of storage at room temperature for 72 h, either as serum or whole blood, on serum insulin concentration. We hypothesised that storage at room temperature for 72 h, either as serum or as whole blood would not significantly reduce serum insulin concentration compared to immediate separation and storage at -20 ˚C.

Blood samples from 14 horses (mean (range) age 14.4 (6-27) years), of mixed breeds, (7 mares, 6 geldings and 1 stallion) presented to the Helsinki University Equine Teaching Hospital, Finland for investigation of endocrine disease based on clinical signs of recurrent laminitis, PPID or phenotypic indicators of EMS between February and July 2008, were included in the study. All blood samples were taken with informed owner consent for the purposes of clinical endocrine testing with aliquots used to perform the same test in triplicate using different storage conditions. Venous blood was collected from the jugular vein by single direct venipuncture into three identical evacuated tubes containing a clot activator (Vacuette, 2 serum clot activator, Greiner Bio-One, Austria) and allowed to clot at room temperature for 45 min. Samples CenRT and CenFr were immediately centrifuged at 2000g for 10 min and the serum separated. Serum from sample CenRT was then maintained at room temperature (22 ˚C) in air-conditioning for 72 h. Serum from sample CenFr was immediately frozen at -20 °C. Sample RTCen was maintained at room temperature (22 °C) for 72 h, and then separated by centrifugation at 2000g for 10 min. After 72 h, all samples were transferred to -80 °C storage until analysis.

All samples were packaged on ice and sent frozen to the same commercial laboratory by next-day delivery (Cambridge Specialist Laboratories, UK). Samples were analysed as a single batch using the DiaSorin S insulin RIA validated for use in horses and previously described in horses (Karikoski et al., 2011).

Statistical analysis was performed using SPSS version 21, with significance set at P<0.05. Comparisons were made between CenRT-Cenfr, RTCen-CenFr and RTCen-CenRT. Agreement between groups was assessed using Bland Altman Plots, including analysis for proportional bias,and Linn’s correlation coefficient with >0.99 indicating almost perfect strength of agreement, 0.95-0.99 substantial, 0.90-0.95 moderate and <0.9 poor. The mean value of the difference between the groups was compared to 0 using a 1-sample T test, to detect the presence of a fixed bias. Linear regression analysis was performed on the Bland Altman plot to detect proportional bias. Dichotomous outcomes were created using cut-off values for serum insulin concentration commonly used in clinical practice to diagnose EMS (30 µIU/mL for basal and 100 µIU/mL at 45 min during a combined glucose-insulin tolerance test (CGIT)). Agreement between groups was analysed using Kappa’s measure of agreement.

Measured insulin concentrations ranged from 6.6 – 310 µIU/mL (Table 1, Fig.1). Data analysis is summarised in Table 2. Bland Altman analysis (Fig. 2) showed a very mild bias for all three comparisons, however, the mean difference between CenRT and RTCen (2.16 µIU/mL) was significantly different from 0 (*P*=0.042). The 95% limits of agreement were narrowest for CenRT-RTCen and wider for the other comparisons, reaching -21.3 – 20.7 µIU/mL for CenFr–CenRT (Fig. 2). Linear regression of the Bland Altman plots showed significant proportional bias between RTCen and CenRT (*P*=0.004). Lin’s concordance correlation coefficient showed almost perfect agreement between all three comparisons. For both 30 µIU/mL and 100 µIU/mL cut-offs Kappa’s measure of agreement was perfect (Kappa statistic = 1).

The agreement between samples in this study support that storage of serum or clotted blood at room temperature for 72 h has a clinically insignificant effect on serum insulin concentration, compared to immediate separation and freezing.

Although the 95% limits of agreement on Bland-Altman plots were quite wide, the mean differences were very small, and the larger variation was consistently found in horses with much higher insulin concentrations. The greatest variation in serum insulin was detected in one outlier with a high insulin concentration of 279-310 µIU/mL. Variation around the diagnostic cut-off values, which for this laboratory were 30 and 100 µIU/mL for basal and dynamic testing, respectively, were low and support the stability of serum insulin concentrations for clinical diagnostic purposes in horses. The mean difference between CenRT and RTcen was detected as a significant effect. This was associated with a mean difference of only 2.16 µIU/mL, which is unlikely to be of clinical significance. This difference supports that separation of serum helps prevent degradation of insulin and should be performed when possible.

Significant reductions in human insulin concentration have been demonstrated after storage for less than 24 h at room temperature (Oddoze et al., 2012). Given that human and horse insulin show substantial homology (Conlon, 2001) this apparent discrepancy is most likely explained by variation in study design (assay and statistical analysis), in the secondary, tertiary or quaternary structure of the different insulin molecules, or in insulin stability offered by human and equine serum.

Although the gold standard for any laboratory assay would be immediate analysis, this is frequently not available to veterinary surgeons in practice. Storage at room temperature for up to 72 h, either as serum or whole blood has minimal effect on serum insulin concentration compared to immediate separation and freezing. Immediate separation of serum is recommended to prevent a small reduction in measured insulin concentration.

**Conflict of interest statement**

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

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**Tables**

**Table 1**

Summary of serum insulin concentration after three different storage and handling conditions in 14 horses.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Serum Insulin (µIU/mL)** | | |
| **CenFr** | **CenRT** | **RTCen** |
| Median | 49.5 | 50.0 | 47.0 |
| Interquartile range | 20.3 – 127.8 | 18.7 – 124.8 | 18.5 – 120.8 |
| Range | 7.2 - 279 | 7 - 310 | 6.6 - 299 |

*CenRT, centrifuged and serum maintained at 22°C for 72 h; CenFr centrifuged and serum immediately frozen at -20 °C; RTCen unseparated sample maintained at 22°C for 72 h*

**Table 2**

Comparison of serum insulin concentrations after three different storage and handling conditions, using Linn’s concordance correlation coefficient, Bland Altman analysis and Kappa’s measure of agreement for cut-offs of 30µIU/ml and 100µIU/ml.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | | **CenFr – RTCen** | **CenFR-CenRT -** | **CenRT - RTCen** |
| Linn’s Concordance Correlation coefficient (95% CI) | | 0.994 (0.983-0.998) | 0.992 (0.977 – 0.997) | 0.999 (0.997 – 0.999) |
| Bland Altman Analysis  Mean difference (µIU/mL) (95% LOA) | | 1.81 (-14.8 – 18.4) (*P*=0.42) | -0.34 (-21.3 – 20.7) (*P* =0.91) | 2.16\* (-4.99 - 9.3) (*P* =0.04) |
| Regression R2 | | 0.063 | 0.19 | 0.51 |
| Unstandardised Coefficient | | -0.03(*P* =0.39) | -0.06 (*P* =0.12) | 0.03 (*P* =0.004) |
| Kappa Measure of agreement value | 30µIU/mL | 1.0 | 1.0 | 1.0 |
| 100 µIU/mL | 1.0 | 1.0 | 1.0 |

*(CenRT, centrifuged and serum maintained at 22°C for 72 h; CenFr centrifuged and serum immediately frozen at -20 °C; RTCen unseparated sample maintained at 22°C for 72 h*

*LOA - limits of agreement*

*\* = mean difference significantly different to 0*.

**Figure Legends**

Fig. 1. Comparison of insulin concentrations in 14 horses after three different storage and handling conditions in low-medium (< 100 µIU/mL, A) and high (> 100 µIU/mL, B) ranges. *CenRT, centrifuged and serum maintained at 22°C for 72 h; CenFr centrifuged and serum immediately frozen at -20 °C; RTCen unseparated sample maintained at 22°C for 72 h*

Fig. 2. Bland-Altman plots of the difference in serum insulin concentration against the mean for different storage and handling in 14 horses. The solid line is the average difference and the dotted lines represent 95% limits of agreement (values in Table 2). *CenRT, centrifuged and serum maintained at 22°C for 72 h; CenFr centrifuged and serum immediately frozen at -20 °C; RTCen unseparated sample maintained at 22°C for 72 h*

