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Pre-analytical stability of sorbitol dehydrogenase in equine heparinized plasma

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ABSTRACT

Sorbitol dehydrogenase (SDH) activity is one of the most sensitive and specific markers for hepatocellular injury in horses, but its reported lability makes it impractical for use in many clinical settings. To date, stability of SDH in equine samples has only been evaluated in a limited number of studies in serum samples of horses with activities within reference intervals. The objective of the study was to determine pre-analytical stability of equine SDH activity in heparinized plasma stored at different temperatures for up to 72 h. Twenty client-owned horses admitted to a veterinary teaching hospital for any reason were included in the study. Blood samples collected in lithium-heparin tubes were immediately centrifuged and SDH activity was analyzed within 1 h of collection (T0). Aliquots of plasma were stored at room temperature, 4 °C and −20 °C and SDH activity was re-analyzed after 4 h (T4), 24 h (T24) and 72 h (T72).

A significant difference from values measured at T0 was found for samples stored at room temperature ($P = 0.022$) and −20 °C ($P < 0.001$), but not at 4 °C. The activity of SDH was within ±20% of that measured at T0 for all samples under all temperature conditions stored for 4 h, and for all samples stored at 4 °C for 24 h. Bland–Altman plots revealed narrow limits of agreement at T4 for all storage temperatures and at T24 for samples stored at 4 °C. The mean absolute percentage error and 95th percentile of the absolute percentage error were lower for samples stored at 4 °C than those stored at room temperature or −20 °C. The activity of SDH has adequate stability for 4 h regardless of storage temperature and 24 h if stored at 4 °C across a wide range of values. Knowledge of the pre-analytical stability of SDH may permit its broader use in assessing hepatic disorders in horses.

Introduction

Hepatic disorders are common in horses (DeNotta and Divers, 2020); diagnosis generally relies on a combination of clinical signs, clinicopathologic findings, ultrasound imaging and liver biopsy (Durham et al., 2003a,b,c). Detection of increased hepatic enzyme activities in serum or plasma is important when establishing a diagnosis of equine hepatopathy and, when evaluated over time, may help predict prognosis (Durham et al., 2003a; DeNotta and Divers, 2020). While elevations in some enzyme activities (alkaline phosphatase, aspartate aminotransferase, lactate dehydrogenase) may indicate hepatobiliary disorders, only γ-glutamyl transferase (gGT), glutamate dehydrogenase (GLDH) and sorbitol dehydrogenase (SDH) are considered liver specific in horses. Both SDH and GLDH are considered specific for hepatocellular damage, and gGT is primarily associated with biliary disease, but may also increase with hepatocellular injury (DeNotta and Divers, 2020).

Sorbitol dehydrogenase (also known as iditol dehydrogenase) is a cytosolic enzyme, found in the highest concentrations in the liver and is a very sensitive and specific marker of liver disease (Bernard and Divers, 1989; El-Kabbani et al., 2004). The half-life of equine SDH is very short and has been reported to be in the range of 12−24 h (Engelking and Paradis, 1987). Given this short half-life, SDH is of potential benefit for monitoring horses with acute hepatic injury (Divers, 2015). Indeed, SDH activity has been shown to increase above baseline as early as 4 h after oral administration of carbon tetrachloride, peak after two days, and decrease to baseline within 5 days (Bernard and Divers, 1989).

Despite these potential advantages, SDH is not offered by many veterinary laboratories because it is known to be very labile. In one

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study, serum SDH activity in 9 horses was stable for only 5 h at room temperature (21 °C), 24 h refrigerated (0–5 °C) and 48 h in frozen samples (−30 °C) (Horney et al., 1993). The same study measured SDH in equine heparinized plasma and found increased activity in samples refrigerated for 5 h, but did not evaluate stability in frozen samples (Horney et al., 1993). Comparable stability of SDH was found in serum from cows and llamas (Horney et al., 1993; Tornquist et al., 2000). However, these studies only included samples with SDH activities within reference intervals, and no study has evaluated the stability of equine SDH in heparinized plasma samples from animals with both normal and increased activities.

The objective of this study was therefore to evaluate the effect of storage time and temperature on SDH activity in equine plasma samples with normal and increased activities stored for up to 72 h at room temperature, refrigerated, and frozen.

Materials and methods

Animals

The study population consisted of horses referred to an equine hospital for any reason, including suspicion of liver disease, to cover a broad range of SDH activities. Horses were enrolled if a biochemistry profile that included SDH activity was requested by the attending clinician for diagnostic work-up and owner consent was obtained to use the remaining portion of samples for research purposes. Ethical approval was waived by the institutional ethics committee as all samples were taken for diagnostic purposes and only leftover material was used in this study. Consent was obtained from the owners for all diagnostic procedures and for the use of leftover biological material for research purposes.

Specimen collection and analysis

Blood (9 mL) was collected into lithium-heparinized blood tubes (S-Monovette Sarstedt) and samples were centrifuged immediately at 1400 × *g* for 10 min at room temperature. Plasma was removed directly after centrifugation and aliquoted. Baseline plasma SDH activity was measured within one hour of sample collection (T0). The remaining aliquots were immediately stored for 4 h (T4), 24 h (T24) and 72 h (T72) at room temperature, 4 °C and −20 °C prior to analysis. Samples were allowed to come to room temperature (30 min) and vortexed for 5 s prior to analysis. Storage duration times were chosen to reflect the three most common scenarios of sample submission by private practitioners to the veterinary laboratory, namely same-day delivery, next-day delivery, or Monday morning delivery of samples taken on Fridays. Temperatures were monitored every 20 min using electronic temperature recorders (TRID30-7, LogTag Recorders Ltd, Spaelti-TS AG) and storage was considered acceptable if temperatures remained within 18–25 °C (room temperature), 2–6 °C (refrigerated at 4 °C) and −18 to −22 °C (frozen at −20 °C). Plasma SDH activity was measured with a commercial biochemical analyzer (Cobas c501, Roche Diagnostics) using a commercial SDH reagent (VetSpec, C432-0B, Catachem), and two levels of controls (VetSpec, C434-41; C434-42, lot DL281801 and lot DA292003, Catachem), following manufacturer's guidelines. Analyzer observed total error (TEobs) was calculated as follows -

$$\text{TEobs} = 2\text{CV} + \text{bias\%}$$ (Harr et al., 2013).

All measurements performed for the same animal at the same time-point were measured in a single run.

Statistical analysis

Data were analyzed using statistical software (Medcalc Statistical Software version 19.6, Medcalc Software). As data was not normally distributed (Shapiro–Wilk), a Friedman test was used to evaluate measurements at different time points for each of the three different storage temperatures. Where significant differences were found, post-hoc analysis was performed using pairwise Wilcoxon signed-rank tests with Bonferroni correction to assess differences between each time point and T0. Bland-Altman plots were created using values at T0 as the reference method and plotting the percentage differences between values at a given time point and the value at T0 against the value at T0. The median absolute percentage error and 95th percentile of the median absolute percentage error were also calculated, whereby the absolute percentage error was calculated as -

$$100 \times (\text{absolute value } [y-x]/x)$$

where y is SDH activity at a given time point and x is SDH activity at T0. The 95th percentile is interpreted as the percentage difference between the activity at a given time point and that at T0 that is not expected, with 95% certainty, to be exceeded. A *P*-value <0.05 was considered significant. Independent of statistical significance, we considered values within ±25% of that measured at T0 to be a "clinically acceptable" total allowable error (Harr et al., 2013).

Results

The study included 20 horses between 1 and 30 years of age (mean ± standard deviation [SD]; 14 ± 8.3 years). There were 8 mares and 12 geldings and the following breeds were represented: Warmblood ($n = 5$), Irish Cob ($n = 3$), Freiberger ($n = 2$), Welsh pony ($n = 2$), Friesian ($n = 2$), Crossbred pony ($n = 2$), Arabian ($n = 1$), German riding pony ($n = 1$), Paint horse ($n = 1$), Shetland pony ($n = 1$).

Baseline SDH activity measured at T0 ranged between 6 and 98 U/L (Table 1). Based on a reference interval of 3–16 U/L, 4 horses had SDH activity within reference limits (range, 6–15 U/L), 9 had values between the upper reference limit (URL) and 2× the URL (range, 18–29 U/L), 3 had values between 2× and 3× the URL (range, 34–44 U/L) and 4 horses had activities over 3× the URL (range, 58–98 U/L).

Controls were within limits published by the manufacturer, and analyzer TEobs was within (20.0%; high level) or very close to (25.3%; low level) the total allowable error of 25%, recommended for SDH (Harr et al., 2013).

The activity of SDH was within ±20% of that measured at T0 for all samples under all temperature conditions stored for 4 h, and for all samples stored at 4 °C for 24 h. No significant difference ($P = 0.060$) was found between values at T0 and values measured after storage at 4 °C (Fig. 1). However, significant differences were found between values at T0 and values measured after storage at room temperature ($P = 0.022$) and −20°C ($P < 0.001$). Post hoc analyses revealed significant differences at T24 and T72 for samples stored at room temperature, and T4

Table 1
Comparison of SDH activities in equine plasma after 0 h (T0), 4 h (T4), 24 h (T24), and 72 h (T72) of storage at different temperatures.

Temperature	Time point	*n*	Median	Interquartile range	Min-Max	*P*[a]
Room temperature	T0	20	27.5	18.5–42.5	6–98	
	T4	20	26.5	18.0–42.5	6–97	0.252
	T24	20	24.0	17.5–45.0	6–85	0.039
	T72	20	24.5	14.0–44.5	6–84	0.010
4 °C	T0	20	27.5	18.5–42.5	6–98	
	T4	20	26.5	18.5–42.5	6–100	n/a
	T24	20	26.0	18.5–43.0	6–91	n/a
	T72	20	25.0	18.0–42.5	6–119	n/a
−20 °C	T0	20	27.5	18.5–42.5	6–98	
	T4	20	26.0	17.5–39.0	7–100	0.031
	T24	20	25.0	18.5–39.0	6–94	0.010
	T72	20	24.5	17.0–38.5	5–126	0.429

Min, Minimum; Max, Maximum; n/a, not applicable.
[a] Difference compared to T0, Wilcoxon signed-rank (Bonferroni corrected).

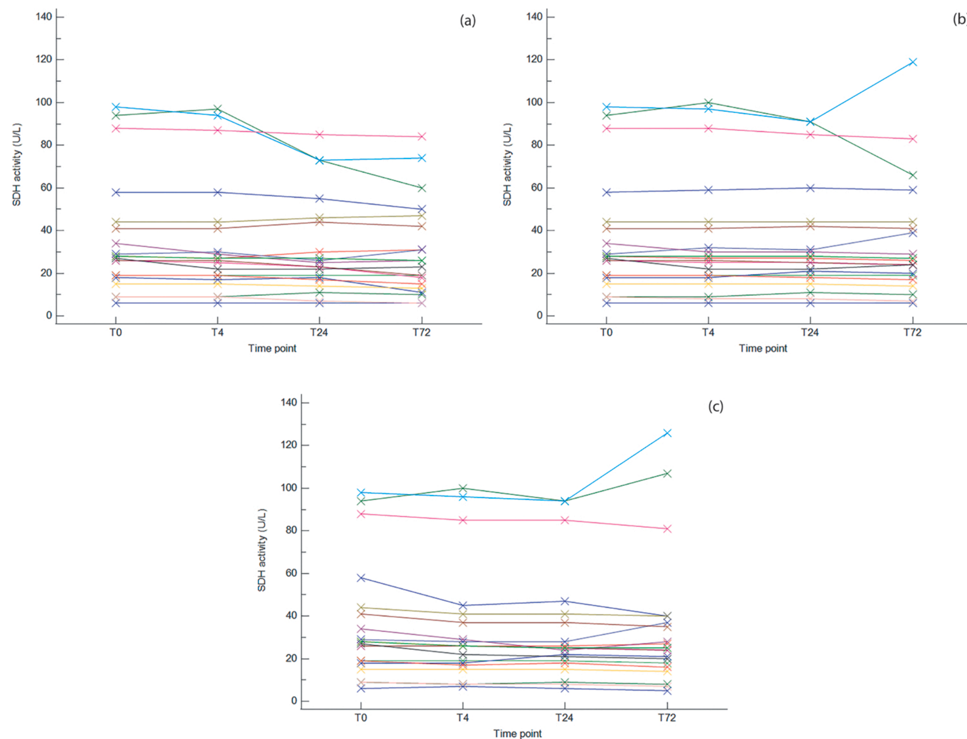

Fig. 1. Dot-and-line diagrams showing sorbitol dehydrogenase (SDH) activities in plasma in 20 horses after 0 h (T0), 4 h (T4), 24 h (T24), and 72 h (T72) of storage at room temperature (1a), 4 °C (1b) and at −20 °C (1c). Reference intervals: 3–16 U/L.

and T24 for samples stored at −20 °C (Table 1). Bland-Altman plots revealed narrow limits of agreement at T4 for all storage temperatures, and at T24 for samples stored at 4 °C (Fig. 2). A negative proportional bias was present with increasing SDH activity after storage at room temperature for 24 and 72 h, and a positive proportional bias was evident after storage at −20 °C for 72 h. The mean absolute percentage error and 95th percentile of the absolute percentage error were lower for samples stored at 4 °C than those stored at room temperature or −20 °C (Table 2). Changes in SDH activity under all investigated storage conditions did not affect the clinical classification regarding presence or absence of hepatocellular disease in any of our studied horses.

Data from the temperature monitoring devices revealed that storage temperatures were within the acceptable range at all time points except for minor fluctuations for refrigerated samples (minimum recorded temperature, 1.8 °C; maximum recorded temperature 7.5 °C).

Discussion

Biochemical markers that clearly differentiate between horses with and without liver disease are currently lacking (Durham et al., 2003b), and a combination of diagnostic tests is needed to identify affected animals. Measurement of SDH activity is generally underused due to concerns regarding its stability, but a greater understanding of SDH stability could allow for wider use of this enzyme in the evaluation of suspected hepatopathies in horses (Pieralisi and Comazzi, 2006).

The results of this study indicate that SDH activity in heparinized plasma may be more stable at 4 °C than at either room temperature or −20 °C if samples are centrifuged, and plasma separated and refrigerated immediately after collection. Analysis after 4 and 24 h storage at 4 °C yielded limits of agreement within ±20% of activity at T0 and may thus be acceptable when used to screen for evidence of equine liver disease. However, the limits of agreement and 95th percentile absolute percentage error were relatively high and increased over time regardless of storage temperature, limiting the use of SDH activity for serial measurements in horses if timely analysis is not possible. Nonetheless, a change in enzyme activity beyond the 95th percentile absolute percentage error found in this study is less likely to be attributable to storage alone, and may therefore still be interpreted as clinically relevant, particularly if changes also exceeded the TEobs of 25%. Furthermore, it is noteworthy that storage conditions investigated herein would not have led to a clinical misclassification of presence or absence of hepatic disease in any of our studied horses.

These findings suggest a higher stability of SDH in equine plasma than previously reported for equine serum samples (Horney et al., 1993). Higher stability of SDH in plasma than in serum after 24 h storage at room temperature was also reported in llamas, although this finding was not confirmed in refrigerated or frozen samples (Tornquist et al., 2000).

Some samples showed higher activity after storage, especially for samples stored at −20 °C. This has previously been reported for SDH (Tornquist et al., 2000) and other biochemical analytes (Ada et al., 2017). It has been hypothesized, that the enzyme became more concentrated or that its activity increased at lower temperatures (Tornquist et al., 2000). Some of the results might also be explained by intra-sample variance as previously described especially when analyzing small data sets (Rendle et al., 2009). Analytical variation, such as reaction temperature, reagent degradation, differences in calibration, instrument imprecision or drift, as well as operator imprecision, may also contribute to the measured differences in SDH activities, but are unlikely to account solely for the large changes observed with prolonged storage at room temperature and −20 °C. Nevertheless, all samples stored for 4 h at all temperatures and all stored for 24 h at 4 °C were both within the 25% total allowable error recommended for SDH (Harr et al., 2013) and our TEobs.

The effect of delayed centrifugation on SDH activity was not investigated in this study, but it has previously been shown to have a significant effect on the activity of enzymes in horses including aspartate dehydrogenase, creatine kinase and lactate dehydrogenase (Rendle et al., 2009). Whether and to what extent delayed centrifugation and plasma separation may affect plasma SDH activity in equine samples

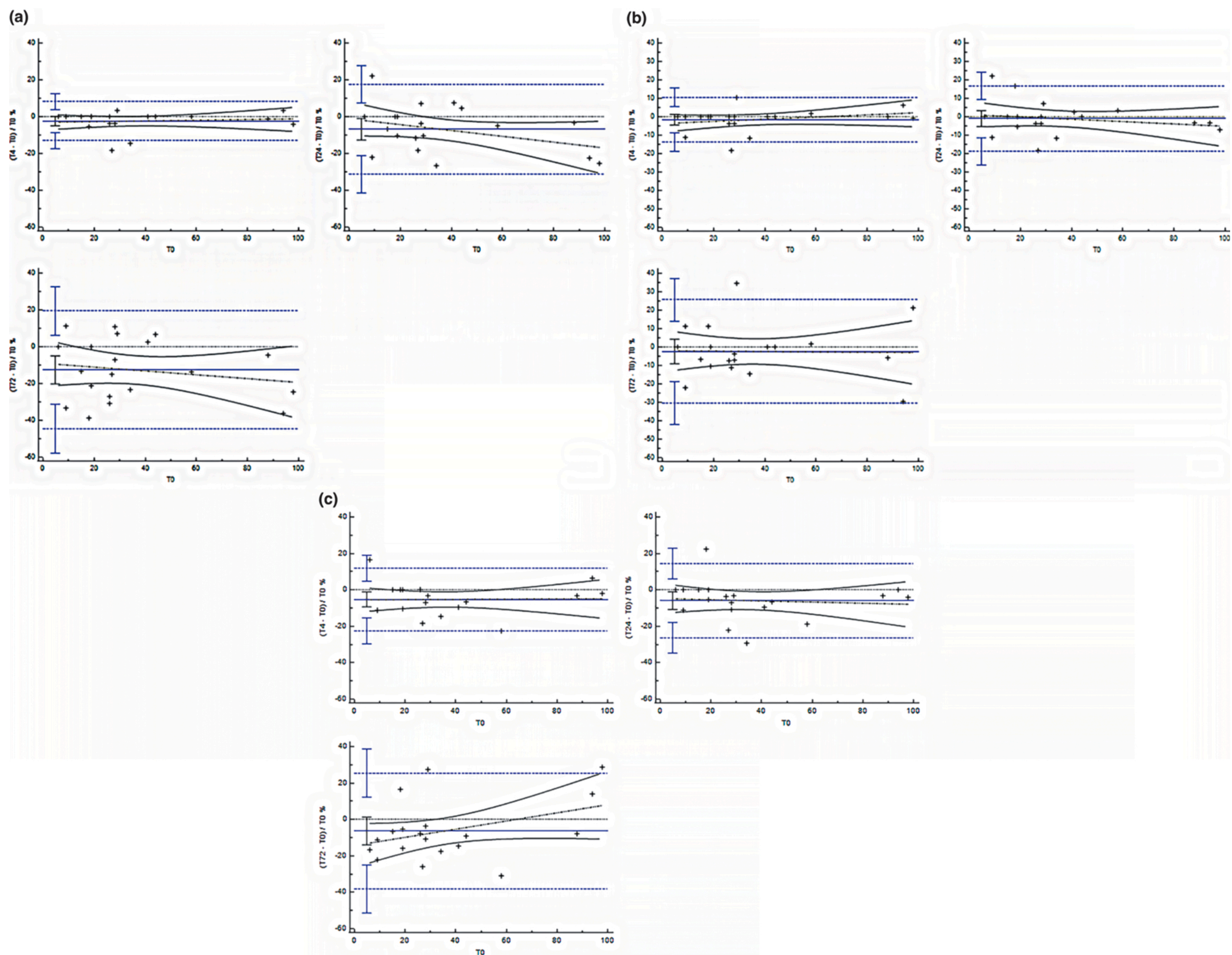

Fig. 2. Bland–Altman plots after storage of equine plasma for 4 h (T4), 24 h (T24) and 72 h (T72) showing sorbitol dehydrogenase (SDH) activities as the percentage difference between values after storage and values prior to storage (T0) plotted against values at T0 at room temperature (2a), 4 °C (2b) and −20 °C (2c). Dotted black line, zero line; solid blue line, average difference; black bar, 95% CI for the average difference; dashed blue lines, limits of agreement; blue bars, 95% confidence intervals (CI) for the limits of agreement; dash/dotted black line, regression line; solid black lines, 95% CI for the regression.

Table 2
Mean percentage difference, limits of agreement, and absolute percentage error of sorbitol dehydrogenase (SDH) activities in equine plasma after storage at room temperature, 4 °C, and −20 °C compared to T0.

Time point	Storage temperature	Mean percentage difference (95% CI)	Lower limit of agreement (95% CI)	Upper limit of agreement (95% CI)	Median absolute percentage error (95% CI)	95th percentile absolute percentage error
T4		−2.42 (−4.94 to 0.12)	−13.00 (−17.39 to −8.61)	8.16 (3.77 to 12.55)	0.57 (0.00 to 3.57)	16.61
T24	Room temperature	−6.83 (−12.66 to −0.99)	−31.27 (−41.41 to −21.12)	17.60 (7.46 to 27.75)	8.83 (4.65 to 17.33)	25.99
T72		−12.54 (−20.17 to −4.91)	−44.50 (−57.77 to −31.23)	19.42 (6.15 to 32.69)	13.56 (6.94 to 24.33)	37.53
T4		−1.57 (−4.48 to 1.34)	−13.76 (−18.82 to −8.70)	10.62 (5.56 to 15.68)	0.00 (0.00 to 3.80)	15.14
T24	4 °C	−1.00 (−5.25 to 3.25)	−18.79 (−23.17 to −11.40)	16.79 (9.40 to 24.17)	3.71 (2.57 to 7.10)	20.37
T72		−2.35 (−9.04 to 4.34)	−30.35 (−41.98 to −18.73)	25.66 (14.03 to 37.28)	7.69 (3.93 to 11.11)	32.13
T4		−5.25 (−9.35 to −1.16)	−22.42 (−29.54 to −15.29)	11.91 (4.78 to 19.03)	6.98 (2.27 to 11.01)	20.47
T24	−20 °C	−5.89 (−10.75 to −1.03)	−26.23 (−34.68 to −17.79)	14.45 (6.01 to 22.90)	4.67 (3.42 to 10.55)	25.82
T72		−6.35 (−13.95 to 1.25)	−38.17 (−51.38 to −24.97)	25.47 (12.26 to 38.68)	14.23 (8.15 to 17.48)	29.80

CI, confidence intervals.

therefore warrants further investigation.

As only SDH and GLDH are considered specific for hepatocellular injury in horses, many veterinary diagnostic laboratories offer measurement of GLDH only, due to concerns regarding the stability of SDH. Rare studies have investigated the relative sensitivities and specificities of these two enzymes or their comparative usefulness as prognostic markers. In one study, both enzymes were found to have comparable sensitivity and specificity, but the assay used for SDH activity differed from that used in the present study (West, 1989). Further investigation and comparison of both enzyme activities in the diagnostic workup of equine hepatopathies are necessary to assess the potential use of measuring SDH or both enzymes compared to GLDH alone.

Limitations of this study include a small sample set with a majority of increased SDH concentrations. A priori estimates for ANOVA repeated measures were not performed to determine the optimal number of study subjects due to the lack of reliable estimates for effect size. In addition, duplicate measurements of each sample may also have given insight into the coefficient of variation due to the method alone. Although there were some fluctuations in refrigeration temperatures, these were very minor and unlikely to have had any marked influence on results. Nevertheless, the study results suggest that SDH can be used in clinical practice if blood samples are centrifuged and plasma separated immediately after venipuncture, and if samples can be analyzed within 4 h or refrigerated and analyzed within 24 h. The results further indicate that results of frozen plasma samples should be interpreted with caution despite previous studies suggesting adequate stability in frozen serum samples.

Conclusions

Equine SDH activity was stable in heparinized plasma stored under all temperature conditions for 4 h and at 4 °C for 24 h, but changes in enzyme activity may only be considered clinically relevant if they exceed 25% of previously measured activity. Results of this study may permit wider use of SDH activity in assessing hepatic disorders in horses.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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