Assessment of beta-hydroxybutyric acid (BHBA) as a plasma biomarker for hepatic lipidosis in bearded dragons *(Pogona vitticeps)*

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Introduction

- Hepatic lipidosis (or fatty liver disease) is characterized by the pathologic accumulation of triacylglycerols in hepatocytes leading to impaired liver function.
- Hepatic lipidosis is one of the most common non-infectious diseases of captive bearded dragons, affecting approximately 38.3% of pet dragons, [1] one of the most common species of pet reptiles.
- Routine hematologic, biochemical, radiographs, and coelomic ultrasound have low sensitivity for diagnosing this disease.[2]
- Currently its diagnosis mainly relies on advanced imaging techniques, such as CT-scanning, or invasive techniques, such as liver biopsy and histopathology.
- A previous pilot study using plasma metabolomics on a small cohort of 14 bearded dragons, with varying degrees of spontaneously occurring hepatic lipidosis, identified β-hydroxybutyric acid (BHBA) as a candidate biomarker for hepatic lipidosis. 2 The main confounding effect was the varying ages between disease groups.

Objectives

Hypotheses:

- Plasma BHBA concentrations are associated with the degree of hepatic lipid accumulation in bearded dragons .
- A BHBA point-of-care analyzer shows good agreement with a reference analyzer.

Our aims:

- Further assess the usefulness of BHBA as a plasma biomarker for the diagnosis and screening of hepatic lipidosis on a larger sample size (n=48) of bearded dragons that were matched for age.
- Assess the reliability of a point-of-care BHBA meter for in-hospital diagnosis.

Methods

- Forty-eight bearded dragons originally selected to be culled were collected from a large breeding facility in Chico, CA. Twenty-four dragons were between 1.5-3 years of age and another twenty-four were between 4-7 years old. There were twenty-five females and twenty-three males.
- Dragons were fasted for 48 hours prior to sample collection.[3]
- 2-3 mL of blood was collected from caudal tail vein or right jugular vein for measurement of plasma BHBA concentration using a point-of-care analyzer on whole blood (NovaVet™ ketone/glucose meter) immediately following blood collection and using a reference analyzer (Vitros 5600 Analyzer, University of Miami Avian and Wildlife Laboratory) on heparinized plasma.
- A hepatic biochemistry panel (AST, ALT, ALP, GGT, GLDH, LDH, Bile acids) was also performed.
- Remaining heparinized plasma was stored at -80°C for additional analyses.
- Dragons were sedated/anesthetized using 10-15 mg/kg alfaxalone SC.
- Whole body CT scans were performed in groups of 8 bearded dragons (Figure 1).
- Hepatic density in Hounsfield units (HU) was determined from standardized ROI from CT images (Figure 2).
- Dragons were humanely euthanized via intracardiac injection of 0.3-0.5 mL of KCl.
- Necropsy and sample collection were performed as well as histopathology and hepatic lipidosis grading using a validated histopathology scoring system.[2]

Methods (continued)

Figure 1: Bearded dragon arrangement in CT machine. Sedated dragons were scanned in groups of 8 at a time.

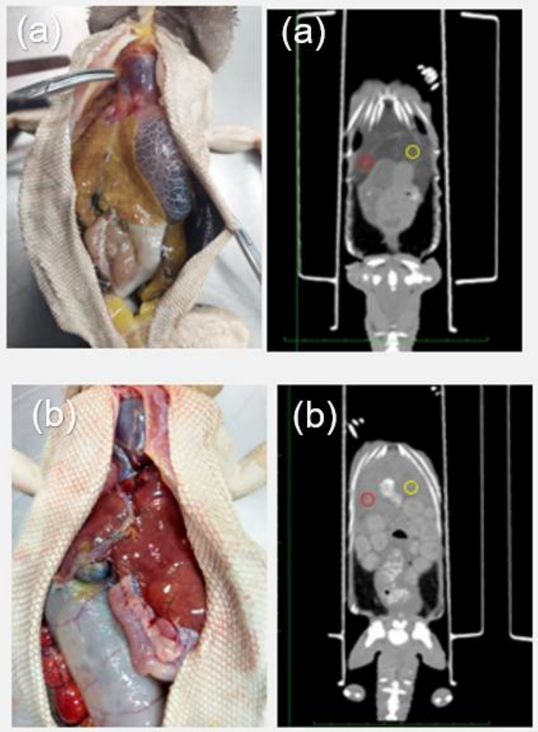

Figure 2: CT and necropsy images of a lipidotic (a) and normal (b) bearded dragon. Left (yellow) and right (red) lobe standard ROIs for determining liver density in HU on CT coronal views. The average between these two lobes was calculated as an estimate of the overall HU of the liver.

Statistical Analysis

- The association between BHBA (log transformed) and hepatic fat content (hepatic density on CT-scan) was evaluated using linear regression analysis (Figure 4). Assumptions of linear regression were evaluated on residual plots. Other analytes from the biochemistry profiles were evaluated using the same approach.
- Imprecision of the analyzers was calculated from running 5 replicates on 5 bearded dragons.
- The bias for BHBA analysis (reference method – POC method) was modelled using linear regression including BHBA values by the reference method as a predictor to estimate the proportional bias. The agreement was further evaluated using a difference plot with the limits of agreement (LOA) calculated as $1.96*SD_{bias}$. Observed total error (TE_{obs}) was determined as 2*CV+bias (%).[3] A total allowable error (TEa) of 30% was considered acceptable for clinical use.[4]

Resullts

There was no significant association between plasma BHBA concentrations and fat liver content (R2=0.05, p=0.12) (Figure 3, figure 4). No significant associations were seen on any of the hepatic biochemical analytes (all p>0.05).

For the POC meter, there was no constant bias, but a significant proportional bias (p<0.001) (Figure 5). The POC meter was found to mostly underestimate BHBA obtained from the reference analyzer. The imprecision of the POC meter was 5.8%, which was considered low. The LOA were 0.52 mmol/L (Figure 5). TEobs was 23.2%, which was lower than TEa but much higher than POC imprecision.

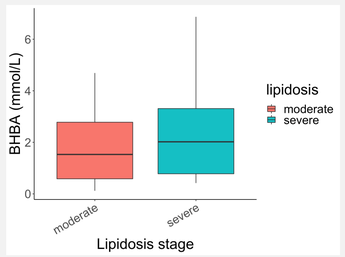

Figure 3: Box and whisker plot depicting the range of BHBA concentration (mmol/L) relative to degree of of hepatic lipidosis (moderate or severe). Median BHBA concentration was 1.53 mmol/L with an interquartile range (IQR) of 2.19 in moderate cases (n=16) and 2.04 with an IQR of 2.56 in severe cases (n=32).

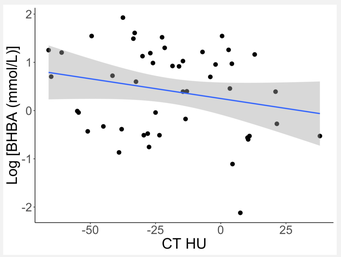

Figure 4: Linear regression comparing each dragon's BHBA (mmol/L) concentration to average HU on CT. No significant correlation was found (R2=0.05, p=0.12).

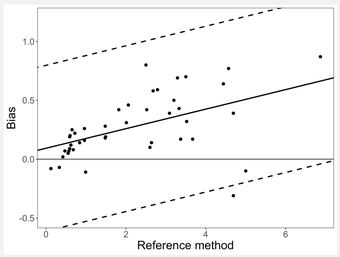

Figure 5: Percent bias of the POC BHBA meter compared to reference analyzer. POC readings tended to underestimate BHBA concentrations determined by the reference analyzer.

Discussion

Our findings regarding BHBA's relationship to hepatic lipidosis are not consistent with the results of the pilot study, as we did not find any significant association. There were several potential factors that may have contributed to this difference, including:

- Captive bearded dragon husbandry practices, diet, or breeding schedule may have predisposed animals to hepatic lipidosis and other conditions, as almost all dragons were found to have moderate to severe hepatic lipidosis.
- Many dragons were thought to be not fully fasted after 48 hours. Plasma lipoprotein profiling using polyacrylamide gel electrophoresis (Lipoprint® Lipoprotein Subfractions Testing System) revealed chylomicrons to present in the plasma of most dragons.

A standard biochemistry panel did not reveal any additional potential analytes for diagnosing hepatic lipidosis.

While the POC BHBA meter showed wide limits of agreement, its precision was considered adequate and observed error were within acceptable values for clinical use. However, a significant proportional bias was found suggesting that the POC analyzer may show higher disagreement in higher plasma BHBA concentrations than seen in this study.

Conclusion and Future Direction

Our data does not support the use of BHBA as a plasma biomarker for the diagnosis of severe hepatic lipid accumulation. The POC BHBA meter may be used in clinics to assess fatty acid metabolism when access to a reference analyzer is limited, but caution is advised when interpreting high BHBA values. To further explore metabolic pathway disruption with hepatic lipidosis in bearded dragons and continue to screen for novel plasma biomarkers, metabolomics data are pending for this cohort.

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